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Molecular Cloning of the *Bombyx mori* Prothoracicotropic Hormone

Atsushi Kawakami, Hiroshi Kataoka, Tadanori Oka, Akira Mizoguchi, Mina Kimura-Kawakami, Takashi Adachi, Masafumi Iwami, Hiromichi Nagasawa, Akinori Suzuki, Hironori Ishizaki*

Prothoracicotropic hormone (PTTH), a brain secretory polypeptide of insects, stimulates the prothoracic glands to produce and release ecdysone, the steroid essential to insect development. The complementary DNAs encoding PTTH of the silkmoth *Bombyx mori* were cloned and characterized, and the complete amino acid sequence was deduced. The data indicated that PTTH is first synthesized as a 224–amino acid polypeptide precursor containing three proteolytic cleavage signals. The carboxyl-terminal component (109 amino acids) that follows the last cleavage signal represents one PTTH subunit. Two PTTH subunits are linked together by disulfide bonds, before or after cleavage from prepro-PTTH, to form a homodimeric PTTH. When introduced into *Escherichia coli* cells, the complementary DNA directed the expression of an active substance that was functionally indistinguishable from natural PTTH. In situ hybridization showed the localization of the prepro-PTTH mRNA to two dorsolateral neurosecretory cells of the *Bombyx* brain.

Ecdysone, the steroid secreted by the prothoracic glands, is required for insect growth, molting, and metamorphosis. The prothoracicotropic hormone (PTTH), a brain neuropeptide, stimulates the prothoracic glands to synthesize and release ecdysone, thereby playing a central role in the endocrine network that controls insect development (*1*). Many efforts have been made to purify this hormone from insects (*1*), but purification has been hampered by the small amount present in the brain and by attendant technical difficulties inherent in protein purification. We previously isolated PTTH from the silkmoth *Bombyx mori* and determined the sequence of 13 amino acids from its NH_2-terminus (*2*). We then purified *Bombyx* PTTH on a far larger scale, concluded that PTTH is a dimeric molecule of relative molecular mass ~30 kD consisting of two identical or very similar subunits which are held together by one or more disulfide bonds, and sequenced the 104 amino acid residues from the NH_2-terminus of the subunit (*3*). The complete sequence was still unknown, however. Here, we report the cloning and characterization of the *Bombyx* cDNA which enabled us to deduce the complete amino acid sequence of PTTH.

By screening a *Bombyx* brain cDNA expression library with a mouse antiserum to PTTH (*4*) and then with an isolated positive clone as a hybridization probe (*5*), we obtained seven PTTH cDNA clones and characterized two types of cDNAs that encode prepro-PTTH, a precursor molecule for PTTH. The P-1 type cDNA contains an open reading frame that encodes a prepro-PTTH of 224 amino acids (molecular mass 26,027) consisting of a putative signal peptide segment containing the signal peptide (29 amino acids), two peptide components tentatively referred to as p2K (21 amino acids) and p6K (57 amino acids), and a PTTH subunit (109 amino acids), in this order from the 5′ end (Fig. 1). Proteolytic cleavage signals bound these components, suggesting that they are separated after translation. We defined the last component as the PTTH subunit because the amino acid sequence of this component up to the 104th residue matched the sequence determined by amino acid sequencing of purified PTTH (*3*), except for the 41st residue, which had not been identified because no phenylthiohydantoin derivatives of amino acids were detected at this cycle of Edman degradation. The 41st residue has now been deduced to be Asn, forming a possible N-linked glycosylation site together with the following two residues (Asn-Lys-Thr). The identification of this glycosylation site agrees with the previous evidence that *Bombyx* PTTH is a glycoprotein (*6*). The calculated molecular mass of the PTTH subunit (12,737) is slightly less than half the apparent molecular mass of purified mature PTTH [molecular mass of *Bombyx* PTTH has been estimated to be 22 kD by molecular sieving (*2*), but is now considered to be 30 kD from SDS electrophoresis data (*3*)]. The sequence length of the prepro-PTTH cDNA is consistent with the brain mRNA size of ~1.0 to 1.2 kb as determined by RNA (Northern) hybridization analysis (*7*). From these results and the previous amino acid sequencing data, we conclude that PTTH is first synthesized as a 224–amino acid polypeptide precursor containing a 109–amino acid PTTH subunit, and then two PTTH subunits are linked by disulfide bonds and glycosylated before or after posttranslational cleavage to generate a homodimeric mature PTTH. Sites of disulfide bonds remain to be determined. Peptide analysis of native PTTH (*3*) disclosed heterogeneity with respect to the chain length at the NH_2-terminus of the PTTH subunit, and possibly at the COOH-terminus also. This heterogeneity may be due to proteolysis during purification, posttranslational deletion of the terminal regions, or the presence of multiple PTTH genes differing in sequence. Two peptide components contained in prepro-PTTH, p2K and p6K, are presumed to be cleaved posttranslationally and may play some unknown physiologically or developmentally important functions.

