A maternal homeobox gene, *Bombyx caudal,* forms both mRNA and protein concentration gradients spanning anteroposterior axis during gastrulation

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SUMMARY

We have isolated a *caudal* (*cad*) homologue from a cDNA library of *Bombyx mori* embryos. The *Bombyx cad* cDNA encodes a protein of 244 amino acids. The homology between *Drosophila* and *Bombyx* homeodomains is 80%. Similar to *Drosophila cad*, there is no YPWM peptide sequence along the upstream of homeodomain. Northern blot hybridization with a *Bombyx cad* probe revealed the presence of single maternal transcript of 2.3 kb. A stronger signal of the transcripts was detected in unfertilized eggs and in eggs up to 36 hours after deposition. The transcripts decreased rapidly by 2 days and a weak signal was maintained until hatching. To analyse its spatial expression pattern, we have established a novel frozen sectioning method for in situ hybridization and immunohistochemistry experiments. The results showed that *Bombyx cad* transcripts accumulated first in the nurse cells and transferred into the oocyte at a defined time during oogenesis. The maternal transcripts of *Bombyx cad* formed a concentration gradient spanning the anteroposterior axis during the gastrulation stage and were restricted to the anal pad, the most posterior domain, after 2 days of embryogenesis; the *Drosophila cad* mRNA revealed the corresponding expression profile during the syncytial blastoderm stage. The Bombyx cad protein was not detected in the ovary and the first 9 hours of eggs, but was first detected evenly during cellular blastoderm stage. During gastrulation, Bombyx cad protein concentration gradients shifted along the anteroposterior axis coinciding with the shifting of the mRNA concentration gradients. This is the first example of a concentration gradient along anteroposterior axis that may be involved in segmentation in insect embryos other than the long germ-band type. A comparison of the timing and conservation of mRNA as well as protein gradient formation in *Drosophila* and *Bombyx* might help to explain differences in the body plans and give some clues to elucidate the mechanism and function related to mRNA and protein concentration gradients.

INTRODUCTION

Body plan in *Drosophila* is embryonically controlled by a hierarchy of interaction among three groups of genes. Those at the top of the hierarchy consist of maternally active genes, which define the spatial coordinates along the anteroposterior and dorsoventral axes of embryos (Gehring, 1973; Nüsslein-Volhard, 1979; Driever and Nüsslein-Volhard, 1988); mutations in these genes cause dramatic changes in the embryonic pattern (Bull, 1966; Anderson and Nüsslein-Volhard, 1984; Mohle and Wieschaus, 1986). Those acting further down, including pair-rule and segment polarity genes, determine the correct number and polarity of the metameric units (Nüsslein-Volhard and Wieschaus, 1980; Nüsslein-Volhard et al., 1982). The final group of genes that specify segment identity are organized into two small clusters: the Antennapedia complex (Kaufman et al., 1990) and the Bithorax complex (Duncan, 1987).

We are interested in studying the maternal homeobox gene *Bombyx mori caudal* (*Bm cad*) for three reasons. (1) Until now, the only reports on maternally active genes are from long germ-band type insects, although several segment polarity and homeotic selective genes have been isolated based on sequence homology (Tear et al., 1990; Sommer and Tautz, 1991; Stuart et al., 1991; Hui et al., 1992; Ueno et al., 1992). *Bombyx* may belong to either the short or intermediate germ-band type but clearly differs from the long germ-band type both in morphology and the *Bm en* stripes expression pattern in segmentation processes during gastrulation (K. Amanai et al., unpublished data). (2) Until now, there is no direct molecular identification of the concentration gradient spanning anteroposterior axis in insects other than the long germ-band type. (3) *Drosophila cad* was the first characterized maternal homeobox gene. It encodes maternal and zygotic transcripts that are derived from different promoters, but both mRNAs contain the same open reading frame. In situ hybridization and antibody staining showed that the maternal RNA and protein accumulate in concentration gradients along the anteroposterior axis during the syncytial

blastoderm stage while the zygotic RNA and protein are present in the primordia of terminal abdominal segment and hindgut and in the posterior midgut rudiment (Mlodzik et al., 1985; Mlodzik and Gehring, 1987). Mutations in the *cad* gene that reduce or eliminate the gradients cause an abnormal zygotic expression of at least one segmentation gene (*fushi tarazu)* and alter the global body pattern (Macdonald and Struhl, 1986). Ectopic expression of cad at the anterior end of cellular blastoderm embryos was found to disrupt head development and segmentation, due to alteration of the expression such as *fushi tarazu* and *engrailed,* as well as repression of head-determining genes such as *Deformed* (Mlodzik et al., 1990). As one of several approaches towards comparing the evolutionary conservation of the gene hierarchy system, the studies on the *Bm cad* will lead to better understanding of the molecular identity of the so-called morphogen gradient (Sander, 1960, 1976; French, 1988) in insects other than the long germ-band type and will further give us some clues to explain the upstream molecular interaction of the many consecutive steps used for segmentation, which differ in long, intermediate and short germ-band types of insect embryos.

Based on such strategy, we have isolated *Bm cad* cDNA, using PCR cloning method, and investigated the temporal and spatial expression pattern during oogenesis and early embryogenesis, using a modified frozen sectioning method for in situ hybridization and immunohistochemistry experiments.

MATERIALS AND METHODS

Animals
Bombyx mori eggs, from a Japanese strain (Kin-Shu), a chinese strain (Sho-Wa) and a hybrid between them (Kin-Shu × Sho-Wa), were purchased from Kanebo Silk Company (Kasugai City, Japan). Larvae were raised aseptically at 27°C on an artificial diet from Nippon Nohsankoh (Funabashi City, Japan). Adults were mated at 25°C for 2 hours, and the eggs newly deposited were collected and developed at 25°C. We sampled the eggs at 1 hour intervals and staged according to the standard method (Takami and Kitazawa, 1960).

