Genet. Res., Camb. (1995), **65**, *pp.* 105–111 *With 3 text-figures*

Linkage analysis of the gene encoding precursor protein of diapause hormone and pheromone biosynthesis-activating neuropeptide in the silkmoth, *Bombyx mori*

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(*Received 22 November 1994 and in revised form 10 January 1995*)

Summary

We have determined the map position of the gene encoding a common precursor protein for diapause hormone and pheromone biosynthesis-activating neuropeptide (the DH-PBAN gene, *Dh*) in the silkmoth, *Bombyx mori*. First we compared the structure of introns in the DH-PBAN gene by the polymerase chain reaction, and found that the *Dh* locus carried three alleles, Dh^{A1}, Dh^{A2} and Dh^{B}. The Dh^{A1} and Dh^{A2} alleles contained a fourth intron consisting of 740 bp, whereas Dh^{B} had a longer fourth intron of 770 bp. Dh^{A1} and Dh^{A2} contained a fifth intron consisting of 940 bp, whereas the fifth intron in Dh^{B} was much longer and consisted of 1700 bp. Dh^{A1} was distinguished from Dh^{A2} by an RFLP in the fifth intron after digestion with *Rsa* I. Linkage analyses using these polymorphisms showed that *Dh* was linked to the *bp* gene on chromosome 11, and independent of markers on chromosomes 1, 2, 3, 4, 5, 6, 7 and 13. To determine the map position, we obtained F_1 hybrids between the n501 strain ($K\ Dh^{A1}$) and the w30 strain ($+^{K}\ Dh^{B}$), and backcrossed the F_1 hybrid to females of the w30 strain. From the segregation of *K* and *Dh* in 864 individuals in the next generation, the recombination value was calculated as 25·5% between *K* and *Dh*. Similarly we obtained backcross progeny between the No. 744 strain ($Bu\ Dh^{A1}$) and the w30 strain ($+^{Bu}\ Dh^{B}$), and calculated the recombination value between *Bu* and *Dh* as 30·4% from 487 progeny. Because *K* and *Bu* had already been mapped at positions 11-23·2 cM and 11-28·7 cM, respectively, we mapped *Dh* at 11--2·2 cM. The *Dh* locus is different from any loci which are known to control diapause, development or growth.

1. Introduction

Embryonic diapause of the silkmoth, *Bombyx mori*, is controlled by both environmental and genetic factors. In typical bivoltine races, high temperature and long photoperiod exposure at the middle to late embryonic stages results in an embryonic diapause in the next generation (Yamashita & Hasegawa, 1985). A neuropeptide, the diapause hormone (DH), has an essential role for the induction of diapause, and is secreted by the suboesophageal ganglion of the female moth (Hasegawa, 1952; Imai *et al.* 1980). Recently Imai *et al.* (1991) and Sato *et al.* (1992) purified DH and determined its primary structure. Sato *et al.* (1993) cloned a cDNA coding for DH, which revealed that DH mRNA encodes a precursor polypeptide from which not only DH but also pheromone biosynthesis-activating neuropeptide (PBAN) (Kitamura *et al.* 1989, 1990; Kawano *et al.* 1993) is released post-translationally along with three other unknown peptides. All five of these peptides share a similar structure at their carboxyl termini, FXPRL-amide. Sato *et al.* (1993) also showed that the DH-PBAN precursor mRNA is expressed only in SG of females and males.

Many variants for diapause, growth and development have been found in *Bombyx*. Genetical experiments have clarified that major factors controlling diapause belong to two loci, *Lm* (Late maturity, 1-2·0) and *V* (Voltinism, 6-21·5) (Nagatomo, 1941; Morohoshi, 1957). Other loci including *pnd* (pigmented, non-diapausing egg, 11-53·7), *pnd-2* (pigmented, non-diapausing egg-2, 12-2·6) and *l-n* (lethal non-diapausing egg, 12-0·0), also affect diapause induction (Katsumata, 1968; Yoshitake & Hashiguchi, 1969; Yamamoto *et al.* 1978). To clarify the

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relationship between these known loci and the DH-PBAN gene, we at first looked for polymorphism of the DH-PBAN gene among different silkworm strains, and mapped it onto a chromosome by crossing experiments between strains carrying different DH-PBAN alleles. We employed the same strategy as described for mapping the prothoracicotropic hormone gene (Shimada *et al.* 1994), using primers for the polymerase chain reaction designed on the basis of the sequence of the genomic DH-PBAN gene (Xu *et al.* in press).

