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Molecular analysis of the deletion mutants in the E homeotic complex of the silkworm *Bombyx mori*

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Summary

The E loci in *Bombyx mori* are expected to contain a homeotic gene complex specifying the identities of the larval abdominal segments. However, the molecular structure of this complex remains to be determined. We have started to analyze the structural changes in the E complex mutations. We used three newly isolated *Bombyx* homeobox genes as probes. These genes are probably homologues of the *Ultrabithorax* (*Ubx*), *abdominal-A* (*abd-A*) and *Abdominal-B* (*Abd-B*) in the *Drosophila* bithorax complex, because the amino-acid sequences of the homeobox regions in these *Bombyx* genes are almost identical to those of *Drosophila* genes. We found that the *Bombyx Ubx* and *abd-A* genes are deleted in the E^N chromosome, and the *Bombyx abd-A* gene is deleted in the E^{Ca} chromosome. From these results, we conclude that the *Bombyx* E complex consists of the *Ubx*, *abd-A* and possibly *Abd-B* genes, which may play similar roles to their homologues in the *Drosophila* bithorax complex.

Key words: *Bombyx mori*, homeotic gene, the E complex, *Ultrabithorax*, *abdominal-A*, *Abdominal-B*.

Introduction

Genetical studies revealed that the identities of the body segments are determined by homeotic genes in many kinds of insects (Ouweneel, 1976). In the fruit fly *Drosophila melanogaster*, the red flour beetle *Tribolium castaneum* (Beeman, 1987) and the silkworm *Bombyx mori* (Hashimoto, 1941), homeotic genes are clustered in homeotic gene complexes. In *Drosophila*, the Antennapedia complex (ANT-C) (Wakimoto and Kaufman, 1981) and the bithorax complex (BX-C) (Lewis, 1978), which are located on the right arm of the third chromosome, specify the identities of the body segments or parasegments. ANT-C determines identities of the head to the middle thoracic segment and BX-C determines identities of the posterior thoracic segment and all the abdominal segments. Molecular studies of the structure of ANT-C and BX-C revealed that these gene complexes consist of homeobox genes (Gehring and Hiromi, 1986). In BX-C, three homeobox genes, *Ubx*, *abd-A* and *Abd-B* are involved in the determination of the abdominal segments in *Drosophila* (Duncan, 1987). In *Tribolium*, similar homeotic genes are known to determine the identities of the body segments. Beeman demonstrated that six loci of homeotic genes are clustered in the second linkage group of *Tribolium* (Beeman, 1987) and these loci include elements with homology to the homeotic genes in the ANT-C and BX-C in *Drosophila*.

The E loci in *B. mori* contain homeotic genes specifying the identities of the larval abdominal segments (Hashimoto, 1941; Tazima, 1964). This homeotic gene complex is thought to be located on the 0.0 locus of the sixth chromosome linkage group in *Bombyx*, and over thirty types of mutations were found and analyzed for pseudoallelism. All of these mutations are dominant and induce expression of the extra markings or the supernumerary legs in the abdominal segments. Most of the mutations in the E complex are lethal in the homozygous condition.

The E complex reveals one interesting aspect different from the homeotic gene complexes of *Drosophila*. Most of the E mutations cause a particular directional shift, anterior to posterior or *vice versa*, on both the dorsal and ventral sides of larvae. However, some E mutations cause shifts in one direction on the dorsal side and in the opposite direction on the ventral side (Itikawa, 1943; Tazima, 1964). Since such an independent determination on the dorsal side and ventral side in larvae has not been observed in *Drosophila*, there must be differences in the regulatory mechanisms that specify abdominal segments between *Bombyx* and *Drosophila*.

To understand how the E complex specifies the identities of body segments on the dorsal side and ventral side of larvae independently, it is necessary to know the molecular structure of the E complex. Since some mutations in the E complex are associated with phenotypes similar to those of the mutations in the bithorax complex, we assumed that the E complex might be analogous to the *Drosophila* bithorax complex. In this report, we describe the isolation of three *Bombyx* genomic fragments that are probably homologues of the *Ubx*, *abd-A* and *Abd-B* in *Drosophila*. Analyses with the two types of mutant chromosomes, E^N and E^{Ca}, showed that the *Bombyx Ubx* and *abd-A* genes are deleted in the E^N chromosome, and the *Bombyx abd-A* gene is deleted in the E^{Ca} chromosome. These results suggest the E complex consists of the homeobox genes and is similar to the bithorax complex in *Drosophila*.

Materials and methods

Animals

Bombyx eggs and larvae of mutant strains f12 carrying E^N chromosome and f21 carrying E^{Ca} chromosome were gifts from Dr H. Doira, Institute of Genetic Resources, Kyushu University. Embryos were developed at 25°C for about 5 days to stages 22 or 25 (Takami and Kitazawa, 1960) and dissected out from eggs under a binocular microscope. Larvae of a commercial strain of *B. mori* (Kin-Shu × Sho-Wa from the Kanebo Silk Co., Kasugai City, Japan) were reared at 27°C on an artificial diet from Kyodo Shiryo Co. (Yokohama, Japan).

Cloning of the Bombyx Ubx, abd-A *and* Abd-B *genes*

Isolation of *Bombyx Ubx* gene was performed following the PCR amplification method as described (Kamb et al., 1989). DNA fragments containing homeobox regions were amplified from genomic DNA with fully degenerated primers that correspond to two consensus amino-acid sequences, 5′ELEK-EFH3′ and 5′IKIWFQN3′, in the homeodomain. The reaction mixture of the PCR amplification was incubated in a Perkin Elmer Cetus thermocycler for 25 cycles; 1 min at 94°C, 2 min at 37°C, and 3 min at 55°C (Saiki et al., 1985). After amplification, the DNA fragments were subcloned into a plasmid vector pGEM3Zf(+). Clones containing *Bombyx Ubx* were identified by sequencing. Clones carrying longer genomic fragments were isolated from a genomic library with a probe that was amplified from the subclone. The *Bombyx* genomic library (2 × 10^5 clones) was constructed by size fractionating a *Mbo*I partial digest of posterior silk gland DNA from *Bombyx* (the Kanebo strain, Kin-Shu × Sho-Wa) and ligating the 30-45 kb fraction into the *Bam*HI site of pWE15 cosmid vector (Stratagene Co.) (Evans and Wahl, 1987). Hybridization was performed at 65°C for 2 hours using Amersham Rapid Hybridization kit. After blots were washed in 2 × SSC containing 0.1% SDS (sodium dodecyl sulfate) at 65°C for 3 hours, positive clones were detected by autoradiography. Since the screening probe hybridized to a 7.0 kb *Eco*RI fragment, this fragment was subcloned into plasmid vector and sequenced with fully degenerated primers that correspond to two consensus amino-acid sequences in homeodomain as described above.