Since there was no detailed description of staging during oogenesis, we sampled oocytes from the sixth day pupa, which includes oocytes of almost all stages, and compared developmental stages of the oocytes according to their sizes. Oocytes for northern hybridization were taken from the abdomens of female adults and unfertilized eggs were deposited during 2 days from female unmated adults.

Preparation of poly(A)+ RNAs and cDNA library
Total RNA was isolated by using an acid guanidinium thiocyanate/phenol/chloroform method (Chomczynski and Sacchi, 1987) and poly(A)+ RNA was enriched by oligo(dT)-cellulose chromatography.

cDNA was synthesized from egg poly(A)+ RNA using a cDNA synthesis kit (Invitrogen). Synthesized cDNA was ligated to λgt11 vector. The ligated materials were packaged in vitro using Gigapack II Gold Packaging Extract (Stratagene).

Cloning and sequencing of *Bm cad* cDNA
Isolation of *Bm cad* cDNA was performed following the PCR amplification method as described (Kamb et al., 1989). DNA fragments containing homeobox regions were amplified from cDNA prepared from the posterior silk gland (PSG) of 2-day-old fifth instar larvae with fully degenerated primers corresponding to two consensus amino acid sequences 5′-ELEKEFH-3′ (sense) and 5′-I/VKI/VWFQN-3′ (antisense) in the homeodomain. This mixture was incubated in a ther-

mocycler for 40 cycles: 1 minute at 94°C, 1 minute at 45°C and 3 minutes at 55°C. After amplification, the DNA fragments were subcloned into a pBluescript II KS(−) vector (Stratagene). One clone containing partial *cad*-like homeobox region was identified by sequencing (Hui and Suzuki, 1994). Clones containing whole open reading frame were isolated from a cDNA library for *Bombyx* embryos of stages 20-25 by using the PCR fragment as a probe. The sequence was determined by the dideoxynucleotide method using the Sequenase 2.0 DNA Sequencing Kit (United States Biochemical).

Southern and northern blotting
10 μg of genomic DNA extracted from PSG of the Sho-Wa or Kin-Shu strain of *Bombyx* was digested with *Bam*HI, *Eco*RI, *Hin*dIII or *Xho*I and fractionated on a 0.8% agarose gel. After electrophoresis, the gel was soaked in 0.2 N HCl for 20 minutes, and 0.5 N NaOH/1.5 M NaCl for 15 minutes sequentially and subjected to blotting onto a nylon membrane (Biodyne Electronics, Santa Monica, CA; Pall). For northern blotting, poly(A)+ RNAs (5 μg per lane) were separated on 1% agarose/1.1 M formaldehyde gels (Ausubel et al., 1987) and transferred onto nylon membranes. Filters were hybridized with a probe specific for *Bm cad* at 42°C, in 50% formamide/5× SSC/5× Denhardt's solution/0.1% SDS/5 mM sodium phosphate (pH 6.5)/denatured salmon testes DNA (250 μg/ml).

A primer of 24 nucleotides corresponding to 5′-TCCTGTGTA-CAATGGAGGGACCAG-3′ sequence of *Bombyx* actin (Mounjer and Prudhomme, 1986) was used as a probe for positive control.

Antibody preparation
Both a 13-mer peptide (HFPKREPGEREIA) and a 12-mer peptide (EEVVMKEKGDHA) corresponding to residue no. 53-65 and 183-194 of Bm cad, respectively, were synthesized on the 8-branched MAP-lysine core (Applied Biosystems). Anti-cad antibodies were obtained by immunizing two white female rabbits with these two types of peptides separately.

Specific antibodies were purified using a peptide-affinity column prepared using ProtOn™ kit 1 (Multiple Peptide Systems), which significantly improved the signal-to-noise ratio for immunohistochemistry experiments.

Western analysis
Bombyx embryos were dissected out of the eggs, cleaned of yolk and then homogenized in SDS sample buffer (Laemmli, 1970). SDS-PAGE (15% polyacrylamide) was performed according to standard procedure. Proteins were electrophoretically transferred to Immobilon™-P transfer membrane (Millipore) in 50 mM Tris, 380 mM glycine, 0.1%(W/V) SDS, 20% methanol, pH 8.0. Membranes were processed with primary antibody (1:1000 dilution antiserum or 1:200 dilution affinity-purified antibody), followed by secondary antibody (1:1000 dilution alkaline phosphatase-conjugated goat anti-rabbit IgG). The alkaline phosphatase IV kit (Vector laboratories) was used for all color development according to the manufacture's instruction.

In situ hybridization to egg sections
We established a stable in situ hybridization system for *Bombyx* egg sections by using a novel frozen sectioning method with a Cryostat Frozen Sectioning Aid (Instrumedics). Eggs were directly snap frozen and embedded in Tissue-Tek (Miles Scientific) and the blocks were transferred into the cryostat chamber. A precooled adhesive tape (Instrumedics) was used to support a 6 μm thickness section as it was being cut; then the still frozen section adhering to the tape was laminated to a cold adhesive-coated slide (Instrumedics). An ultraviolet flash polymerized the adhesive coating into a hard solvent-resistant plastic, tightly anchoring the section to the slide. Finally, the tape was removed and the slide was immersed in cold acetone (−25°C) where the ice was dissolved not <u>melted</u> (freeze substitution). This method gave the best retention of morphology and the results were 100% reproducible with the highest signal-to-noise ratio. After trans-

ferring slides from cold acetone to 4% paraformaldehyde (PFA)/80% alcohol in cryostat chamber, we treated the slides in above fixative for 20 minutes at room temperature (22°C). Then the slides were immersed in 0.3% Triton/PBS for 10 minutes, 0.2 N HCl for 20 minutes and digested with 5 mg/ml proteinase K/PBS (Boehringer Mannheim Biochemica) for 5-10 minutes at 37°C. After being postfixed in 4% PFA/PBS for 10 minutes at room temperature (22°C), the slides were immersed in 2 mg/ml glycine for 1 minute twice and dehydrated through 70%/90%/100%/100% alcohol series. Sections were hybridized at 50°C for 16-24 hours in solution containing 50% formamide, 1× Denhardt's solution, 2× SSC, 10 mM EDTA, 10% dextran sulfate, 500 μg/ml yeast tRNA, 500 μg/ml salmon sperm DNA, 10 mM DTT.

