The Remarkable Conservation of Corticotropin-Releasing Hormone (CRH)-Binding Protein in the Honeybee (*Apis mellifera*) Dates the CRH System to a Common Ancestor of Insects and Vertebrates

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CRH-binding protein (CRH-BP) is a key factor in the regulation of CRH signaling; it modulates the bioactivity and bioavailability of CRH and its related peptides. The conservation of CRH-BP throughout vertebrates was only recently demonstrated. Here we report the presence of CRH-BP in the honeybee (*Apis mellifera*) and other insects. Honeybee CRH-BP resembles previously characterized vertebrate CRH-BP sequences with respect to conserved cysteine residues, gene organization, and overall sequence identity. Phylogenetic analyses confirm the unambiguous orthology of insect and vertebrate CRH-BP sequences. Soon after their discovery, it was noted that insect diuretic hormone-I (DH-I) and its receptor share similarities with the vertebrate CRH family and their receptors. Despite these similarities, demonstration of common ancestry of DH-I and the vertebrate CRH family is still speculative: the mature neuropeptides are short, and their genes differ substantially with regard to the number of coding exons. Moreover, DH and CRH receptors belong to the much larger family of G protein-coupled receptors. In contrast, the unique and conspicuous features of CRH-BP greatly facilitate the establishment of orthology over much larger evolutionary distances. The identification of CRH-BP in insects clearly indicates that this gene predates vertebrates by at least several hundred million years. Moreover, our findings imply that a CRH system is shared by insects and vertebrates alike and, consequently, that it has been present at least since the common ancestor to both phylogenetic lines of proto- and deuterostomians. (*Endocrinology* 146: 2165–2170, 2005)

CRH-BINDING PROTEIN (CRH-BP) is a 322-amino-acid soluble protein that is structurally unrelated to the CRH receptors. It is unique with respect to its 10 cysteine residues that form five consecutive disulfide bonds (1). CRH-BP was initially discovered in late gestational maternal plasma (2), where it prevents hypothalamo-pituitary-adrenal axis activation by the high concentrations of placenta-derived CRH that circulate around parturition (3). Human CRH binds to CRH-BP with a considerably higher affinity than to either CRH receptor type 1 or 2 (4, 5). It has been suggested that the binding of CRH to CRH-BP protects the former from degradation and by doing so acts as a delivery system in a fashion similar to that described for the various IGF-BP (6). However, upon bolus injection of CRH, CRH-BP/CRH complexes are rapidly cleared from circulation (7), indicating an antagonistic role of CRH-BP in CRH signaling. Indeed, CRH-BP abrogates CRH-induced ACTH release *in vitro* (8, 9). Besides its well-documented role in hypothalamo-pituitary-adrenal axis regulation, CRH-BP recently received interest as a potential therapeutic target in the treatment of anorexia nervosa, obesity, depression, and Alzheimer's disease, disorders that are associated with dysregulated brain CRH signaling (10–13).

Only recently, the CRH-BP gene was cloned in several nonmammalian vertebrates, including bony fish (14, 15), confirming that the CRH-BP gene is conserved throughout vertebrate evolution. Moreover, in common carp (*Cyprinus carpio*), CRH-BP and CRH-positive nerve fibers project onto the pars distalis and prominently onto the pars intermedia of the pituitary gland, and the hypothalamic expression of both corresponding genes is subject to regulation during acute restraint stress (15). Collectively, it seems that the CRH system (comprising CRH, CRH-BP, and CRH receptor type 1) is involved in the regulation of the stress response throughout the vertebrate lineage.