For the isolation of *Bombyx abd-A* and *Abd-B* genes, the *Bombyx* genomic cosmid library was screened by cross-hybridization with the homeobox regions of the *abd-A* and *Abd-B* genes of *Drosophila*. The homeobox region of *Drosophila abd-A* was amplified from *abd-A* genomic DNA p53 with two primers, 5′CCCCCCCAACGGCTGTCCACGAAG3′ and 5′CTCACCTGTTCATTTATTTCCTTG3′ (Regulski et al., 1985). *Drosophila Abd-B* probe was amplified from *Abd-B* cDNA E61 by PCR with two primers 5′CAGGTGTCCGTCCGGAAAAAGCGC3′ and 5′GTTCAGGTGGTGGCCGCTGTGGTG3′ (Zavortink and Sakonju, 1989). The hybridization conditions were the same as described above. The *Drosophila abd-A* probe hybridized to a 2.5 kb *Hin*dIII fragment, and the *Drosophila Abd-B* probe hybridized to a 5.0 kb *Hin*dIII fragment. Therefore these fragments were subcloned and sequenced as described above.

Extraction of DNA from the posterior silk glands of the fifth instar larvae and whole embryos

The heterozygous $E^N/+$ and $E^{Ca}/+$ DNAs and the wild-type +/+ DNA for Southern blot analysis were extracted from the posterior silk glands of the fifth instar larvae by the method described previously (Gross-Bellard et al., 1973). We started DNA extraction from two pairs of the posterior silk glands which were stored at −80°C. Posterior silk glands were ground under liquid nitrogen and suspended in 500 μl of digestion buffer (100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 25 mM EDTA (pH 8.0), 10% SDS and 0.1 mg/ml Proteinase K) and incubated at 50°C for 12 hours. DNAs were extracted with phenol-chloroform-isoamylalchol (25:24:1). After 1/2 volume of 7.5 M ammonium acetate and 2 volumes of ethanol were added to DNA solution, long-size DNAs were wound up with a pipette tip and dissolved in TE buffer (10 mM Tris-HCl (pH 8.0) and 1 mM EDTA). Quantities of DNA were estimated by diphenylamine reaction (Burton, 1956).

For PCR analysis, DNAs were prepared from the homozygous E^N/E^N and E^{Ca}/E^{Ca} embryos and the wild-type embryos by the method described (Jowett, 1986). After the embryos were dissected under a binocular microscope in 1 × SSC at room temperature, mutant and wild-type embryos were washed in 1 × SSC twice and stored at −80°C. Since embryos with the +/+ genotype or $E^N/+$ or $E^{Ca}/+$ genotype cannot be distinguished, we extracted DNA from individual embryos of apparent wild-type phenotype (either $E^N/+$ or +/+, $E^{Ca}/+$ or +/+) and apparent E^N/E^N or E^{Ca}/E^{Ca} phenotype. Each embryo was suspended in 25 μl of a buffer containing 10 mM Tris-HCl (pH 7.5), 60 mM NaCl, 50 mM EDTA, 0.15 mM spermine and 0.15 mM spermidine, and ground with a pipette tip. After 25 μl of a solution containing 1.25% SDS, 0.3 M Tris-HCl (pH 9.0), 0.1 mM EDTA, 5% sucrose, and 0.75% freshly added diethylpyrocarbonate were added, solutions were incubated for 40 minutes at 60°C. SDS and protein were precipitated by centrifugation after addition of 30 μl of 8 M potassium acetate and cooling for 45 minutes on ice. DNAs were precipitated by adding 2 volumes of ethanol and dissolved in 25 μl of TE buffer.

Southern blot analysis of heterozygous mutant DNA

After each 3 μg of DNA extracted from the $E^N/+$ or $E^{Ca}/+$ and +/+ larvae were digested with *Eco*RI, Southern blot analyses were performed sequentially with randomly primed specific DNA probes which are located 3′ to each homeodomain. A 296-bp DNA probe was prepared for the analysis of *Bombyx Ubx* by PCR amplification from cloned *Bombyx Ubx* DNA with two primers, 5′TGAACGAGCAGGAGAAACAGGCGC3′ and 5′TCCAACAGTTTATTGTTCGTATCA3′. A 341-bp DNA was amplified for the analysis of the *Bombyx abd-A* gene with two primers 5′AACAGGCTCGCCGCGAAAGAGAGG3′ and 5′CCTTCACAACAAGACGACACTTCC3′. A 287-bp DNA was amplified for the analysis of the *Bombyx Abd-B* gene with two primers 5′AACAACAATTCAAATGCGAACAAC3′ and 5′GCATACAACGTTCTCCGGTCAATT3′. Hybridization was performed at 65°C for 2 hours with Amersham Rapid

Hybridization kit and blots were washed in 0.3 × SSC buffer containing 0.1% SDS at 65°C. Hybridization patterns were analyzed and extents of hybridization were estimated with a Bio-Image Analyzer (BAS 2000, Fuji Photo Film Co., LTD).