A. Kawakami, T. Oka, A. Mizoguchi, M. Kimura-Kawakami, T. Adachi, M. Iwami, H. Ishizaki, Department of Biology, School of Science, Nagoya University, Nagoya 464-01, Japan.
H. Kataoka, H. Nagasawa, A. Suzuki, Department of Agricultural Chemistry, Faculty of Agriculture, The University of Tokyo, Tokyo 113, Japan.

*To whom correspondence should be addressed.

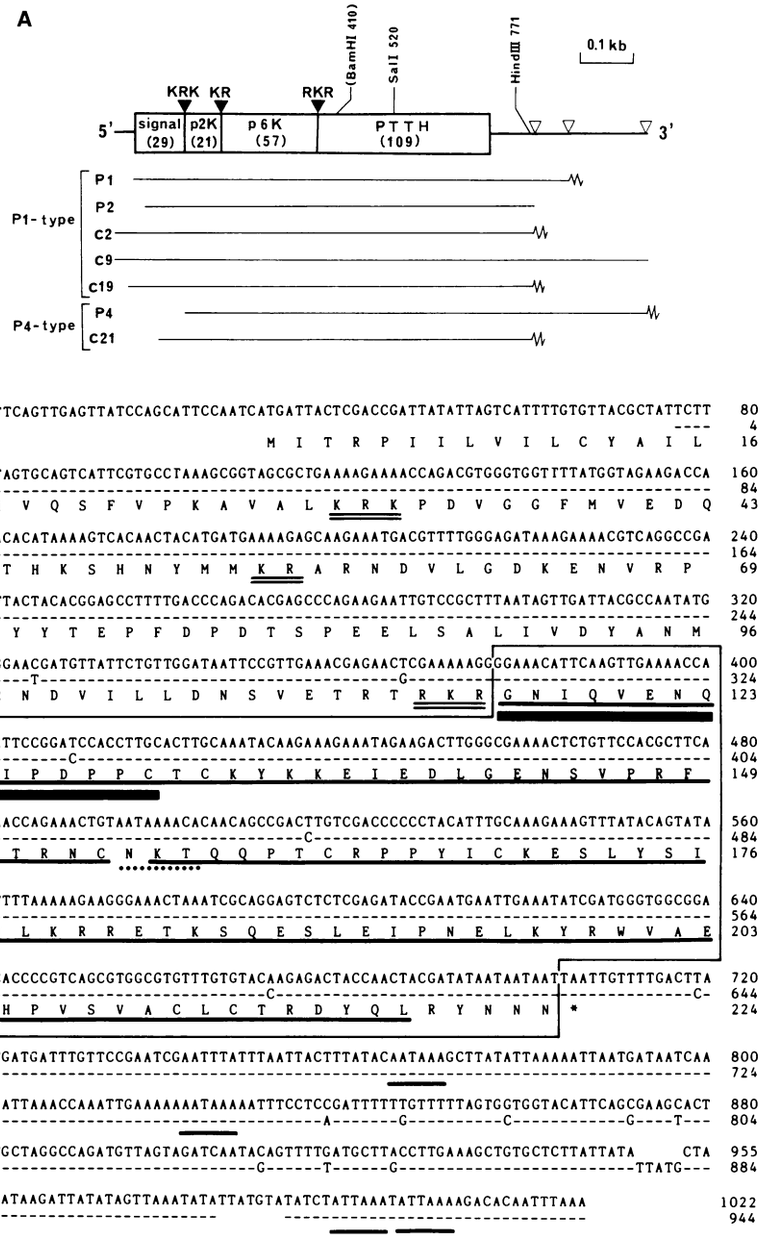

Fig. 1. Prepro-PTTH cDNA structure. (**A**) Schematic representation of the cDNA and overlapping clones used for sequencing. The coding region is indicated by a box with partitions that bound a putative signal peptide or a segment containing it (signal), two peptide components of molecular size, 2 kD and 6 kD (p2K and p6K), and the PTTH subunit (PTTH). Numerals in parentheses indicate the number of amino acids in the respective components. Putative proteolytic cleavage signals are indicated by KRK, KR, and RKR (K, Lys; R, Arg). Restriction sites are indicated. A parenthesis for Bam HI indicates that this restriction site occurred only in clones P4 and C19. Triangles at the 3′ region and waved lines on the clones represent the polyadenylate tract. (**B**) Nucleotide sequences of P-1 type (upper) and P-4 type (lower) PTTH cDNAs with their predicted amino acid sequence. Dashes in the P-4 sequence represent the nucleotides identical to those of the P-1 sequence. Proteolytic signals are double-underlined. The presumed PTTH sequence is boxed. The amino acid sequence from the purified PTTH is underlined. Thick underline indicates the sequence used as an antigen for PTTH antibody production. The stop codon is marked with asterisk. A possible N-linked glycosylation site is indicated by a dotted underline. Polyadenylation signals are underlined.