Insects too have a neuropeptide that complies with the CRH family motif (PS00511): diuretic hormone-I (DH-I) (16). Insect DH-I is released from a pair of endocrine glands, the corpora cardiaca, that receive input from the insect brain. The corpus cardiacum is a neurohemal organ where the products of neurosecretory cells from the pars intercerebralis are released, and the insect pars intercerebralis-corpus cardiacum axis is regarded as an analog of the vertebrate hypothalamo-pituitary axis (17). The effect of DHs is mediated via diuretic hormone receptors that belong to the seven-helix transmembrane G protein-coupled receptor (GPCR) superfamily (18). Insect DH-I and its cognate receptor have been hypothesized to share a common ancestor with the vertebrate CRH system (19, 20). Despite the general similarities of insect DH-I and

DH receptors with the vertebrate CRH family and CRH receptors, establishing orthology is complicated for three major reasons. First, the mature neuropeptides are short in length; second, DH-I and the vertebrate CRH family members differ vastly in gene structure; and third, the DH and CRH receptors are only a few members of the much larger GPCR family. In contrast, the evolutionary well-conserved CRH-BP does not bear appreciable sequence similarity to any other protein and appears to constitute an autonomous protein family. Therefore, CRH-BP is far better suited to establish the age of the CRH system.

We here report the presence of CRH-BP in insects. We cloned the complete coding sequence of the CRH-BP gene from the honeybee (*Apis mellifera*). Inspection of both the honeybee gene, as well as the corresponding protein, reveals striking similarities to vertebrate CRH-BP sequences. For completeness, we confirm that the honeybee, like many other insect species, possesses a DH-I sequence. Collectively, our findings strongly support the notion that the endocrine CRH signaling system, including its binding protein, is shared by insects and vertebrates and has been present since the common ancestor to both phyla.

Materials and Methods

Animals

Honeybees (*Apis mellifera*) were obtained from the Dutch beekeeping expertise center 'Het Bijenhuis' in Wageningen where they were housed according to standard beekeeping practice. Animals were rapidly sedated on ice preceding dissection.

RNA isolation and first-strand cDNA synthesis

Organs for RNA isolation were flash-frozen in liquid nitrogen. RNA isolation was conducted with Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Total RNA was precipitated in ethanol, washed, and dissolved in water. All reagents for cDNA synthesis were obtained from Invitrogen, and cDNA synthesis was carried out as previously described (15).

Cloning and sequencing

PCR was carried out with bBP.fw1 (AATGACAATGAGGAG-GTCTGT) and bBP.rv1 (TTCATACCGATATTTTTACCACA) primers based on a honeybee expressed sequence tag (BI514351). The sequence encoding the remaining N-terminal part of the sequence as well as a short stretch of 5′ untranslated region was obtained by PCR on cDNA from the head of a single bee with bBP.fw4 (GGATTCTTGAGGTT-TCATTAGAA) and bBP.rv2 primers. Similarly, the C-terminal part of the sequence as well as a partial 3′ untranslated region were obtained by PCR with bBP.fw2 (TCAACTTCATTACTTTTGATATACC) and bBP.rv7 (GATAAATTTATGAAAGACATCTAG) primers. For the assessment of CRH-BP gene expression we used the following primers: bBP.fw3 (CTGGAGATCGTTTCTCAAAGG) and bBP.rv3 (GAGCGC-GACATAAGTGCAATT). Honeybee actin (XM_393368) and 40S ribosomal protein S11 (XM_394541; not shown) were used as internal reference genes, and results were very similar after comparison with either gene. Reference gene primers were actin.fw1 (CCTAGCACCATCCAC-CATGAA), actin.rv1 (GAAGCAAGAATTGACCCACCAA), 40S.fw1 (CCCAAAAGACGGAAGCCTATG), and 40S.rv1 (AAGAATGCGTC-CTCTAATAGAAATGTT). The mature honeybee DH-I sequence was amplified with bDH-I.fw2 (GAAACGTCTTGAATCAAAACGTATC) and bDH-I.rv2 (CTTTTTCCAATCGTCTCCAAAAG) primers based on a honeybee genomic sequence retrieved from the Baylor College of Medicine honeybee genome project (assembly Amel 1.2). All oligonucleotides were obtained from Eurogentec (Seraing, Belgium). PCRs were performed with 0.2 μl *Taq* DNA polymerase (Goldstar, Eurogentec) supplemented with 1.5 mM MgCl$_2$, 200 nM dNTPs, and 400 nM of each

primer in a final volume of 25 μl. Cycling conditions were 94 C for 2 min, 30–35 cycles of 30 sec at 94 C, 30 sec at 55 C, and 1 min at 72 C followed by 10 min at 72 C, and PCRs were carried out on a GeneAmp PCR System 9700 (PE Applied Biosystems, Foster City, CA). PCR products were ligated in the pGEM-T-easy vector (Promega, Madison, WI) and cloned in JM-109 cells according to the manufacturer's protocol. Plasmid DNA was isolated with the Qiaprep Spin Miniprep kit (QIAGEN, Chatsworth, CA), and sequence reactions were carried out with the ABI Prism Bigdye terminator cycle sequencing ready reaction kit according to the manufacturer's protocol and analyzed with an ABI 377 sequencer.