PCR analysis of homozygous mutant DNA

DNAs extracted from individual embryos were subjected to 25 cycles of amplification by PCR, at an annealing temperature of 45°C, using the mixed primers used in the preparation of the probes just 3′ to the homeodomains of the *Bombyx Ubx*, *Bombyx abd-A* and *Bombyx Abd-B* genes as described above. After amplified products were separated by electrophoresis on a 2% agarose gel and transferred to membrane filter by Vacuum Blot (LKB Co.), blots were used for re-hybridization with the probes for the *Bombyx Ubx*, *Bombyx abd-A* and *Bombyx Abd-B* genes. Hybridization was performed at 65°C for 2 hours and blots were washed in 2 × SSC containing 0.1% SDS for 30 minutes.

Results

The phenotypes of E^N *and* E^{Ca} *mutations*

Itikawa (1943) reported that embryos homozygous for the E^N (new additional crescent) mutation express many thoracic-type appendages in would-be abdominal segments and die at the late embryonic stages. As shown in Fig. 1B, homozygous E^N/E^N embryos express thoracic-type legs from the first to the seventh abdominal segments (A1 to A7) and intermediate thoracic/abdominal-type legs in the A8 segment. Itikawa (1943) also reported that the nerve commissures and the tracheae in the first to the eighth abdominal segments in the embryos homozygous for E^N show the patterns similar to those in the thoracic segments of normal embryos. From these observations, Itikawa proposed that the first to the seventh or eighth abdominal segments in homozygous E^N/E^N embryos are transformed to the thoracic-type segments.

In *Drosophila*, the bithorax complex consists of three homeobox genes, the *Ubx*, *abd-A* and *Abd-B*. Lewis reported that the functional deficiency of the *Ubx* and *abd-A* ($DfUbx^{109}$) causes the transformation of most abdominal segments to the thoracic-type segments in *Drosophila* (Lewis, 1978). From the similarity of the phenotype between the homozygous E^N embryo and $DfUbx^{109}$ embryo, we assumed that the E complex has a similar structure and function to the bithorax complex in *D. melanogaster*.

Itikawa (1943) also reported that the embryos homologous for E^{Ca} (additional crescent) reveal three pairs of normal thoracic legs in the thoracic segments, but no abdominal legs in the abdominal segments. The abnormality of the homozygous E^{Ca} embryos is shown in Fig. 1C. We speculated that the A3 to A6 segments, which normally reveal abdominal legs, might have transformed to other abdominal segments that have no leg. Which abdominal segment repeats at the position of the A3 to A6 segments in the embryos homozygous for E^{Ca} was not determined.

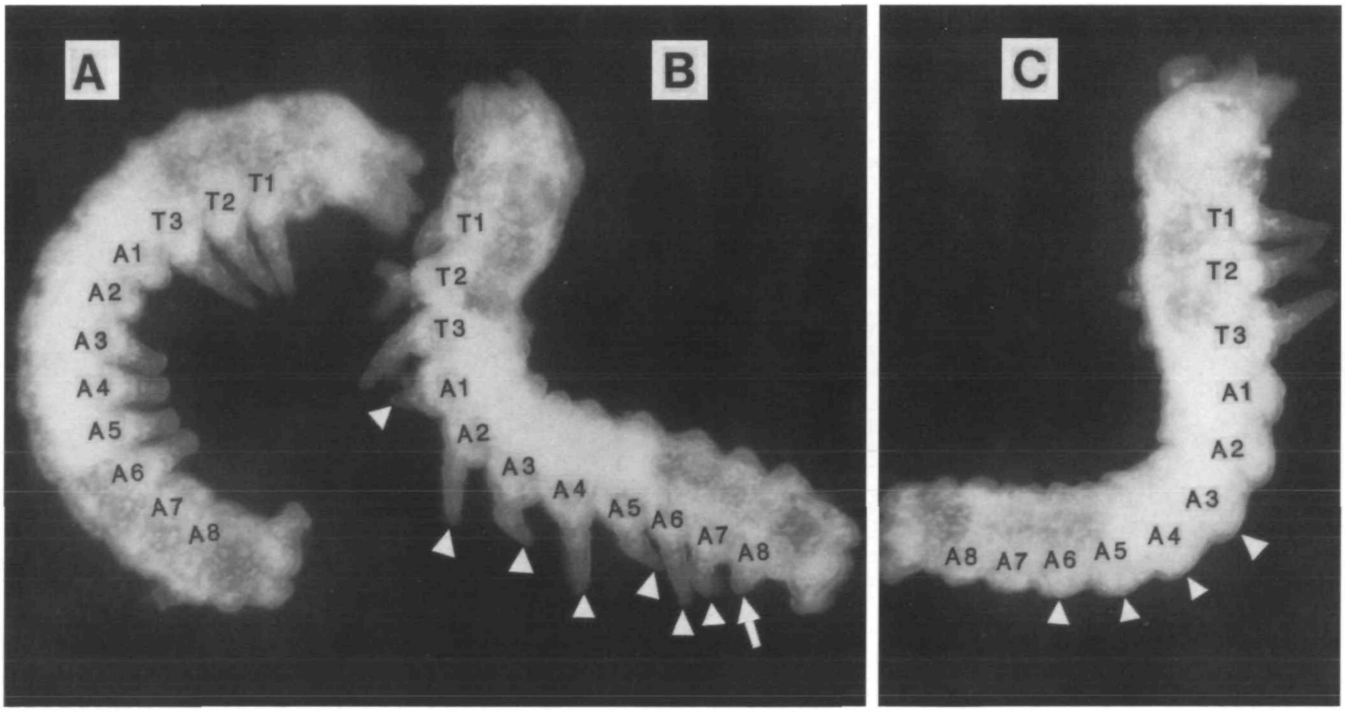

Fig. 1. Phenotypes of *Bombyx mori* (A) wild-type, (B) homozygous E^N/E^N and (C) homozygous E^{Ca}/E^{Ca} embryos. The wild-type embryo has three pairs of thoracic legs from the first to the third thoracic segments (T1 to T3) and four pairs of abdominal legs from A3 to A6 segments. The embryo homozygous for E^N expresses thoracic-type legs from A1 to A7 segments (arrowheads) and intermediate thoracic/abdominal-type legs on A8 segment (arrow). The homozygous E^{Ca} embryo does not express any abdominal legs (arrowheads). Embryos were approximately 2 mm long.