The other cDNA, a P-4 type, which apparently lacks the 5′ portion, differs from the P-1 type cDNA by only five nucleotides in the coding region. These substitutions are silent, coding for the same amino acid sequence as that of the P-1 type cDNA. The 3′ noncoding region of the P-4 type cDNA differs in sequence considerably from that of the P-1 cDNA, suggesting that the two cDNAs were derived from two distinct genes. Preliminary experiments with the use of Southern hybridization suggested that the *Bombyx* haploid genome contains a single PTTH gene. The presence of the two types of cDNA is not unexpected, however, since the mRNA for the cDNA library was derived from a racial hybrid (Kinshu × Showa) of *Bombyx*. The nucleotide and amino acid sequences of prepro-PTTH show no homology with those of bombyxin, a 5-kD *Bombyx* brain peptide with a PTTH-like function (*8*). Our database search did not reveal homology with any other protein (*9*).

When introduced into *Escherichia coli* (*10*), a portion of the cDNA encoding the PTTH subunit directed the expression of an active peptide that was functionally indistinguishable from natural PTTH, proving that the cDNA that we cloned actually encodes the *Bombyx* PTTH and that glycosylation is not essential for biological activity. Thus, extracts of the recombinant *E. coli* induced adult development when injected into debrained *Bombyx* pupae, and exhibited a dose-response relation that was the same as natural PTTH (*11*). Extracts from *E. coli* carrying a control plasmid were ineffective. An *E. coli* extract derived from a 400-ml culture contained 10^5 *Bombyx* units of PTTH activity, which is equivalent to the amount contained in ~10^4 *Bombyx* pupal brains.