Bioinformatics

Multiple sequence alignments were carried out using ClustalW 1.82. The organization of the honeybee CRH-BP gene structure was carried out by comparison of the complete cDNA sequence with the honeybee genome sequence at the Ensembl site (http://www.ensembl.org/). Other nonvertebrate CRH-BP sequences were retrieved via BLAST searches. Phylogenetic trees were constructed on the basis of amino acid differences (p-distance) by the neighbor-joining method using MEGA version 3.0 (21). Reliability of the tree was assessed by bootstrapping, using 1000 bootstrap replications.

Results

The honeybee ortholog of vertebrate CRH-BP sequences was amplified from cDNA of the head of a single worker bee. The complete coding sequence measures 999 nucleotides and encodes a 332-amino-acid protein, which is 10 amino acids longer than most vertebrate CRH-BPs. Honeybee CRH-BP shares highest identity (up to 33%) with two automatically annotated dipteran CRH-BP sequences of the malaria mosquito (*Anopheles gambiae*) and the fruit fly (*Drosophila melanogaster*) (Table 1). Amino acid identity with the more distantly related CRH-BP sequences of various vertebrate species is slightly lower at 25–29%. Eight of the 10 cysteine residues that characterize vertebrate CRH-BP sequences are conserved and identically spaced in honeybee CRH-BP, but the final pair of cysteines is absent (Fig. 1). Furthermore, throughout the alignment, several short stretches of amino acids are identical in all CRH-BP sequences. When honeybee CRH-BP is subjected to a BLAST search to identify the sequences in the Swissprot database that are most similar, the only significant hits are other CRH-BP sequences (Table 2). This once again illustrates the uniqueness of CRH-BP and provides further testimony of the unambiguous orthology of honeybee and vertebrate CRH-BP sequences.

The honeybee CRH-BP gene consists of seven exons, as is the case for all vertebrate CRH-BP genes that have been characterized (Fig. 2). Furthermore, exon sizes correspond well to the lengths of each of the seven vertebrate exons, with honeybee exons two, three, and four each extending merely one triplet over the sizes of their corresponding vertebrate exons. Also the distribution of the conserved cysteine residues over the exons is highly similar in honeybee and vertebrates, and all honeybee introns contain well-recognizable 5′ donor (GT) and 3′ acceptor (AG) splice sites.

Phylogenetic analyses corroborate the notion that all CRH-BP sequences conform to the accepted patterns of animal evolution, with sequences from more distantly related species clustering less proximately (Fig. 3). Inclusion of the human IGF-BP family as an outgroup results in a phylogenetic tree where all CRH-BP sequences form a stable cluster, separate from the IGF-BP sequences (not shown).

TABLE 1. Percentages of amino acid sequence identity for CRH-BP sequences of various animal species

	Honeybee	Malaria mosquito	Fruit fly	Sea squirt	Carp 1	Carp 2	Pufferfish	*Xenopus*	Chicken	Sheep	Rat	Mouse	Human
Honeybee	100												
Malaria mosquito	33.1	100											
Fruit fly	29.8	46.3	100										
Sea squirt[a]	26.8	28.0	28.6	100									
Carp 1	29.0	22.1	24.0	33.9	100								
Carp 2	28.3	23.3	24.6	33.9	97.8	100							
Pufferfish	26.6	23.7	23.3	33.3	67.8	68.4	100						
Xenopus	24.9	21.5	25.6	31.6	58.9	59.2	54.7	100					
Chicken	26.1	21.5	24.3	34.5	59.8	59.5	57.2	72.0	100				
Sheep	26.5	24.3	23.6	33.9	57.0	57.0	55.3	64.5	70.8	100			
Rat	26.7	21.5	23.6	33.9	58.3	58.6	55.0	63.9	71.1	77.3	100		
Mouse	26.7	21.8	24.3	32.1	60.8	60.4	55.9	67.6	73.0	79.8	93.8	100	
Human	28.9	24.3	24.6	31.6	61.7	62.0	58.1	68.2	73.9	85.1	84.5	87.3	100