Sense and anti-sense digoxigenin (DIG)-labeled RNA probes were prepared from the 2167 bp cDNA insert, which was subcloned into pBluescript II KS(−) plasmid by using DIG RNA Labeling Kit (Boehringer Mannheim Biochemica). Following coloring and washing, sections were mounted in clearing solution (2 parts benzyl-benzoate : 1 part benzyl alcohol). Photographs were taken using bright-field optics on a Zeiss Axiophot. The slides were scanned by using a Nikon LS-3510AF 35 mm Film Scanner and analyzed according to NIH Image 1.49 software.

Preparation of *Bombyx* egg sections for antibody staining

10-20 μm thickness sections for *Bombyx* eggs were obtained following the same procedure as above. After transferring slides from cold acetone to 2% paraformaldehyde (PFA)/80% alcohol in cryostat chamber, we treated the slides in above fixative for 2-10 minutes at room temperature (22°C) and blocked in 1% normal goat serum for 1 hour. Then the sections were incubated with 1:200 dilution affinity-purified primary antibodies at 4°C overnight followed by alkaline phosphatase detection with Vector lab 'ABC' system. Both affinity-purified antibodies showed the same expression pattern (data not shown).

RESULTS

Isolation and structural analysis of *Bm cad* cDNA clones

By screening a primary embryonic cDNA library (stages 20-25), we obtained a cDNA clone that contained a partial *cad*-like homeobox sequence. After further screening of an amplified library of the above with a DNA fragment from the afore-mentioned clone, we obtained three independent positive cDNA clones. The nucleotide sequence analysis as summarized below revealed that it corresponds to the *Bombyx* homologue of *Drosophila cad.*

The nucleotide and amino acid sequences of a composite cDNA molecule of *Bm cad* are shown in Fig. 1. The 732 base-paired sequence reveals an open reading frame that yields a protein of 244 amino acids with a deduced molecular mass of about $28 \times 10^3 M_r$. As shown in Fig. 2, the amino acid sequences of *Bm cad* and *Drosophila cad* homeodomains are 80% identical. Interestingly, such homology between hamster *cdx-3* (German et al., 1992) and *Bm cad* is as high as 91% (data not shown). The immediate 5′ upstream region of home-odomain is also highly conserved (Fig. 1). Since the putative amino acid sequence encoded by the *Bm cad* gene in the 5′ upstream region of homeobox was homologous to that of *Drosophila cad* gene, the *Bm cad* gene may also contain an intron at the same position as indicated in *Drosophila cad* (Mlodzik and Gehring, 1987). Similar to *Drosophila cad, Bm cad* lacks the sequence corresponding to the YPWM peptide

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1    TCGTCCGTAGCGTGTCTCGTGATCGGAGCGGTGGTGTTGTTTGTGACGGCGAGTGTGTGT    60
61   GCAATGCTCGGACGGTGATCGAAGCATGGTGAATTACGTCAATCCGCTCGCCATGTACCA   120
1                        M  V  N  Y  V  N  P  L  A  M  Y  Q       12

121  AGGCAAGGGCGGGCAATACGGCGGTGGCTGGTACGGCTGGCAGCATCAGAACTTGGAAGA   180
13    G  K  G  G  Q  Y  G  G  G  W  Y  G  W  Q  H  Q  N  L  E  E    32

181  GCAACAGTGGTGCGCATGGAACGGCGCACCGGCTACGGGGGAATGGACTCCGGATCCGCA   240
33    Q  Q  W  C  A  W  N  G  A  P  A  T  G  E  W  T  P  D  P  H    52

241  TCATTTTCCGAAAAGGGAACCAGGAGAACGAGAGATAGCCGATATGCCGTCGCCGGCCAG   300
53    H  F  P  K  R  E  P  G  E  R  E  I  A  D  M  P  S  P  A  R    72

301  GGGGGATTTGGCGAGTCCAGGAGAAGGTTCTCCAGGCTCAGGATCTCGGCCTTCACAACC   360
73    G  D  L  A  S  P  G  E  G  S  P  G  S  G  S  R  P  S  Q  P    92

361  ACCGGGCCCGCCGCGGTCGCCATACGAGTGGATGAAAAAACCAAACTATCAGACCCAACC   420
93    P  G  P  P  R  S  P  Y  E  W  M  K  K  P  N  Y  Q  T  Q  P   112

421  AAATCCAGGTAAAACGCGCACTAAAGACAAATACCGCGTGGTGTACAGTGATCATCAGCG   480
113   N  P  G  K  T  R  T  K  D  K  Y  R  V  V  Y  S  D  H  Q  R   132

481  GCTGGAGCTCGAGAAGGAATTTCACTACAGTCGTTACATCACTATTCGCCGTAAAGCGGA   540
133   L  E  L  E  K  E  F  H  Y  S  R  Y  I  T  I  R  R  K  A  E   152

541  ATTAGCCGTCAGCCTTGGTCTCTCGGAACGACAGGTAAAAATTTGGTTTCAAAATCGACG   600
153   L  A  V  S  L  G  L  S  E  R  Q  V  K  I  W  F  Q  N  R  R   172

601  CGCCAAGGAAAGGAAGCAAGTCAAAAAACGTGAAGAGGTGGTGATGAAGGAAAAGGGCGA   660
173   A  K  E  R  K  Q  V  K  K  R  E  E  V  V  M  K  E  K  G  D   192

661  TCACGCTTCGTTGCAGCACGCGCAACTCCACCACGCGACAATGTTACACCACCAGCAGAT   720
193   H  A  S  L  Q  H  A  Q  L  H  H  A  T  M  L  H  H  Q  Q  M   212

721  GATGAACGGTATGATGCACCACCACCATTACCATCAAGGAGTACTACAAGGCGTGCCCGA   780
213   M  N  G  M  M  H  H  H  H  Y  H  Q  G  V  L  Q  G  V  P  E   232