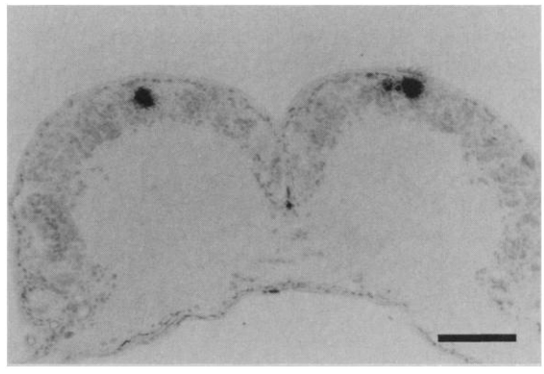

Fig. 2. Cellular localization of PTTH mRNA in the brain of a *Bombyx* fifth instar larva as detected by in situ hybridization. Transverse section. Bar, 100 μm. In this photograph, hybridization is shown in one neurosecretory cell in the left hemisphere of brain and one cell and the edge of another cell in the right hemisphere. Serial sections revealed, however, the presence of two positive neurosecretory cells in each hemisphere.

Finally, we performed in situ hybridization with a ^{35}S-labeled complementary RNA probe (*12*) to examine the expression of the PTTH gene in the brain of fifth instar

Bombyx larva. The PTTH gene transcript was localized to two pairs of dorsolateral neurosecretory cells of *Bombyx* brain (Fig. 2), indicating that PTTH is synthesized by these cells. The same cells react immunohistochemically with a monoclonal antibody that recognizes PTTH (*13*). In *Manduca sexta*, two pairs of brain neurosecretory cells at a similar position have been immunohistochemically identified as the PTTH-producing cells (*14*).

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5. The library was further screened with a ^{32}P-labeled DNA fragment that was derived from the P1 clone and contained nucleotides 34 to 776 of Fig. 1b and an extra 34-bp sequence at the 5′ end, an inverted duplicate of the nucleotides 34 to 67 generated as a cloning artifact. Hybridization and washing were performed at 60°C in the presence of 6× standard saline citrate (SSC) (0.9*M* NaCl, 0.09*M* sodium citrate) and 2× SSC, respectively. Four positive clones (C2, C9, C19, and C21) were obtained. Nucleotide sequencing of these clones disclosed two types of cDNA, P1-type (P1, P2, C2, C9, and C19) and P4-type (P4 and C21). Sequencing was done by the dideoxynucleotide method.
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10. The plasmid expression vector used was pKK233-2 [E. Amann and J. Brosius, *Gene* **40**, 183 (1985)] which provided *trp-lac* fusion promoter and the *lacZ* ribosome-binding site. The DNA fragment coding for the PTTH subunit was excised from clone P1 by digesting with Bam HI and Hind III. This DNA fragment, devoid of the sequence encoding the NH_2-terminal portion of the PTTH subunit, was ligated along with a synthetic oligonucleotide adaptor (designed to code for the NH_2-terminal region of the PTTH subunit and having Met as a start codon) to the pKK233-2 vector digested with Nco I and Hind III. The expression construct (pKKPTTH) or control plasmid was introduced into *E. coli* strain JM109. These cells were cultured at 37°C in 400 ml of LB medium containing ampicillin (0.05 mg/ml) to an OD_{600} of 0.5. After addition of 1 m*M* isopropyl-β-D-thiogalactopyranoside and further incubation for 1 hour, the cells were harvested by centrifugation at 8000 rpm for 15 min at 4°C, washed with 10 m*M* phosphate-buffered saline (PBS), *p*H 7.0, and suspended in 20 ml of 50 m*M* sodium phosphate, *p*H 7.0, 10 m*M* EDTA, and 10 m*M* 2-mercaptoethanol. The suspensions were allowed to stand on ice for 30 min with the addition of 10 mg of lysozyme. The *E. coli* cells were frozen and thawed repeatedly and disrupted by sonicating five times for 20 s each. After centrifugation, the supernatants were dialyzed against 10 m*M* PBS and assayed for biological activity.
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12. *Bombyx* brains were fixed for 0.5 to 1 hour in a fixative containing 85% ethanol, 4% formaldehyde, and 5% acetic acid at room temperature, and washed in PBS containing 15% sucrose at 4°C overnight [C. E. Bandtlow, R. Heumann, M. E. Schwab, *EMBO J.* 6, 891 (1987)]. After dehydration, the brains were embedded in paraffin wax, sectioned at 7 μm, and pretreated for hybridization as described [E. Hafen, M. Levine, R. L. Garber, W. J. Gehring, *ibid.* **2**, 617 (1983); P. W. Ingham, K. R. Howard, D. Ish-Horowicz, *Nature* **318**, 439 (1985)]. The DNA fragment of clone P1 that was used for screening the library (see legend to Fig. 1) was subcloned into Eco RI–Hind III sites of Bluescript-SK vector (Stratagene), and ^{35}S-labeled RNA was synthesized with T7 RNA polymerase. Final specific activity was 1.0×10^9 dpm/μg. The probe was used at a final concentration of 200 ng per milliliter in 50% formamide, 5% dextran sulfate, 2× SSC, 1× Denhardt's solution, 10 m*M* dithiothreitol (DTT), and yeast tRNA at 0.1 mg/ml. After hybridization to probe, the slides were washed in 0.1× SSC for 1 hour at 50°C and treated with ribonuclease (RNase) A at 20 μg/ml in 0.5*M* NaCl, 10 m*M* tris-HCl, *p*H 8.0, and 1 m*M* EDTA for 30 min at 37°C. Subsequently, the slides were washed in 0.1× SSC for 1 hour at 65°C. After dehydration, slides were coated with Sakura NR-M2 emulsion, exposed at 4°C for 5 days, and developed. The tissue was counterstained with hematoxylin.
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Isolation of a cDNA from the Virus Responsible for Enterically Transmitted Non–A, Non–B Hepatitis