2. Materials and methods

(i) *Insects*

Eighteen strains of *Bombyx mori* and two strains of *Bombyx mandarina*, the wild species of *B. mori*, were used in this study. They are listed in Table 2. They were raised at 25 °C with mulberry leaves by conventional methods.

(ii) *DNA extraction*

DNA was extracted from posterior silk glands of fifth instar larvae or from pupal whole bodies. A single pair of posterior silk glands from the larva or the pupal whole body was homogenized with 2 ml of lysis buffer (1 % Cetyltriammonium bromide, 0·75 M NaCl, 10 mM EDTA and 50 mM Tris-HCl, pH 8·0), and genomic DNA was prepared according to Hunt & Page (1992).

(iii) *Primers*

We synthesized four primers for polymerase chain reaction (PCR) to detect polymorphism of the DH-PBAN gene. Their sequences were as follows: DH13: 5′-CTACGCCAGCTGACCAGGA-3′ [4062 → 4080, forward]; DH14: 5′-ATTTCTTCGGGGT-CAGGTTG-3′ [4804 → 4785, reverse]; DH15: 5′-CTGGGAAGGGAGCTTTCGTA-3′ [4866 → 4885, forward]; DH16: 5′-GCAACCCTATATTTTGTA-GG-3′ [5805 → 5786, reverse]. Numbers indicate the corresponding bases in the DH-PBAN gene sequence of the Tokai × Asahi strain (Xu *et al.* in press). They were synthesized with a Cyclon Plus DNA Synthesizer (Millipore Corp., Bedford, USA).

(iv) *Polymerase chain reaction (PCR)*

In PCR, each reaction mixture was 30 μl in volume and contained 0·2 μg of template DNA, 0·2 mM dNTP (Pharmacia-LKB Biotech.), 0·5 mM reverse primer, 0·5 mM forward primer and 3·0 units of *Taq* polymerase (Wako Pure Chemical Industries, Osaka, Japan). Amplification consisted of 30 cycles of 1 min at 94 °C, 1 min at 50 °C and 2 min at 72 °C. The reactions were performed with the Zymoreactor II machine (Atto Co. Ltd, Tokyo, Japan). PCR products were analysed on 2% agarose gels. PCR products were ethanol precipitated before digestion with restriction endonucleases.

(v) *Cloning and sequencing*

The PCR product was purified by extraction with phenol/chloroform (1:1), concentrated by ethanol precipitation, and dissolved in TE buffer (10 mM Tris-HCl, pH 7·6; 1 mM EDTA). DNA ends were blunted using T_4 DNA polymerase in a DNA blunting kit (Takara Shuzo Co. Ltd, Kyoto, Japan) according to the manufacturer's instructions. The blunted DNA was extracted with phenol/chloroform and recovered by ethanol precipitation. The plasmid, pBluescript II SK+ (Stratagene Cloning System, La Jolla, USA), was digested with the endonuclease *Sma* I (Nippon Gene Co. Ltd, Tokyo, Japan), ligated with the blunted PCR product, and used to transform *E. coli* JM109. Plasmids were isolated from transformants and purified by CsCl-gradient ultracentrifugation (Sambrook *et al.* 1989). Double-stranded plasmids were denaturated with alkali and sequenced by the dideoxy chain termination method of Sanger *et al.* (1977) using the Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical Corp., Cleveland, USA) and commercial primers.