781  GCCTCTAGTCGCAGGGGTACCGCCGGTCCCTCTACTGTGACCACATCAACAGGACTATTG   840
233   P  L  V  A  G  V  P  P  V  P  L  L  *                       245

841  CGACTACAGTGCCACTTTTACACAGTGACCGCTCCAGTGATTTCATGTTATGACCATATT    900
901  ATGAACGAATATCCCGATAGGGCCATCGATAGTATGTGTGACATGATTTAATTAAATGTG    960
961  TGATTTCGTGCAATCTCGTGAAACACGTTCCGATTTGGCATCATTATAATATTTGATACA   1020
1021 TTCAATACTCTTAGTATAGGCGCTACTTCGGTAGTTACCTTGCTGTAAGCGGGACACTTG   1080
1081 TTTAAGTTATAGCGTTTAGGTTTTTTTCTTTGATGAGAAACAATACATGAAAGTATATGAA   1140
1141 TAATTTCTACAAACTACGATGACAATTAAAGGAAAAACATTAGCGCAATAACTGTTACGT   1200
1201 CCGTCGTTAATAATGCCTTTTGTGAAGTAGCTAAAATGAAGTAGGTATATTCTAAGTGTT   1260
1261 CACGCCTTATAGCATTTTAGTGTTTAGTACATTGGTTGCAAAGTACGAATAATTATTCCA   1320
1321 ATATGAATTTTGTCACTTGACTGTAAATATAGATAACTATATTTTTATATTATTATTGAT   1380
1381 AACAATTAAGTATTAATTAATAATTGTAAGTGATTGTTTCTCAATGTTACTAGCACATTC   1440
1441 ACGAAGAAAGTAATTGCAAAATTAGTTGTAAAAATATTGGTTTCACGTAGTATTGTTTTA   1500
1501 TTCATAGCCTTTTCGCCTCGCCGTATATGATAAATGTATGATTTTTTTTGTCAAA       1560
1561 TTATTGGATTCAAATAATGTTTTATTTAAATTTTCCGTACAGGTGTTGTTTCTAGAGAAT   1620
1621 CTCAACTACAAAAGCTGATTAAATCCGTTAATTTCAAATTAAAAGCCTGTAACATTGAGA   1680
1681 ACTGCAAATGATTTTTTACGTTGAATGAGATTATTTGTAATTTAAGATTTCCTGTAAATA   1740
1741 ATAGCTTAATTAATAGATTAAATTATTTTACGCCGGTAATTTTTCACTTTTCACATTGTA   1800
1801 ACTTCGACTTTGTAAAATCATTAGTTACATAGATTGAGGAAGGTTTGTAAAAGCATTTTA   1860
1861 ACGAAAAGAATGATAGGTTAAACTGAATTATTTGTTAAATTTAACAATATTTATTGATTC   1920
1921 ACAAACCGTGGAACTAGGAAATCTTGTTTATCGTTTGTTTTGTGATATATTATGATGTAT   1980
1981 AAGTTAAAATAGGGGTGCTAAAAGAATTTTTTTCTTTTCTAAAACAATTTGTGCAGAAAT   2040
2041 TCTCGAGTTGTAAAAATAGAGTTGTGTTGATTTTTAAATGAATGAATTATCGTTTGTAAC   2100
2101 ACACGAATTGTTAGTATTTCTTGTACGTAGCACTATCACAAAACACATTTGGTAAACTAA   2160
2161 ATAAAAT                                                       2167
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Fig. 1. The nucleotide sequence and deduced amino acid sequence of *Bm cad* are shown and numbered left to right. The amino acids are numbered starting with the putative initiating methionine residue. The homeobox region is indicated by underlining. The amino acid sequence in the upstream of homeodomain conserved between *Bombyx* and *Drosophila cad* is indicated with double underlines. The putative polyadenylation signal is shown by a wavy line. The position of an intron is shown by an arrowhead by the analogy with the *Drosophila* sequence.

which is conserved in the upstream of most homeobox genes (Fig. 1).

Southern genomic hybridization was carried out using a 250 bp cDNA fragment as a probe. As shown in Fig. 3, only one band was strongly detected for every digestion tested. From the simplicity of the Southern hybridization and the intensity of the band, we estimate that one copy of *Bm cad* gene is present in the haploid genome.

mRNA expression pattern during embryonic development

We have examined embryonic developmental expression

	1	11	21	31	41	51
Bombyx cad	KDKYRVVYSD	HQRLELEKEF	HYSRYITIRR	KAELAVSLGL	SERQVKIWFQ	NRRAKERKQV
Drosophila cad	--------T-	F--------Y	CT---------	-S---QT-S-	----------	-------TSN

Fig. 2. Comparison of the homeodomain of *Bombyx cad* with that of *Drosophila cad*. The identical residues are shown by a dash(−). Between the homeodomains of *Bobyxm cad* and *Drosophila cad*, 48 amino acids residues (80%) are identical.
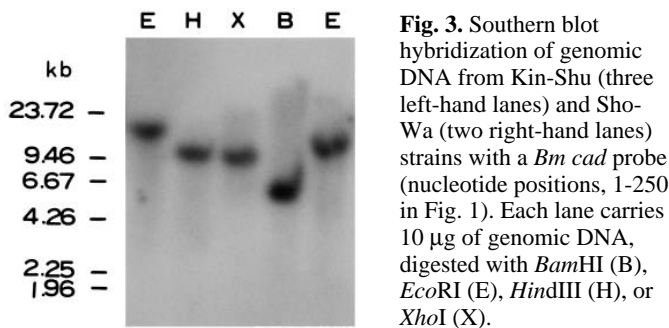

Fig. 3. Southern blot hybridization of genomic DNA from Kin-Shu (three left-hand lanes) and Sho-Wa (two right-hand lanes) strains with a *Bm cad* probe (nucleotide positions, 1-250 in Fig. 1). Each lane carries 10 μg of genomic DNA, digested with *Bam*HI (B), *Eco*RI (E), *Hin*dIII (H), or *Xho*I (X).