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                10        20        30        40        50        60
                                AlaAsnGlyLeuArgArgArgGlyArgGlnThrTyr
Bm    ATCGTTGCTTTTATTGTTACAGGAGCGAACGGCCTCAGGAGACGAGGAAGACAAACCTAC
                            :  : :: :: ::  : ::::: ::  :::: :: :::
Dm    TCACTTCTACCAGACTGGCTAGGTACAAATGGTCTGCGAAGACGCGGCCGACAGACATAC
                            ↑   ThrAsnGlyLeuArgArgArgGlyArgGlnThrTyr
                            |
                70        80        90       100       110       120
      ThrArgTyrGlnThrLeuGluLeuGluLysGluPheHisThrAsnHisTyrLeuThrArg
Bm    ACTAGATATCAAACGCTAGAATTAGAGAAAGAGTTCCACACGAACCACTACCTTACGCGA
      ::  : :: :: ::::: ::  : ::::: :::::::::::::: :: :: :: :: ::
Dm    ACCCGCTACCAGACGCTCGAGCTGGAGAAGGAGTTCCACACGAATCATTATCTGACCCGC
      ThrArgTyrGlnThrLeuGluLeuGluLysGluPheHisThrAsnHisTyrLeuThrArg

               130       140       150       160       170       180
      ArgArgArgIleGluMetAlaHisAlaLeuCysLeuThrGluArgGlnIleLysIleTrp
Bm    AGGAGACGCATAGAGATGGCGCACGCGTTGTGCCTCACGGAGAGGCAAATCAAAATATGG
      ::  :  : :: :::::: :::::::: : ::::: :::::: :::: ::::: :: :::
Dm    AGACGGAGAATCGAGATGGCGCACGCGCTATGCCTGACGGAGCGGCAGATCAAGATCTGG
      ArgArgArgIleGluMetAlaHisAlaLeuCysLeuThrGluArgGlnIleLysIleTrp

               190       200       210       220       230       240
      PheGlnAsnArgArgMetLysLeuLysLysGluIleGlnAlaIleLysGluLeuAsnGlu
Bm    TTCCAGAACCGAAGGATGAAGTTAAAGAAAGAGATCCAGGCTATAAAGGAGTTGAACGAG
      :::::::::::  : :::::: : ::::: ::::::::::: :: :::::: :::::::
Dm    TTCCAGAACCGGCGAATGAAGCTGAAGAAGGAGATCCAGGCGATCAAGGAGCTGAACGAA
      PheGlnAsnArgArgMetLysLeuLysLysGluIleGlnAlaIleLysGluLeuAsnGlu

               250       260       270       280       290
      GlnGluLysGlnAlaGlnArgGluLysAlaAspAlaCysSerArgAlaGlySerCys
Bm    CAGGAGAAACAGGCGCAGCGCGAGAAGGCCGACGCGTGCTCGCGGGCCGGCTCGTGC
      :::::::: :::::::::  : ::::::: :  :::    ::   :: :   ::
Dm    CAGGAGAAGCAGGCGCAGGCCCAGAAGGCGGCGGCGGCAGCGGCTGCGGCGGCGGCG
      GlnGluLysGlnAlaGlnAlaGlnLysAlaAlaAlaAlaAlaAlaAlaAlaAlaAla
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Fig. 2. Comparison of *Bombyx Ubx* genomic sequences (upper, Bm) with cDNA sequences of *Drosophila Ubx* (lower, Dm). Sequences encoded by the *Ubx* from *Drosophila* are from the reference by Kornfeld et al. (1989). Positions of nucleic acid identity and amino acid identity are marked by colons and underlines, respectively. The homeoboxes are typed in italic character and the position of an intron in the *Drosophila* sequence is indicated by an arrow. The sequence has been entered in the EMBL database, accession no. X62618.

Isolation of Bombyx *homologues of* Drosophila Ubx, abd-A *and* Abd-B

To determine whether the E complex consists of homeobox genes similar to *Drosophila Ubx*, *abd-A* and *Abd-B*, we screened the *Bombyx* genome to isolate the homologues. First we performed PCR amplification to isolate of *Bombyx* homeobox genes using the degenerated oligonucleotides primers corresponding to the consensus amino-acid sequences in homeodomain (see Materials and Methods). With this method, we expected to obtain homologues of *Drosophila Ubx* and *abd-A* genes, because *Ubx* and *abd-A* genes contain the consensus amino-acid sequences. Although we sequenced many clones prepared by subcloning of the amplified DNA, we could obtain only the gene that seems to be a homologue of the *Drosophila Ubx* gene. Using the 112 bp DNA fragment amplified from the subclone, we screened the *Bombyx* genomic library and isolated longer DNA fragments which contain the corresponding homeobox region.

Fig. 2 shows the comparison of the nucleotide and amino-acid sequences between genomic fragment of *Bombyx Ubx* and cDNA of *Drosophila Ubx*. The nucleotide sequences of the homeobox region and the flanking regions of *Bombyx Ubx* (nucleotide position 23 to 276) were 77% homologous to those of *Drosophila Ubx*. The 80 amino acids for the homeodomain and the flanking regions encoded by both *Ubx* genes were identical. The *Drosophila Ubx* gene contains an intron just 5′ to the homeobox as indicated in Fig. 2 (Kornfeld et al., 1989). Since the putative amino acid sequences encoded by the *Bombyx Ubx* following the corresponding position were homologous to those of *Drosophila Ubx*, the *Bombyx Ubx* gene might also contain an intron at the same position.