3. Results

(i) *Polymorphism in intron length of the DH-PBAN gene*

We surveyed the polymorphisms in intron length of the DH-PBAN gene in 18 strains of *B. mori* and 2 strains of *B. mandarina* (Fig. 1 and Table 1) by amplifying DH-PBAN genes by the PCR technique.

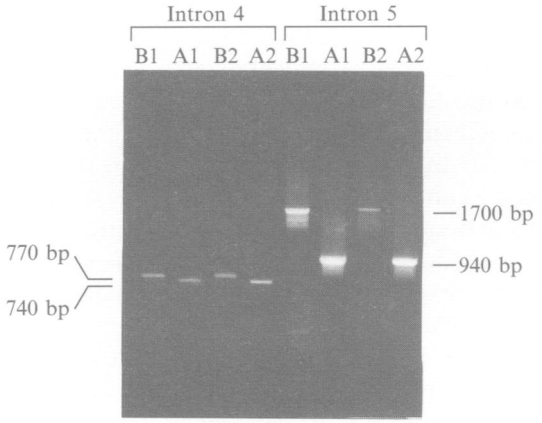

Fig. 1. Variation in the length of the 4th and 5th introns in the DH-PBAN gene. B1, w30 strain of *B. mori* and B2, *B. mandarina* in Sakado (Japan) have Dh^B. A1, No. 912 strain of *B. mori* and A2, *B. mandarina* in Hangzhou (China) have Dh^A.

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Table 1. *Distribution of* Dh *alleles*

Strain	Maintaining institution	*Dh* allele
UT18 E^{Kp} *os-e re q*	Univ. Tokyo[1]	Dh^{A1}
848 *pnd-K*	NISES[2]	Dh^{A5}
944 *bp mp*	NISES	Dh^{A}
501 *K lem*	NISES	Dh^{A}
744 *Bu*	NISES	Dh^{A}
927 *K-mp*	NISES	Dh^{A} and Dh^{B}
912 *pre-re ch*	NISES	Dh^{A2}
UT16 N_4	Univ. Tokyo	Dh^{A1}
312 Kansen	NISES	Dh^{A1}
319 Oha	NISES	Dh^{A1}
322 Sekko	NISES	Dh^{A1}
751 E^{Ns}	NISES	Dh^{A1}
502 Ascoli	NISES	Dh^{A1}
604 Mysore	NISES	Dh^{A1}
p50 Daizo	Kyushu Univ.	Dh^{A1}
NIG785 C108	NIG[4]	Dh^{A1}
u10 *bp mp*	Kyushu Univ.[3]	Dh^{A}
w30 p^{M} *Ze L q bp*	Kyushu Univ.	Dh^{B}
UTW1 *B. mandarina* from Sakado	Univ. Tokyo	Dh^{B}
UTW2 *B. mandarina* from Hangzhou	Univ. Tokyo	Dh^{B}

For each strain, 1–3 individuals were examined.

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[5] 'Dh^{A}' means that we have not determined whether it is Dh^{A1} or Dh^{A2}.

The primer set of DH13 and DH14 was used to look for variations of the fourth intron. It amplified a PCR product consisting of 740 bp from 17 strains of *B. mori* and the Chinese strain of *B. mandarina*. This size coincides with that expected from the sequence of the Tokai × Asahi strain (Xu *et al.* in press). The same primer set amplified a fragment of 770 bp from one strain, w30, of *B. mori*, and the Japanese strain of *B. mandarina*. To confirm that the 770 bp DNA fragments corresponded to the fourth intron of the DH-PBAN gene, we cloned the PCR product from w30 and partly determined the sequence. It coincided with the fourth intron of Tokai × Asahi but contained 7% exceptional substitutions indicating that the 770 bp fragment includes also part of the DH-PBAN gene. We designated the DH-PBAN gene (*Dh*) from which 740 bp PCR product was obtained by DH13/14 as allele Dh^{A}, and that from which the 770 bp product was obtained as allele Dh^{B}. Another strain, No. 927, was heterozygous for Dh^{A} and Dh^{B}.