Fig. 4. Developmental expression of *Bm cad* gene. For each lane 5 μg of poly(A)$^+$ RNA was applied. Lane 1, oocytes; lane 2, unfertilized eggs; lanes 3 and 4 were eggs deposited in 30 minutes and 2 hours, respectively; lane 5, 24-25 hours; lanes 6-14, 2-10 day eggs, respectively. The 1.65 kb band was detected by *Bombyx* actin oligoprobe as a positive control.

pattern of *Bm cad* gene. Poly(A)$^+$ RNAs were prepared from eggs at various stages and subjected to northern blot hybridization. As shown in Fig. 4, a single band of 2.3 kb transcript was detected. During early embryonic development, the 2.3 kb transcript was most strongly expressed until 36 hours (lanes 3-5; data of 36 hours not shown here). The signal decreased precipitously by 2 days (lane 6) and remained weakly during the rest of embryogenesis stages (lanes 7-14). The experiments were repeated with different RNA samples and probes more than 5 times and the data were essentially reproducible (data not shown). Because of the strong signal observed both in oocytes and unfertilized eggs (lanes 1 and 2), as well as in eggs before pronuclear fusion (lanes 3 and 4), we conclude that *Bm cad* is a maternal gene.

Transcript localization during oogenesis and early embryogenesis

The ovary of *Bombyx* consists of 8 ovarioles. In the upper part of the ovariole, free stem cells generate cystoblasts, which divide into eight cells; seven of these become nurse cells and the eighth develops into an oocyte. The oocyte and the nurse cells are connected to each other via cytoplasmic bridges, which resulted from incomplete cytogenesis during cell division. This group of cells originating from the germ line is surrounded by somatic follicle cells, together constituting the egg chamber (King and Akai, 1971; Kawaguchi and Fujii, 1983; Kawaguchi et al., 1985; 1988).

Bm cad transcripts were first detected in the younger nurse cells in ovary of the 6-day pupa (Fig. 5A). The signal was also confirmed by northern hybridization results (data not shown). The transcripts were shifted from the more developed nurse cells into the oocyte (Fig. 5B). It is consistent with the previous observation that the oocyte is transcriptionally inactive throughout oogenesis while most materials are produced in the nurse cells and transferred into the oocyte during a definite period of oogenesis (Kawaguchi and Fujii, 1983; Kawaguchi et al., 1985; 1988). The signals appearing in follicle cells were due to non-specific hybridization since they were also detected
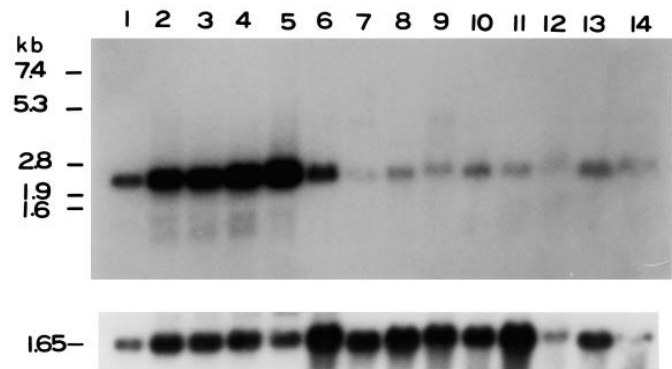
in the experiments using a sense strand probe (unpublished observation).

Generally, a newly laid *Bombyx* egg will finish pronuclear fusion in 120-140 minutes at 25°C. Following the fusion, the zygotic nucleus undergoes more than 10 cycles of syncytial nuclear divisions. During the same period, cytoplasm along with nucleus migrates into cortical layer in an anteroposterior direction. Around 12-13 hours after oviposition, the cellular blastoderm is formed. The ventral plate appears during 14-20 hours and the early germ-band formation is initiated. Later, gastrulation takes place and segments appear gradually between 24 and 48 hours after the egg laying. Unlike *Drosophila*, there are no visible oosome and pole cells observed in *Bombyx* during early embryogenesis (Ohtsuki, 1965; Ohtsuki and Murakami, 1968).

As shown in Fig. 5, in situ analysis of eggs during early embryogenesis revealed that the expression pattern of *Bm cad* gene was dramatically changed. Before the nuclear migration, *Bm cad* transcripts are homogeneously expressed in freshly laid eggs (data not shown). Then the transcripts in cytoplasm inside the egg comigrate with nucleus to the cortical cytoplasm. In *Bombyx*, such migration takes place anteriorly at first. Consequently, the signals become stronger in the anterior region than in the posterior region (Fig. 5C). At the end of migration, the transcripts are evenly distributed again while those left inside the egg are concentrated in the cytoplasm extruding from the yolk (data not shown). Continuously, cells aggregate together and form the ventral plate. At that time, *Bm cad* transcripts were detected more on the ventral side (more cells) than on the dorsal side (less cells; Fig. 5D). After gastrulation commenced, the difference in amounts of *Bm cad*