To isolate of *Bombyx* homologues of *abd-A* and *Abd-B* genes, the genomic library was screened by cross-hybridization with the homeobox regions of the *abd-A* and *Abd-B* genes of *Drosophila*. Figs 3 and 4 show the comparison of the nucleotide and amino-acid sequences between *Bombyx* genes and *Drosophila* cDNAs. The nucleotide sequences of the homeobox and flanking regions of the *Bombyx abd-A* (nucleotide position 13 to 276) were 77% homologous to those of the *Drosophila abd-A* gene. The 86 amino acids encoded by the *Bombyx abd-A* gene were identical to those of *Drosophila abd-A* cDNA (Karch et al., 1990). A homologue of *abd-A* was isolated from the locust *Schistocerca gregaria* and the amino-acid sequences including the homeodomain and its flanking regions were found identical to the corresponding regions of *Drosophila abd-A* (Tear et al., 1990). Although the *Drosophila abd-A* gene has a short intron at the position just 3′ to the homeobox as indicated in Fig. 3, this intron was absent in the *abd-A* homologue from *Schistocerca*. Since the putative amino-acid sequences in the 3′ flanking region of the *Bombyx abd-A* gene were also identical to the sequences of *Drosophila abd-*

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            10        20        30        40        50        60
                  ProAsnGlyCysProArgArgArgGlyArgGlnThrTyrThrArgPhe
Bm     TTGTTTCCAGGTCCAAACGGATGTCCGCGAAGGCGAGGACGACAAACTTACACTAGGTTC
                   :: ::::: ::::: :::::::: :: :: :: :: ::: :: : :::
Dm     GATTTCAACGGCCCCAACGGCTGTCCACGAAGGCGCGGTCGCCAGACCTACACTCGCTTC
                  ProAsnGlyCysProArgArgArgGlyArgGlnThrTyrThrArgPhe

            70        80        90       100       110       120
       GlnThrLeuGluLeuGluLysGluPheHisPheAsnHisTyrLeuThrArgArgArgArg
Bm     CAGACTCTAGAACTAGAAAAGGAATTCCACTTCAATCATTATCTGACGCGCCGCCGCAGG
       ::::: :: ::::: :: ::::: ::::::::::: :: ::  : :: :: ::  :  :
Dm     CAGACCCTCGAACTGGAGAAGGAGTTTCACTTCAACCACTACTTAACTCGGCGAAGGCGC
       GlnThrLeuGluLeuGluLysGluPheHisPheAsnHisTyrLeuThrArgArgArgArg

           130       140       150       160       170       180
       IleGluIleAlaHisAlaLeuCysLeuThrGluArgGlnIleLysIleTrpPheGlnAsn
Bm     ATAGAAATAGCACACGCGCTATGCCTCACCGAGAGACAGATCAAAATATGGTTTCAAAAT
       :: :: :: ::::: :: :: ::::: :::::: ::::::::: :: :::::::: ::
Dm     ATCGAGATCGCACATGCCCTCTGCCTGACCGAGCGACAGATCAAGATCTGGTTTCAGAAC
       IleGluIleAlaHisAlaLeuCysLeuThrGluArgGlnIleLysIleTrpPheGlnAsn

           190       200       210       220       230       240
       ArgArgMetLysLeuLysLysGluLeuArgAlaValLysGluIleAsnGluGlnAlaArg
Bm     CGACGCATGAAACTAAAGAAAGAACTCCGAGCCGTGAAAGAGATAAACGAACAGGCTCGC
       :: :::::::: :: ::::: ::  : :::::::: :: :: ::::: :::::::: ::
Dm     CGTCGCATGAAGCTGAAGAAGGAGTTACGAGCCGTCAAGGAAATAAATGAACAGGCGCGA
       ArgArgMetLysLeuLysLysGluLeuArgAlaValLysGluIleAsnGluGlnAlaArg

           250       260       270       280       290       300
       ArgGluArgGluGluGlnAspArgMetLysGlnGlnGlnGlnGluLysGlnAlaLysLeu
Bm     CGCGAAAGAGAGGAACAGGACAGAATGAAGCAGCAGCAACAAGAGAAGCAGGCCAAGCTG
       :::::  ::::::: ::::: : :::::::    ::: :      :::    ::: ::: :
Dm     CGCGATCGAGAGGAGCAGGAGAAAATGAAGGCCCAGGAGACGATGAAATCCGCCCAGCAG
       ArgAspArgGluGluGlnGluLysMetLysAlaGlnGluThrMetLysSerAlaGlnGln
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Fig. 3. Comparison of *Bombyx abd-A* genomic sequences (upper, Bm) with cDNA sequences of *Drosophila abd-A* (lower, Dm). Sequences encoded by the *abd-A* gene from *Drosophila* are from the reference by Karch et al. Markings are the same as in Fig. 2. The sequence has been entered in the EMBL database, accession no. X62620.

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            10        20        30        40        50        60
       SerSerAsnProLeuAspTrpThrGlyGlnValThrValArgLysLysArgLysProTyr
Bm     TCCTCGAACCCTCTGGATTGGACTGGTCAAGTGACGGTGCGCAAAAAACGCAAACCATAC
             :     :  :  :: ::::: ::::: ::: : :: :: ::::::::::: :: :::
Dm     AATCCCGGACTGCACGAGTGGACCGGTCAGGTGTCCGTCCGGAAAAAGCGCAAGCCGTAC
       AsnProGlyLeuHisGluTrpThrGlyGlnValSerValArgLysLysArgLysProTyr

            70        80        90       100       110       120
       SerLysPheGlnThrLeuGluLeuGluLysGluPheLeuPheAsnAlaTyrValSerLys
Bm     TCGAAATTTCAAACTCTCGAACTGGAAAAGGAATTCCTTTTCAACGCGTATGTCTCCAAA
       :: :: :: :: :: :: :: ::::: ::::: :: :::::::: ::::::::  ::::
Dm     TCCAAGTTCCAGACCCTGGAGCTGGAGAAGGAGTTTCTTTTCAATGCGTATGTTTCCAAG
       SerLysPheGlnThrLeuGluLeuGluLysGluPheLeuPheAsnAlaTyrValSerLys