The fifth intron was amplified by using a set of primers, DH15 and DH16 which amplified a fragment of 940 bp from 10 strains of *B. mori* and the Chinese strain of *B. mandarina*. Another fragment of 1700 bp was amplified from the w30 strain in *B. mori* and the Japanese strain of *B. mandarina*. All of the strains which had a 940 bp fifth intron carried a 740 bp fourth intron corresponding to Dh^{A}, whereas both strains which had a 1700 bp fifth intron contained a 770 bp fourth intron. We also looked for restriction fragment length polymorphism (RFLP) in the PCR products of the 740 bp fifth intron, and found two types of restriction fragment patterns. Digestion with *Rsa* I resulted in 4 fragments consisting of 140, 160, 350, 380 bp from 9 of 10 strains; the other strain, No. 912, showed a different pattern of fragments consisting of 150, 350, and 540 bp. We designated the former subtype of Dh^{A} whose fifth intron was cleaved into 4 fragments by *Rsa* I as Dh^{A1}, and the latter subtype of Dh^{A} whose fifth intron was cleaved into 3 fragments by *Rsa* I as Dh^{A2}. We cloned the PCR product from the fifth intron of w30, and determined its terminal sequences. They were approximately 92% identical to the sequence of the fifth intron of Tokai × Asahi.

(ii) *Linkage group to which the DH-PBAN gene belongs*

To determine the linkage group to which the DH-PBAN gene belongs, we performed some mating experiments using visible chromosome markers. To amplify the fourth and fifth introns, we used the primer sets DH13/DH14 and DH15/DH16, respectively. The mating schemes and the results are summarized in Table 2. First, we crossed No. 912 *pe-re ch* Dh^{A2} ♀ with w30 p^{M} *Ze L q bp* Dh^{B} ♂, No. 912 *pe-re ch* ♀ with UT18 E^{Kp} *os-e* ♂, and UT18 ♀ with No. 912 ♂. The *Dh* genotypes in the F_1 hybrid showed that *Dh* is not linked to sex. Next, we backcrossed the F_1 ♀ between No. 912 ♀ and UT18 ♂ with No. 912 ♂, and in the same manner, we crossed the F_1 ♀ between No. 912 ♀ and w30 ♂ with No. 912 ♂. The results of these crosses indicated independent assortment for p^{M} (Moricaud, chromosome 2-0·0 cM), *Ze* (Zebra, chr. 3-20·8 cM) and *L* (Multilunar, chr. 4-15·3 cM), *pe* (pink-eyed white egg, chr. 5-0·0 cM), E^{Kp} (Kp extra-legs, chr. 6-0·0 cM), and *ch* (chocolate, chr. 13-9·6 cM), as shown in Table 2. Fortunately, we found a linkage between *Dh* and *bp* (black pupa, chromosome 11-40·3 cM) in the cross (No. 912 $+^{bp}$ Dh^{A2} ♀ × w30 *bp* Dh^{B} ♂) ♀ × w30 ♂, since all the black pupae were homozygous for Dh^{B}, and normal pupae were heterozygous for Dh^{A2} and Dh^{B}. To reconfirm complete linkage between *Dh* and other visible markers on chromosome 11, we performed the cross (No. 848 *K* Dh^{A} ♀ × w30 $+^{K}$ Dh^{B} ♂) ♀ × w30 ♂, and (No. 744 *Bu* Dh^{A} ♀ × w30 $+^{Bu}$ Dh^{B} ♂) ♀ × w30 ♂. We obtained 30 *K* $Dh^{A/B}$ individuals and 46 $+^{K}$ Dh^{B}, and did not obtain other phenotypes from the former cross (Fig.