Fig. 5. Location of *Bm cad* transcripts during oogenesis and embryogenesis. In these longitudinal series sections, the anterior points always to the left and the dorsal up, except dorsoventral direction in A and B which we did not check in detail. (A) Follicles at an early stage; (B) follicles at a slightly later stage (both from the sixth day pupa and compared by measuring sizes). Eggs laid between (C) 10-11 hours; (D) 15-16 hours; (E) 20-21 hours; (F) 24-25 hours; (G) 30 hours; (H) 35 hours; (I) 2 days and (J) 4 days of embryo corresponding to shortened germ-band stage. fc, follicle cell; n, nurse cell; o, oocyte; ect, ectoderm; mes, mesoderm. The horizontal scale bars represent a length of 0.25 mm (A,C,D,I,J); 0.20 mm (B) and 0.16 mm (E-H), respectively.
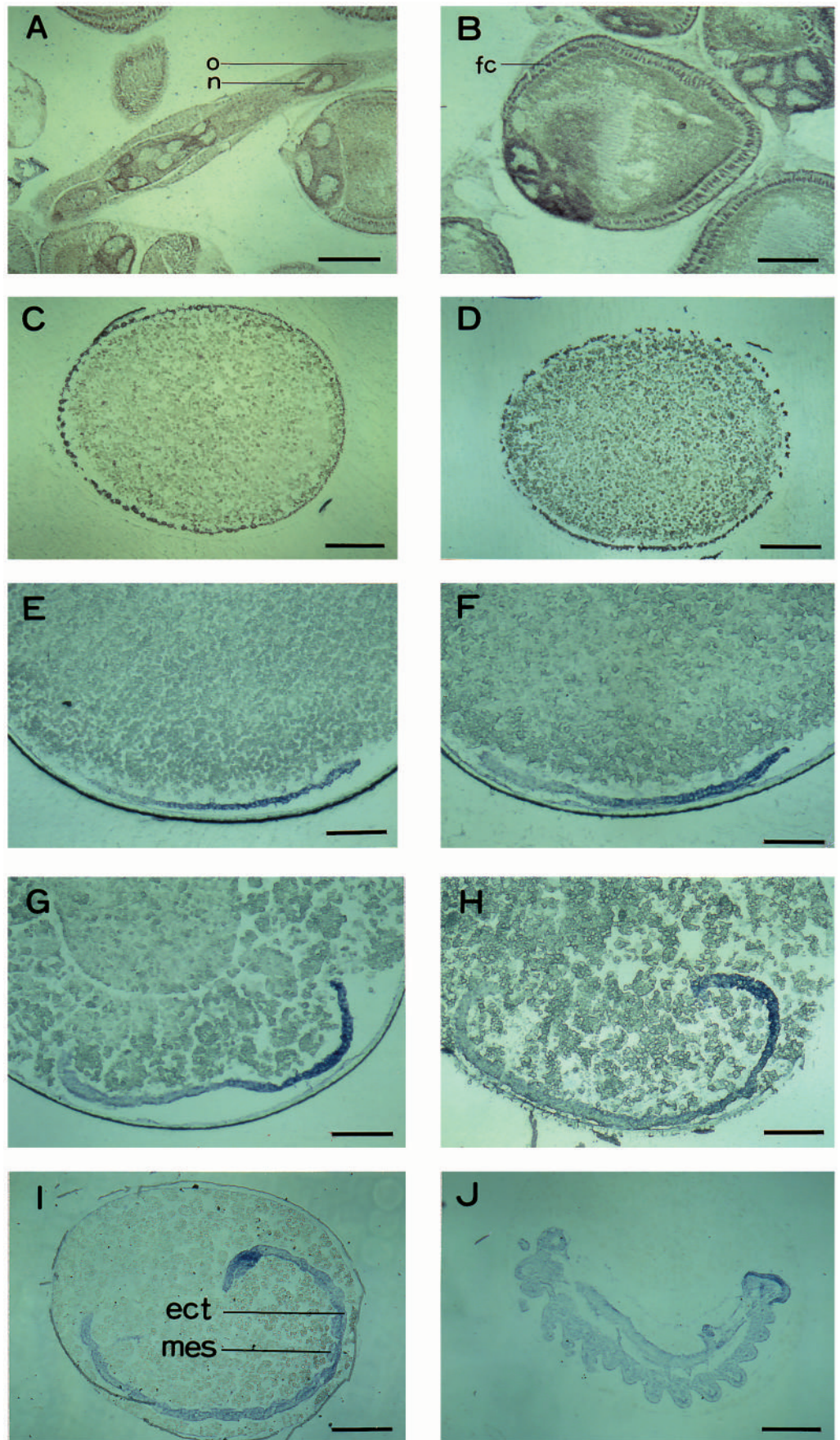
transcripts between the anterior and posterior poles became more marked. The more gastrulation proceeds, the steeper the concentration gradient spanning the anteroposterior axis appears (Figs 5E-H, 6A,B). Once the mesoderm became obvious, *Bm cad* transcripts were limited to 5-10% embryo length and later were restricted to anal pad, the most posterior region (Fig. 5I,J). Unlike the zygotic transcript of *Drosophila cad*, we can not detect strong signals distinguishable from background in the hindgut and posterior midgut rudiment (data not shown).

Bombyx cad protein distribution during early embryogenesis

In order to examine the Bm cad protein distribution during early embryogenesis and characterize the endogenous Bm cad protein, we produced Bm cad-specific antibodies. To prove the specificity of the polyclonal antiserum, we examined embryo extracts at several developmental stages by western blot analysis.

As indicated in Fig. 7, two types of antibodies specifically

A

B
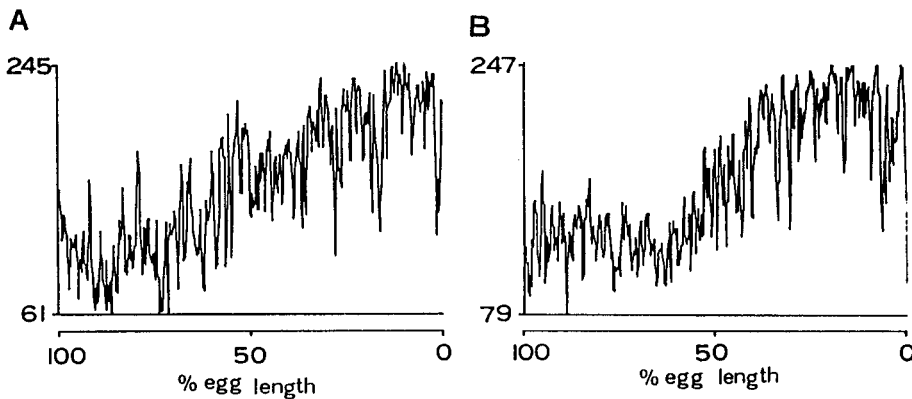
Fig. 6. Concentration gradients of *Bm cad* transcripts shifting along anteroposterior axis during gastrulation. Scanning (A) from Fig. 5F and (B) from Fig. 5G. Vertical axis: intensity of *Bm cad* transcripts, indicated with 0-255 pixels. In the horizontal axis, 0% represents the posterior and 100% the anterior pole of the embryo.