[a] Partial sequence only, potentially influencing the percentages amino acid identity with other sequences.

Because of its CRH motif, insect DH-I would be a likely candidate to bind to insect CRH-BP. Despite its identification in many different insect species, DH-I has not yet been reported in the honeybee. To confirm the presence of DH-I in the honeybee, we amplified the DNA sequence encoding the honeybee mature DH-I peptide. This peptide resembles previously identified DH-I peptides from other insect species (Fig. 4). Moreover, a honeybee DH-receptor

sequence has been automatically annotated (XP_397268) from the honeybee genome.

To learn whether insect CRH-BP, like its vertebrate orthologs, is largely centrally expressed, we established the expression pattern of CRH-BP mRNA in the three major body regions of the honeybee. CRH-BP gene expression occurs in the head of the honeybee (Fig. 5) and to a lesser extent in the abdomen but is absent from the thorax.

FIG. 1. Amino acid alignment of honeybee CRH-BP with CRH-BP sequences of various vertebrate species. Cysteine residues that are involved in the formation of disulfide bonds are *shaded*. Asterisks indicate amino acid identity, whereas *colons* and *dots* indicate decreasing degrees of amino acid similarity. Accession numbers are as follows: honeybee (*Apis mellifera*), AJ780964; carp (*Cyprinus carpio*), AJ490880; *Xenopus* (*Xenopus laevis*), Q91653; chicken (*Gallus gallus*), BU358572 and BU367671; mouse (*Mus musculus*), Q60571; and human (*Homo sapiens*), P24387.

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honeybee CRH-BP   MFLNGSLYCFARLFFIIGFFNAISGAIKGNDYQRYHQQISGDRFSKDFYRQDTKNNLLNA
carp CRH-BP       ----MSGTSRAQLCFLLLSVTALRGHAR---FLDIQDNEISPEGLLSLLSSELKRELPEE
Xenopus CRH-BP    ----MTPASRPDWCLILLFLAVLRGESR---YIQMR--EAAEDALFLLN-SDFKRELSEG
chicken CRH-BP    ----MPSAFQLQCHLVLILLAASKGDTR---YLEVR--DAGEDEPFLLLSEDLKRELSAG
mouse CRH-BP      ----MSPNFKLQCHFILILLTALRGESR---YLEVQ--EAAVYDPLLLFSANLKRDLAEE
human CRH-BP      ----MSPNFKLQCHFILIFLTALRGESR---YLELR--EAADYDPFLLFSANLKRELAGE
                  .          ::: .. *   : :  :      .  :   : *.:*