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Table 2. *Linkage screening of the DH-PBAN gene*

Mating scheme	No. of progenies				Marker (chromosome) and linkage
	Dh^{A1}/Dh^{A2}	Dh^{A2}/Dh^{B}	Dh^{A2}	Dh^{B}	
912 *pe ch* Dh^{A2} ♀ × w30 p^{M} *Ze L* Dh^{B} ♂					
F_1 ♀		3			sex(1) No
F_1 ♂		1			
912 *pe ch* Dh^{A2} ♀ × UT18 UT18 E^{Kp} *os-e* Dh^{A} ♂					
F_1 ♀ *os-e*	4				sex(1) No
F_1 ♂ $+^{os\text{-}e}$	4				
UT18 E^{Kp} *os-e* Dh^{A} ♀ × 912 *pe ch* Dh^{A2} ♂					
F_1 ♀ *os-e*	4				sex(1) No
F_1 ♂ $+^{os\text{-}e}$	4				
(912 *pe ch* Dh^{A2} ♀ × UT18 E^{Kp} *os-e* Dh^{A} ♂) ♀ × 912 ♂					
E^{Kp}	11		15		E^{Kp}(6) No
$+^{E}$	13		11		
(912 *pe ch* Dh^{A2} ♀ × w30 p^{M} *Ze L* Dh^{B} ♂) ♀ × 912 ♂					
pe		16	16		*pe*(5) No
$+^{pe}$		15	17		
ch		14	18		*ch*(13) No
$+^{ch}$		17	15		
p^{M}		12	20		p^{M}(2) No
$+^{pM}$		19	13		
Ze		20	12		*Ze*(3) No
$+^{Ze}$		11	21		
L		16	16		*L*(4) No
$+^{L}$		14	18		
(912 + ^{q} Dh^{A2} $+^{bp}$ ♀ × w30 *q* Dh^{B} *bp* ♂) ♀ × w30 ♂					
q		4		8	*q*(7) No
$+^{q}$		4	10		
bp		0		18	*bp*(11) Yes
$+^{bp}$		8		0	
(848 *K* Dh^{A} ♀ × w30 $+^{K}$ Dh^{B} ♂) ♀ × w30 ♂					
K		30		0	*K*(11) Yes
$+^{K}$		0		46	
(744 *Bu* Dh^{A} ♀ × w30 $+^{Bu}$ Dh^{B} ♂) ♀ × w30 ♂					
Bu		50		0	*Bu*(11) Yes
$+^{Bu}$		0		50	

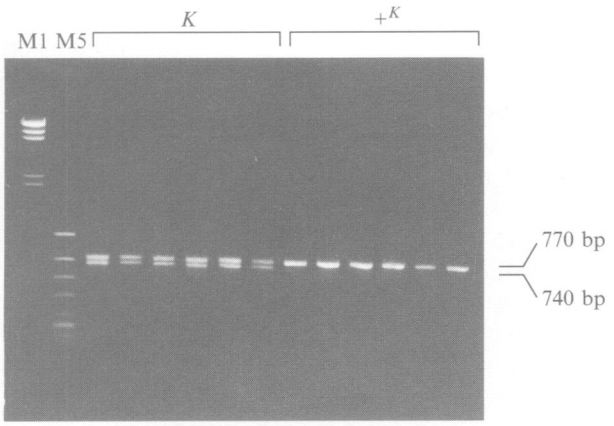

Fig. 2. Co-segregation of *Dh* and *K* phenotypes. Mating scheme was: (No. 848 KDh^{A} × w30 $+^{K}$ Dh^{B}) × w30 + ^{K} Dh^{B}. Six *K* individuals and six $+^{K}$ individuals are shown.

2). Similarly, we obtained 50 *Bu* $Dh^{A/B}$ individuals and 50 $+^{Bu}$ Dh^{B}, and did not obtain other phenotypes from the latter cross. These results clearly prove that *Dh* is linked not only to *bp* but also to *K* and *Bu* on chromosome 11. Therefore, the *Dh* locus is located on this chromosome.