           130       140       150       160       170       180
       GlnLysArgTrpGluLeuAlaArgAsnLeuAsnLeuThrGluArgGlnValLysIleTrp
Bm     CAAAAGAGATGGGAGCTCGCGAGAAATCTGAATCTAACCGAGAGACAAGTGAAGATATGG
       :::::: : :::::  : :: :::::: :: : :: :::::: :::: :: :::::::::
Dm     CAAAAGCGTTGGGAATTGGCCAGAAATTTGCAGCTGACCGAGCGACAGGTCAAGATATGG
       GlnLysArgTrpGluLeuAlaArgAsnLeuGlnLeuThrGluArgGlnValLysIleTrp

           190       200       210       220       230       240
       PheGlnAsnArgArgMetLysAsnLysLysAsnSerGlnArgGlnAlaAlaGlnAlaAla
Bm     TTCCAGAATAGACGAATGAAAAATAAGAAGAACTCACAGCGACAGGCGGCGCAAGCTGCC
       ::::::::: : :: ::::: :: :::::::::::::::: :::::      ::      :
Dm     TTCCAGAATCGGCGCATGAAGAACAAGAAGAACTCACAGCGCCAGGCCAATCAGCAGAAC
       PheGlnAsnArgArgMetLysAsnLysLysAsnSerGlnArgGlnAlaAsnGlnGlnAsn
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Fig. 4. Comparison of *Bombyx Abd-B* genomic sequences (upper, Bm) with cDNA sequences of *Drosophila Abd-B* (lower, Dm). Sequences encoded by the *Abd-B* gene from *Drosophila* are from the reference by Zavortink and Sakonju. Makings are the same as in Fig. 2. The sequence has been entered in the EMBL database, accession no. X62619.

A cDNA, the *Bombyx abd-A* gene probably does not have an intron at the 3′ flanking region of the homeobox.

The homology of the nucleotide sequences between *Bombyx Abd-B* gene (nucleotide position 19 to 234) and *Drosophila Abd-B* cDNA was as high as those of the *abd-A* and *Ubx* genes (79% homologous). In the homeodomains of both *Abd-B* genes, only one amino acid was different, but the asparagine residue in *Bombyx* has a similar property to the glutamine residue

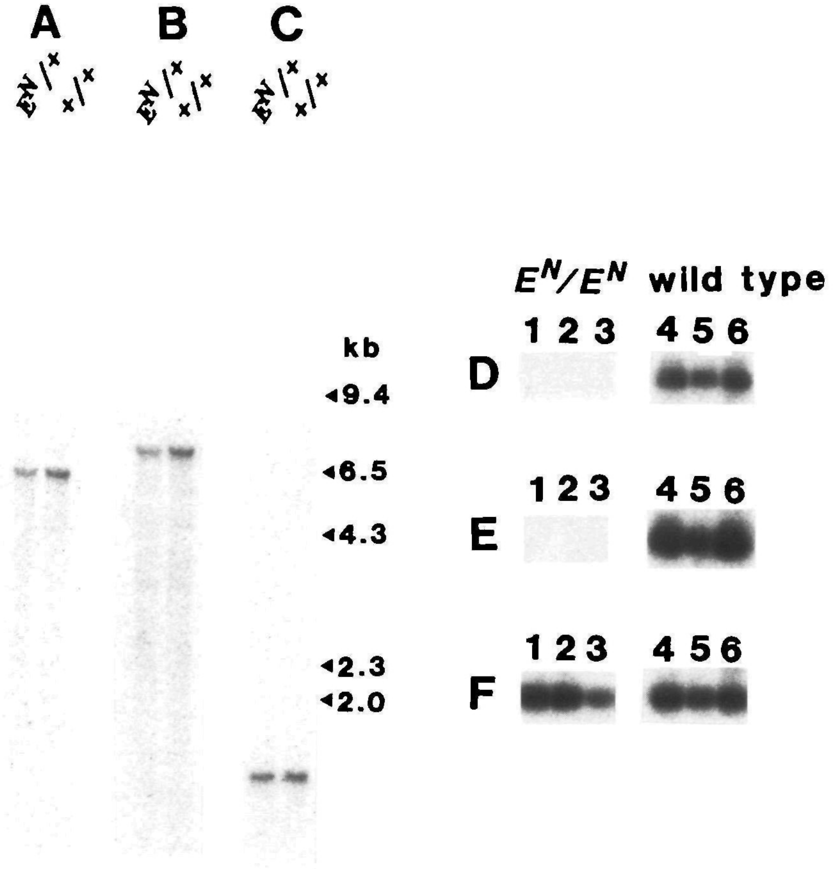

Fig. 5. Analyses on the heterozygous and homozygous E^N chromosomes. (A-C) Southern blot analysis of the heterozygous E^N chromosome (E^N/+) and wild-type chromosome (+/+) with probe fragments just 3′ to the homeodomains of (A) *Bm Ubx*, (B) *Bm abd-A* and (C) *Bm Abd-B* genes. (D-F) PCR analysis of *Bombyx* homologues on the homozygous E^N/E^N embryos and wild-type embryos. DNA fragments just 3′ to the homeodomains of (D) *Bm Ubx*, (E) *Bm abd-A* and (F) *Bm Abd-B* genes were amplified from DNAs from three individual homozygous embryos (lanes 1-3) and from three individual wild-type embryos (lanes 4-6).

in *Drosophila*. The *Drosophila Abd-B* gene also contains an intron in the homeobox region (DeLorenzi et al., 1988). Since the putative amino-acid sequences in the homeobox region of *Bombyx Abd-B* gene were identical to the sequences of cDNA of the *Drosophila Abd-B* gene, the *Bombyx Abd-B* gene probably does not have this intron. Compared with the similarities of the *Ubx* and *abd-A* genes between *Bombyx* and *Drosophila*, the homology of the amino-acid sequences of the 3′ flanking region of the *Abd-B* genes was not so high.

These conservations of the homeodomain amino-acid sequences between *Bombyx* and *Drosophila* suggest that these *Bombyx* homologues may have functions analogous to their *Drosophila* counterparts. Therefore, we name these genes *Bm Ubx*, *Bm abd-A* and *Bm Abd-B*, respectively.