GREGORY R. REYES,* MICHAEL A. PURDY, JUNGSUH P. KIM, KA-CHEUNG LUK, LAVONNE M. YOUNG, KIRK E. FRY, DANIEL W. BRADLEY

Major epidemic outbreaks of viral hepatitis in underdeveloped countries result from a type of non–A, non–B hepatitis distinct from the parenterally transmitted form. The viral agent responsible for this form of epidemic, or enterically transmitted non–A, non–B hepatitis (ET-NANBH), has been serially transmitted in cynomolgus macaques (cynos) and has resulted in typical elevation in liver enzymes and the detection of characteristic virus-like particles (VLPs) in both feces and bile. Infectious bile was used for the construction of recombinant complementary DNA libraries. One clone, ET1.1, was exogenous to uninfected human and cyno genomic liver DNA, as well as to genomic DNA from infected cyno liver. ET1.1 did however, hybridize to an approximately 7.6-kilobase RNA species present only in infected cyno liver. The translated nucleic acid sequence of a portion of ET1.1 had a consensus amino acid motif consistent with an RNA-directed RNA polymerase; this enzyme is present in all positive strand RNA viruses. Furthermore, ET1.1 specifically identified similar sequences in complementary DNA prepared from infected human fecal samples collected from five geographically distinct ET-NANBH outbreaks. Therefore, ET1.1 represents a portion of the genome of the principal viral agent, to be named hepatitis E virus, which is responsible for epidemic outbreaks of ET-NANBH.

THE ABILITY TO SEROLOGICALLY DIagnose viral hepatitis caused by infection with hepatitis A virus (HAV) or hepatitis B virus (HBV) led to the recognition of other viral hepatitis agents transmitted either by the percutaneous (blood) or the fecal-oral routes (*1*). Viral hepatitis resulting from viruses other than HAV or HBV, other well-characterized viruses, or predisposing conditions has been referred to collectively as non–A, non–B hepatitis (NANBH) and until recently has been a clinical diagnosis of exclusion. The molecular cloning of a parenterally transmitted form of viral NANBH, referred to as hepatitis C virus (HCV), has recently been de-

G. R. Reyes, J. P. Kim, K.-C. Luk, L. M. Young, K. E. Fry, Molecular Virology Department, Genelabs Incorporated, Redwood City, CA 94063.
M. A. Purdy and D. W. Bradley, Hepatitis Branch, Division of Viral Diseases, Centers for Disease Control, Atlanta, GA 30333.

*To whom correspondence should be addressed. Address after 9 April: Syva Company, 900 Arastradero Road, P.O. Box 10058, Palo Alto, CA 94303.