Table 3. *Recombination value between* K *and* Dh

w30 $+^{K}$ Dh^{B} ♀ × (n501 *K* Dh^{A} ♀ × w30 $+^{K}$ Dh^{B} ♂) ♂

	Dh^{A}/Dh^{B}	Dh^{B}/Dh^{B}
K	241	81
$+^{K}$	139	403

Recombination value between *Dh* and *K* is 220/864 = 25·5%.

(iii) *Localization of the DH-PBAN gene on chromosome 11*

To find the position of the *Dh* locus on chromosome 11, we performed two crosses using *K* (11-23·2) and *Bu* (11-28·7) as markers (Doira, 1983; Doira *et al.* 1992). Primers DH13 and DH14 were used to amplify the fourth intron. The mating schemes of the crosses were the following: (1) w30 $+^{K}$ Dh^{B} ♀ × (n 501 *K* Dh^{A} ♀ × w30 $+^{K}$ Dh^{B} ♂) ♂ and (2) w30 $+^{Bu}$ Dh^{B} ♀ × (No. 744 *Bu* Dh^{A} ♀ × w30 $+^{Bu}$ Dh^{B} ♂) ♂. Numbers of individuals scored in the next generation are shown in Tables 3 and 4. The recombination value between *Dh*

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Table 4. *Recombination value between* Bu *and* Dh
w30 $+^{Bu}$ Dh^B ♀ × (744 *Bu* Dh^A ♀ × w30 $+^{Bu}$ Dh^B ♂) ♂

	Dh^A/Dh^B	Dh^B/Dh^B
Bu	123	55
$+^{Bu}$	93	206

Recombination value between *Dh* and *Bu* is 148/487 = 30·4%.

Table 5. *Recombination value between* mp *and* Dh
944 *mp* Dh^A ♀ × (944 *mp* Dh^A ♀ × w30 $+^{mp}$ Dh^B ♂) ♂

	Dh^A/Dh^B	Dh^B/Dh^B
mp/mp	25	25
mp/+	26	15

Recombination value between *Dh* and *mp* is 40/91 = 44·0%.

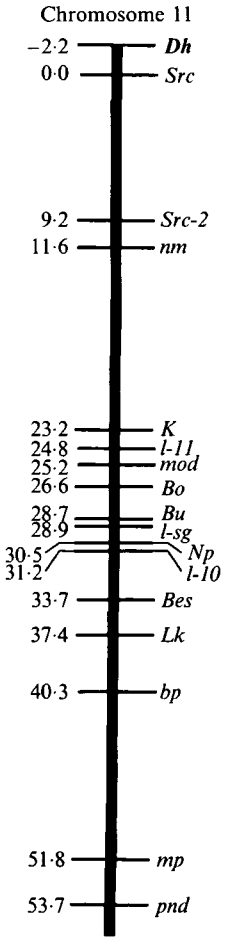

Fig. 3. Location of the *Dh* locus on chromosome 11. See text for symbols.

and *K* was calculated as 25·5% from 864 individuals, and that between *Dh* and *Bu* as 30·4% from 472 individuals. To confirm the map order of *Dh*, *K*, and *Bu*, we also performed another cross, No. 944 *mp* Dh^A ♀ × (No. 944 *mp* Dh^A ♀ × w30 $+^{mp}$ Dh^B ♂) ♂ and calculated the recombination value between *Dh* and *mp* as 44·0% (Table 5). Because *K*, *Bu*, and *mp* had already been mapped at positions 11-23·2, 11-28·7 and 11-51·8 cM, respectively (Japanese Society of Sericultural Science, 1986; Doira *et al.* 1992), we determined the order of loci as *Dh-K-Bu-mp*. We assigned the map position of *Dh* as 11--2·2 cM on the basis of the distance between *K* and *Dh* (Fig. 3).