$M_r \times 10^{-3}$
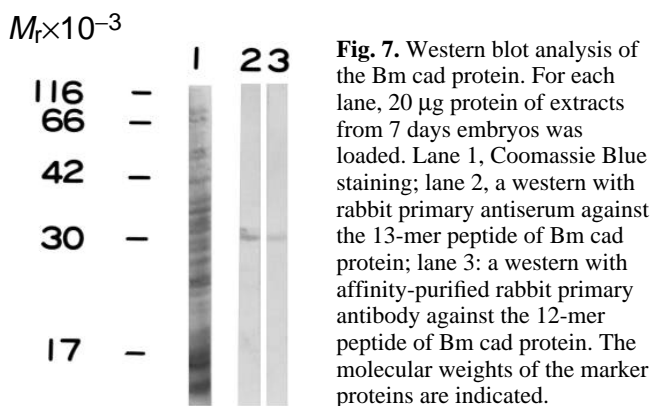
Fig. 7. Western blot analysis of the Bm cad protein. For each lane, 20 μg protein of extracts from 7 days embryos was loaded. Lane 1, Coomassie Blue staining; lane 2, a western with rabbit primary antiserum against the 13-mer peptide of Bm cad protein; lane 3: a western with affinity-purified rabbit primary antibody against the 12-mer peptide of Bm cad protein. The molecular weights of the marker proteins are indicated.

recognized a common band in the 7-day embryo extracts. (Similar results with embryonic extracts of 29 hours, 6, 8, 9, and 10 days were obtained, data not shown). These analyses revealed a band of 31×10^3 M_r, which is in good agreement with the predicted relative molecular mass of 28×10^3. The 3×10^3 difference in the size of the predicted and observed relative molecular mass of the Bm cad protein could be due to the higher proline content (9.80%) (See and Jackowski, 1989).

It was of particular interest to investigate the distribution of the Bm cad protein translated from maternal transcripts and to determine whether or not the protein product was also present in a concentration gradient corresponding the mRNA gradient shifting along the anteroposterior axis during gastrulation (Fig. 5E-H).

We could not detect any visible cad protein signal (data not shown) in the ovary of 6-day pupa or very young embryos about 9 hours after deposition. The Bm cad protein became evenly visible during cellular blastoderm stage (Fig. 8A), and then concentrated on the ventral side following the ventral plate formation (Fig. 8B). The first Bm cad protein concentration gradient was detected along the anteroposterior axis at early gastrulation stage, 20 hours after deposition (Fig. 8C). The protein gradient became sharper at the middle gastrulation stage of 35 hours (Fig. 8D). However, in the most posterior region, the signal was weaker (Fig. 8D). Later, Bm cad protein was limited to the posterior region and finally only at anal pad area (Fig. 8E,F).

DISCUSSION

Evolutionary conservation of the concentration gradient formation in *Bombyx* and *Drosophila*

In contrast to *Drosophila cad,* whose transcript and protein form concentration gradients during syncytial blastoderm stage, *Bm cad* produces noticeable mRNA and protein gradients along anteroposterior axis during gastrulation stage. It has been known that the gradient is important during the segmentation processes (Macdonald and Struhl, 1986; Mlodzik and Gehring, 1987; Driever and Nüsslein-Volhard, 1988). The coincidence between the segmentation process and the concentration gradient formation suggests that some similar signals released from genes upstream to *Bm cad* and passed through *Bm cad* to the downstream genes may share common identities with those of *Drosophila*.

The mechanisms both for the rapid degradation in early embryogenesis and maintenance of stable but weak signals of *Bm cad* transcript remain enigmatic. We assume it may involve a specific degradation of the transcripts at the anterior pole rather than a specific transport from the anterior to posterior, since there is no corresponding increase in the intensity at the posterior pole (scanning data not shown). Nevertheless, the mechanism delaying the concentration gradient formation from the syncytial blastoderm stage in *Drosophila* to the gastrulation stage in *Bombyx* seems rather intriguing. Moreover, the coincidence between *Bm cad* mRNA and protein concentration gradients may imply that *Bm cad* transcript concentration gradient is probably involved in Bm cad protein gradient production. However, further careful investigation and comparison in other insects is absolutely necessary. In addition, functional evidence should be directly obtained by transgenic assays, although we do not have such in vivo techniques in *Bombyx* yet.