Analysis of the E^N *chromosome*

To examine the abnormalities in the E^N chromosome, we performed a Southern blot analysis of the heterozygous E^N/+ chromosome and an amplification analysis by PCR of the homozygous E^N/E^N chromosome. The homozygous E^N/E^N chromosome is the best for analyses of chromosomal abnormalities, but sufficient quantities of DNA for a Southern analysis cannot be easily obtained because of the embryonic lethality of homozygosity. Therefore, first we performed a Southern blot analysis on heterozygous E^N/+ DNA. We used a probe fragment just 3′ to the homeodomain of the *Bombyx* genes (Fig. 5A-5C) to prevent a cross-hybridization. The *Bm Ubx* probe hybridized to 6.7 kb fragments from both heterozygous E^N/+ and wild-type +/+ DNAs digested with *Eco*RI, but a quantitative analysis revealed that the extent of hybridization (as determined by the intensity of signals from hybridized bands) with heterozygous E^N/+ DNA was about a half of that with wild-type +/+ DNA (Fig. 5A). Also, the heterozygous E^N/+ DNA contained only a half amount of a 7.4 kb fragment hybridized with *Bm abd-A* probe compared to wild-type +/+ DNA (Fig. 5B). However, the extent of hybridization with the *Bm Abd-B* probe was almost the same for E^N/+ and wild-type DNAs (Fig. 5C).

These results strongly suggested that the *Bm Ubx* and *abd-A* regions are deleted in the E^N chromosome of *Bombyx*. To confirm this further, we examined whether the DNA fragments just 3′ to the homeodomains of *Bombyx* genes could be amplified from the homozygous E^N/E^N chromosome by PCR (Fig. 5D-5F). Amplified DNA fragments were detected by Southern blot analysis with specific probes to the *Bombyx* homeobox genes. In the case of *Bm Ubx* gene, the DNA fragment was amplified from DNAs extracted from three individual wild-type embryos, while no appropriate product was amplified from DNAs from three individual embryos homozygous for E^N/E^N. Similar results were

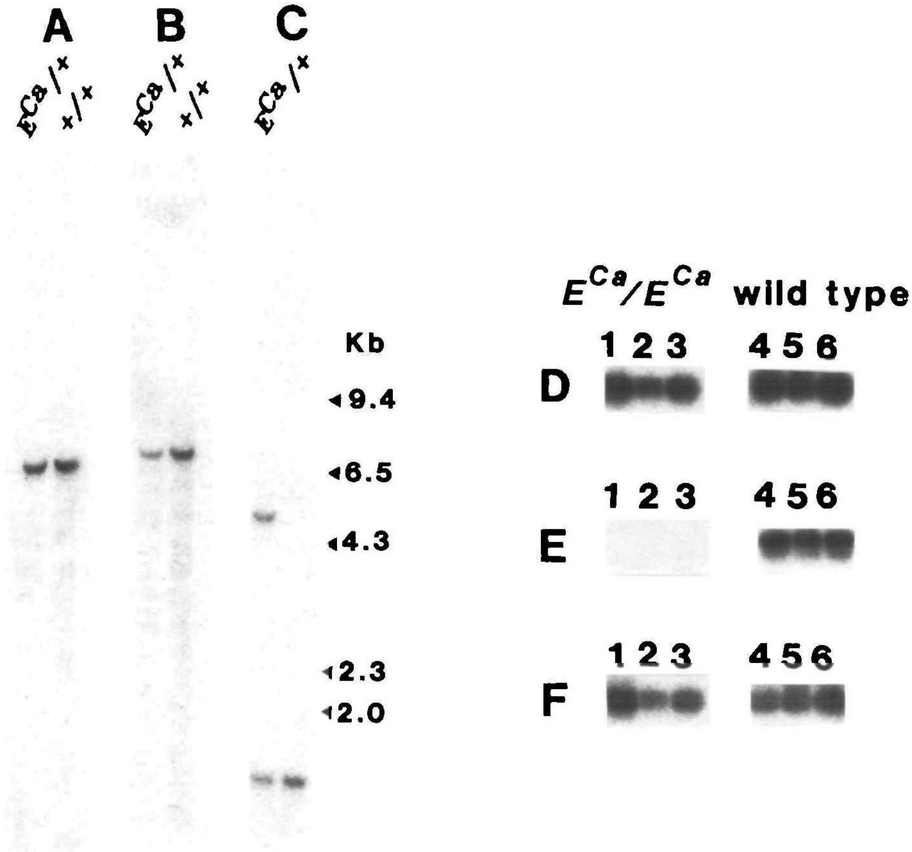

Fig. 6. Analyses on the heterozygous and homozygous E^{Ca} chromosomes. (A-C) Southern blot analysis of the heterozygous E^{Ca} chromosome (E^{Ca}/+) and wild-type chromosome (+/+) with probe fragments just 3′ to the homeodomains of (A) *Bm Ubx*, (B) *Bm abd-A* and (C) *Bm Abd-B* genes. (D-F) PCR analysis of *Bombyx* homologues in the homozygous E^{Ca}/E^{Ca} embryos and wild-type embryos. DNA fragments just 3′ to the homeodomains of (D) *Bm Ubx*, (E) *Bm abd-A* and (F) *Bm Abd-B* genes were amplified from DNA from three individual homozygous embryos (lanes 1-3) and from three individual wild-type embryos (lanes 4-6).

obtained in the case of the *Bm abd-A* gene; no amplified product was detected from DNAs extracted from embryos homozygous for E^N/E^N. However, amplifications of the *Bm Abd-B* gene were detectable from DNAs extracted from embryos homozygous for E^N.