4. Discussion

We found two length variants in the fourth and the fifth introns of the DH-PBAN gene. Dh^B has the longer fourth intron and the longer fifth intron, and was found in only two of 20 strains in *B. mori*. A Japanese strain of *B. mandarina* also carries Dh^B, whereas a Chinese strain of *B. mandarina* has another allele. This evidence suggests that these variants might have already existed in the original population of *B. mandarina* and were introduced to *B. mori* during domestication (Shimada *et al.* 1994); alternatively Dh^B might have been transferred from a Japanese population of *B. mandarina* into *B. mori* in the history of Japanese sericulture. We are now comparing sequences of introns among alleles, and hope that it will clarify the evolution of the *Dh* locus.

Dh is located on chromosome 11 (see Fig. 3). This chromosome carries many marker loci including *Src* (Sericin complex, 11-0·0), *nm* (non-molting, 11·6), *K* (Knobbed, 11-23·2), *l-11* (lethal 11, 11-24·8), *mod* (dimolting, 11-25·2), *Bo* (Bamboo, 26·6), *Bu* (Burnt, 11-28·7), *l-sg* (lethal slow growth, 11-28·9), *Np* (Non-preference, 11-30·5), *Bes* (Blood esterase, 11-33·9), *bp* (black pupa, 11-40·3), and *pnd* (pigmented and non-diapausing egg, 11-53·7) (Japanese Society of Sericultural Science, 1986; Doira *et al.* 1992). The mutant *pnd* is presumed to be involved in a deficiency in the signaling pathway of DH in oocytes or embryos (Yoshitake & Hashiguchi, 1969; Yamamoto *et al.* 1978; Sonobe *et al.* 1986; Sonobe & Odake, 1986; Sonobe, 1989). Since *pnd* expresses its function zygotically, it is improbable that this locus encodes DH. The present study shows that *Dh* is very far from *pnd* on the linkage map, suggesting that *Dh* is not related to *pnd*. The expression of another mutant, *bp*, is affected by rearing temperature, which may be comparable to the induction of diapause (Harizuka, 1947). We, however, think that the *bp* gene has no direct relation with *Dh* because their locations are quite different. Although *nm* (Shimizu *et al.* 1980), *l-11*, *mod* (Ninaki *et al.* 1980), and *l-sg* also control development and growth at embryonic and young larval stages, their map positions are different from that of *Dh*. It is well known that diapause is controlled mainly by two loci, *Lm* and *V* (Nagatomo, 1941; Morohoshi, 1957). They, however, have not been mapped on chromosome 11 but on chromosomes 1 (Z) and 6, respectively. *pnd-2* (12-2·6) and *l-n* (12-0·0) are also known to control diapause, but again have been mapped on a different chromosome. We conclude

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that none of the known mutations and variants that affect diapause, growth and differentiation involve functional defects of the DH-PBAN gene. Therefore, it is likely that all of the genes described above control processes other than synthesis of DH, for example, the release of DH from the SG in the female pupa, or reception of DH and signal transduction in oocytes and developing embryos.

We mapped the *Ptth* locus encoding prothoracicotropic hormone onto chromosome 22 in *B. mori* (Shimada *et al.* 1994), which is different from the chromosome to which *Dh* belongs. Recently, amino acid sequences of many other neuropeptides have been reported from *B. mori*, and their genes have been isolated (Nagasawa, 1992, 1993). We should continue mapping of such peptides, and clarify the relationships between molecular and genetic factors that control development and growth by utilizing knowledge accumulated on both endocrinological molecules and classical genetics in *B. mori*. We have constructed a new linkage map of RAPDs (random amplified polymorphic DNAs) and RFLPs (Promboon *et al.* in press), which will be helpful for quick mapping of the neuropeptide genes.

We express sincere appreciation to Dr Marian R. Goldsmith, University of Rhode Island, for her continuous encouragement and critical reading of the manuscript. We wish to thank Professor Hiroshi Doira, Kyushu University, for providing many genetic stocks and useful suggestions. Dr Masataka Ozaki, University of Tokyo, and members of the National Institute of Sericultural and Entomological Sciences also generously supplied genetic stocks. This work was supported by the Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan (Nos. 03304016, 04404010, 04660058, 06660063), and the Fujisawa Foundation, Osaka, Japan (in 1993).

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