Based on indirect evidence in the form of abnormal patterns resulting from embryological manipulations, Sander (1960) first suggested that the pterygote eggs contain anterior (A′) and posterior (P′) determinants in meroistic ovaries while only single posterior (P′) determinant in panoistic ovaries. Here we provide the first evidence of a direct molecular gradient in a non-*Drosophila* insect. Although the results from the in situ hybridization and immunohistochemistry experiments may be a little fragmented and incomplete, we believe the approach is an essential one and continuing such studies, assisted by molecular techniques, should give us a
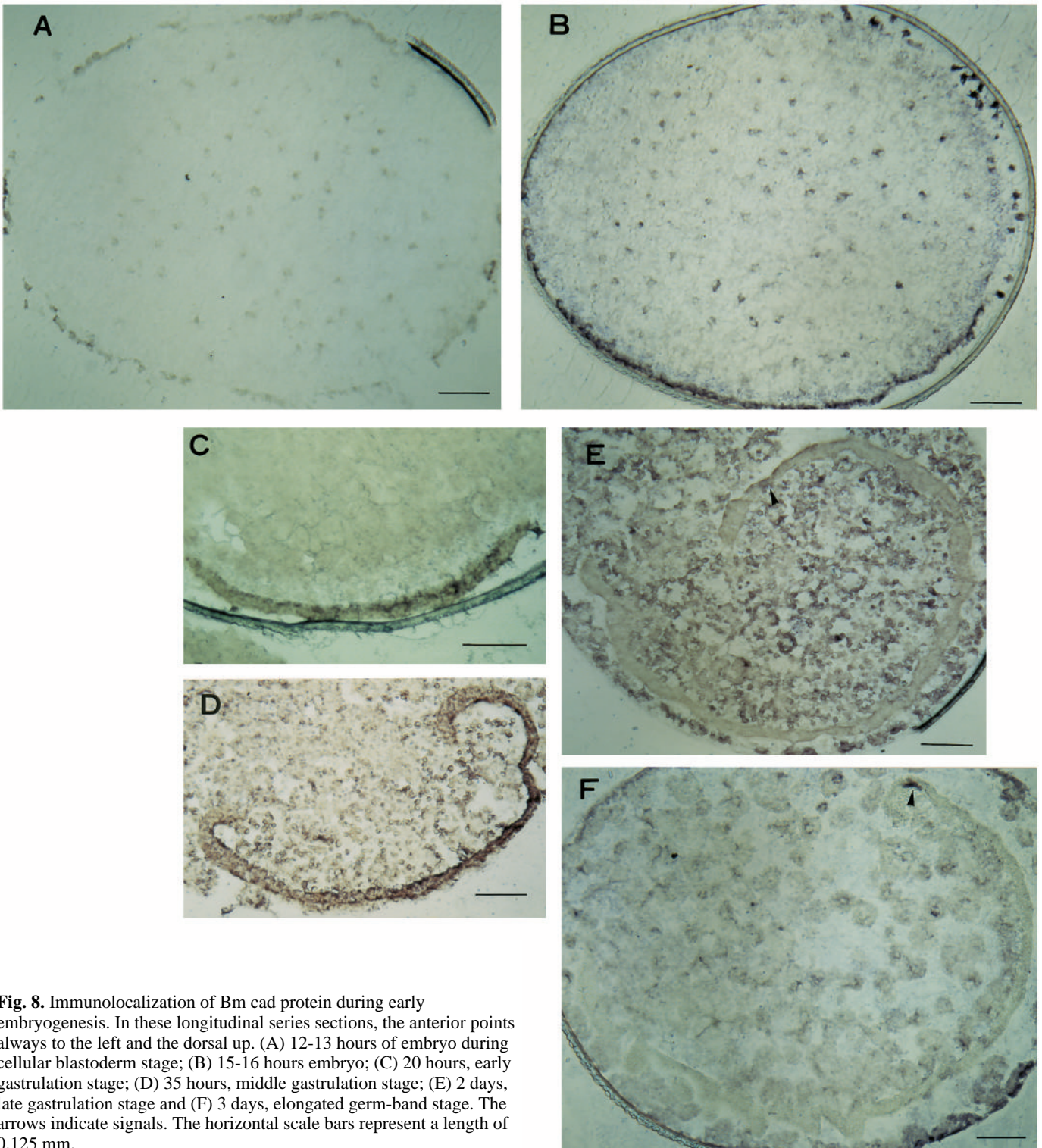
Fig. 8. Immunolocalization of Bm cad protein during early embryogenesis. In these longitudinal series sections, the anterior points always to the left and the dorsal up. (A) 12-13 hours of embryo during cellular blastoderm stage; (B) 15-16 hours embryo; (C) 20 hours, early gastrulation stage; (D) 35 hours, middle gastrulation stage; (E) 2 days, late gastrulation stage and (F) 3 days, elongated germ-band stage. The arrows indicate signals. The horizontal scale bars represent a length of 0.125 mm.

complete picture of specification of body plan for different types of insect.

Similar but distinct genetic hierarchy in *Bombyx* and *Drosophila*

It is particularly interesting to investigate how general are the mechanisms of spatial patterning of early insect embryos

during insect evolution process. The *Schistocerca* homologue of the *Drosophila* pair-rule gene *even-skipped* does not serve a pair-rule function in early development, although it does have a similar function during neurogenesis later in development (Patel et al., 1992). In contrast, the spatial relationships of the expression domains of *hairy* and *krüppel* as well as their relative locations with respect to the developing segments are

conserved between *Drosophila* and *Tribolium* (Sommer and Tautz, 1993).

Our analysis on the *Bm cad* revealed very similar but distinct features compared to *Drosophila*. In *Drosophila*, there are two kinds of *cad* transcripts expressed during different embryogenesis stages (Mlodzik et al., 1985). It was very surprising that only one kind of *Bm cad* maternal transcripts was detected during whole embryogenesis stage in *Bombyx*. The *Bm cad* may act as a gap gene during gastrulation stage and function as a homeotic selective gene during the rest of the embryogenesis. To determine whether it is a general or just an exceptional case that cad may function on two different orders in one genetic hierarchy, studies on the *cad* homologues in other types of insect should be undertaken.

During the *Bm cad* study, we have also cloned the homologues including one of *Gli* family gene (*ci*) (P.-X. Xu, unpublished data), *engrailed* (*en*), *invected* (*in*) (Hui et al., 1992), *Antennapedia* (*Antp*) (T. Nagata et al., unpublished data), and BX-C (Ueno et al., 1992). In situ hybridization results on *Antp* (X. Xu et al., unpublished data), *en* (K. Amanai et al., unpublished data) as well as *cad* shown here revealed patterns similar to but distinct from those in *Drosophila*. The rough conservation implies a similar but evidently distinct genetic hierarchy may exist in the body plan determination in *Bombyx*. Interestingly, segment formation in *Bombyx*, as observed by *Bm en* expression (K. Amanai et al., unpublished data), appears to follow directly the shift in *Bm cad* transcript and protein gradients during gastrulation (data not shown), while all the segmentation in *Drosophila* can be observed more-or-less synchronously only after finishing such shifting (Kornberg et al., 1985). We assume control of or by some intermediate genes like pair-rule or gap genes may have been slightly modified in *Bombyx*. Detailed comparison of such homologues with those in *Schistocerca*, *Tribolium* and *Drosophila* will prove fruitful.

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