Analysis of the E^{Ca} *chromosome*

Structural and functional analysis of the E complex was carried out also on E^{Ca} chromosome. Southern blot hybridization revealed that the extent of hybridization with the *Bm Ubx* probe in the heterozygous E^{Ca}/+ DNA was the same as in wild-type +/+ DNA (Fig. 6A), while that of the *Bm abd-A* in the heterozygous E^{Ca}/+ DNA was a half of that of wild-type +/+ DNA (Fig. 6B). For the *Bm Abd-B* gene from the heterozygous E^{Ca}/+ chromosome, two fragments (5.0 kb and 1.5 kb) of a comparable intensity were detected (Fig. 6C). Since the total intensity of *Bm Abd-B* hybridization on the 5.0 kb and 1.5 kb fragments from the heterozygous chromosome was the same as the intensity of 1.5 kb fragment from the wild-type chromosome, restriction polymorphisms with the heterozygous chromosome might have caused the two fragments. By PCR amplification on the homozygous E^{Ca}/E^{Ca} chromosome, no product was detected for the *Bm abd-A* gene (Fig. 6E), while appropriate products were detected for the *Bm Ubx* (Fig. 6D) and *Abd-B* genes (Fig. 6F). These results suggest that the *Bm abd-A* gene is deleted in the E^{Ca} chromosome.

Discussion

Conservation of amino-acid sequences of the homeodomains between Bombyx *and* Drosophila

We have isolated *Bombyx Ubx*, *abd-A* and *Abd-B* genes, and shown that the amino-acid sequences including the homeodomain and its flanking regions of these genes are almost identical to those of *Drosophila Ubx*, *abd-A* and *Abd-B*. The conservation of the homeodomain amino-acid sequences between *Bombyx* and *Drosophila* suggests that *Bombyx* homologues may have similar functions to those of *Drosophila*.

We have also isolated the *Antennapedia* (*Antp*), *Sex combs reduced* (*Scr*), *engrailed* (*en*), *invected* (*in*) homologues from *Bombyx* (W. Hara and Y. Suzuki, unpublished data; K. Ueno, unpublished data; Hui et al., 1991). The homeodomain sequences of the *Bombyx Antp* and *Scr* were also homologous to those of *Drosophila Antp* and *Scr*, respectively. Four highly conserved domains, including the homeodomain, were identified in *en* and *in* proteins from *Bombyx* and *Drosophila* (Hui et al., 1991). These conservations suggest that the common amino-acid sequences of the homeodomain and flanking regions are necessary for specifying the body segments.

The E^N *and* E^{Ca} *mutations and their phenotypes*

Since the homeodomain is important to express the function of the homeoprotein and our results indicate that the homeodomain regions of the *Bm Ubx* and *abd-*

A genes are deleted in the E^N/E^N chromosome, we speculate that complete functional deficiency of the *Bm Ubx* and *abd-A* genes may have caused the transformation of the A1 to A7 segments to the thoracic type segments in the homozygous E^N embryos (Fig. 1B). Observation of intermediate thoracic/abdominal-type legs in the A8 segment suggests that the *Bm Abd-B* gene may be functional in the homozygous E^N embryos.

In *Drosophila*, the deletion mutations of the *Ubx* and *abd-A* genes such as $DfUbx^{109}$ cause the transformation of the A1 to A7 segments to the thoracic-type segments (Lewis, 1978), and the A8 segment expressed abdominal-type phenotype in $DfUBx^{109}$ mutant. The similarity of the phenotypes of the homozygous E^N/E^N embryos and the $DfUbx^{109}$ embryos suggests that the *Bm Ubx*, *abd-A* and *Abd-B* genes have similar functions to the *Drosophila* genes.

In *Drosophila*, functional deficiency of the *abd-A* gene is thought to cause the transformation of the A2-A7 segments to A1 type segments (Lewis, 1978). By analogy, we infer that a complete deficiency of *abd-A* function in homozygous E^{Ca}/E^{Ca} embryos may have caused the transformation of the A2-A7 segments to A1 type segments, which have no abdominal legs.

The structure of the E complex

These analyses on E^N and E^{Ca} chromosomes suggested that the deletions of *Bm Ubx* and/or *abd-A* genes are responsible for the phenotypes of these E mutants and functions of these genes are analogous to those of homologues in the BX-C. No chromosomal abnormality was detected at the homeobox region of the *Bm Abd-B* gene in either the E^N or E^{Ca} chromosome. Nevertheless, we speculate that the E complex also contains the *Bm Abd-B* gene for the following reasons. It is known that the E^{Ds} (Double stars) causes abnormal specification of segments flanking the A5 segment (Takasaki, 1947). This phenotype seems to resemble the phenotype associated with *Abd-B* mutation in *Drosophila* (Duncan, 1987). Therefore, the E complex in *Bombyx* probably consists of *Ubx*, *abd-A* and *Abd-B* genes and the functions of these homeobox genes resemble those of the *Drosophila* BX-C.

Unity and diversity of the homeotic gene complex between Bombyx *and* Drosophila

From our results, we think that the E loci accommodate a gene complex analogous to BX-C. Lewis has suggested the hypothesis that 'leg-suppressing' genes ancestral to the bithorax complex are responsible for removing the legs from abdominal segments of millipede-like ancestors during the evolution of the arthropod (Lewis, 1978). The E^N/E^N embryo with its many thoracic-type legs may appear to express atavic characteristics by the deletion of some 'leg-suppressing' genes.

Recently, we demonstrated that the *Nc* locus at 1.4 genetic units from the E loci probably contains *Bombyx Antp* gene (T. Nagata, unpublished data). This observation suggests that the *Nc* locus may constitute a complex analogous to *Drosophila* ANT-C. Therefore, *Bombyx* also has two homeotic gene complexes corresponding to the ANT-C and BX-C of *Drosophila*.

The E complex is different in one important respect from the *Drosophila* BX-C. Some *E* mutations like E^{Kp}/E^{Kp} (Kp supernumerary legs) (Hashimoto, 1941) and $E^D/+$ (Double crescents) (Hashimoto, 1930) cause shifts in one direction on the dorsal side and to the opposite direction on the ventral side (Tazima, 1964). Since such an independent determination of the two sides is not seen in *Drosophila*, there must be differences between the *Bombyx* E complex and the bithorax complex of *Drosophila* in the regulatory mechanisms that specify abdominal segments. Further detailed analyses are required to determine these differences.

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