honeybee CRH-BP   YVRFKLVTDCIFVTSEPGYFLYTSKNDNEEVCGIYFLAEPDQKIEINFITFDIPCEHRGL
carp CRH-BP       FV-YRRALRCLDMVAIEGQFTFTAERP-QLNCAVFFIGEPSDIISIEYDSVNIDCRGGDF
Xenopus CRH-BP    QI-YRRSLRCIDMLSIEGQFTFQADRP-QLHCALFLIGEPEEFIIIEYNFVNIDCIGGDI
chicken CRH-BP    HI-YRRCIDMLSIEGQFTFTADQP-QLHCATFFIGEPEELLTIEYDFVNIDCQGGDF
mouse CRH-BP      QP-YRRALRCLDMLSLPGQFTFTADRP-QLHCAAFFIGEPEEFITIHYDLVSIDCQGGDF
human CRH-BP      QP-YRRALRCLDMLSLQGQFTFTADRP-QLHCAAFFISEPEEFITIHYDQVSIDCQGGDF
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honeybee CRH-BP   VSIIDGWELNGEVFPSKMDHQLPLKQRSSEFCGKNIGMKRIFTSSQNIAVIEYRIPKSGK
carp CRH-BP       IKVFDGWVMKGEKFPSTQDHPLPLYKRYSDYCETGV--TRPIVRSSQNVAMLFFRLHQSGS
Xenopus CRH-BP    LKVFDGWIIKGEKFPSSLDHPLSTMERYTDICEDGD-VGSITRSSQNVAMIFFRVQQPGH
chicken CRH-BP    LKVFDGWILKGEKFPSSLDHPLPTSQRYTDFCESGA-VQRSIRSSQNVAMIFFRIHQPGN
mouse CRH-BP      LKVFDGWILKGEKFPSSQDHPLPTMKRYTDFCESGL-TRRSIRSSQNVAMVFFRVHEPGN
human CRH-BP      LKVFDGWILKGEKFPSSQDHPLPSAERYIDFCESGL-SRRSIRSSQNVAMIFFRVHEPGN
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honeybee CRH-BP   GFSLFARFLKNPRFCNVLATSLTEPYTLRNYGRRINCTYVALYPSSVQVIALGVGVSNFL
carp CRH-BP       SFTVTFRKLINPFPCNVVSQTPEGSFTMIIPQQHRNCSFSIIYPVEIQIGELSLGQHNDL
Xenopus CRH-BP    GFTLTIRKIPNLFPCNVISQSMNGRFTMITPHQHRNCSFSIIYPVVIKIFDLTLGHFNEL
chicken CRH-BP    GFTITVKKSANLFPCNVISQTPSGRFTMVIPHQHRNCSFSIIYPVVIKISDLILGHLNGL
mouse CRH-BP      GFTIITIKTDPNLFPCNVISQTPSGRFTLVVPYQHQNCSFSIIYPVAIKISDLTLGHLHGL
human CRH-BP      GFTLTIKTDPNLFPCNVISQTPNGKFTLVVPHQHRNCSFSIIYPVVIKISDLTLGHVNGL
                  .*::   :  *    * ****:: :    :*:    :: **::  :** ::: * :* . *

honeybee CRH-BP   SSTRTAETGTIRKCDESSPHDQVIIGGSNGLDTSKVHIIDSICGIDSKPDYRELTEYSVT
carp CRH-BP       KR---SILG----CAGS--GDFVELLGGNGMDTSKMYPMADLCYSFNGP-AQMKVGCDNT
Xenopus CRH-BP    QLKKPPPKG----CGDA--GDFVELLGGAGLDPSKMFPLADLCHSFHGS-AQMKIGCDNT
chicken CRH-BP    FLKNPS-VG----CAGV--GDFVELLGGTGLDPSKMFPLADLCHSFHGS-AQMKIGCDNT
mouse CRH-BP      QLKKPA-AG----CGGT--GDFVELLGGTGLDPSKMMPLADLCYPFLGP-AQMKISCDNA
human CRH-BP      QLKKSS-AG----CEGI--GDFVELLGGTGLDPSKMTPLADLCYPFHGP-AQMKIGCDNT
                  .  *       *          * *  : *. *:*.**: :  .:*      .  .  . : :

honeybee CRH-BP   SVRLISSGFFDNFVTVQIQPLKNELFNANIGI----------
carp CRH-BP       VIRMVSSGKFVNRVSFQYRLLGHQELQQMKGNSVEDVCLRA-
Xenopus CRH-BP    VVRMVSSGNFINRVTFEYNQLD-RQLEKKQGNSVEEACFPSD
chicken CRH-BP    VLRMVSSGKHINRVTFEYYQLDLQEIENRKENSIEEFCFPGI
mouse CRH-BP      VVRMVSSGKHINRVTFEYRLEPFELETSTGNSIPEYCLSSL
human CRH-BP      VVRMVSSGKHVNRVTFEYRQLEPYELENPNGNSIGEFCLSGL
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TABLE 2. List of BLAST hits after comparison of honeybee CRH-BP to the Swissprot database

Accession number	Species	Description	E value
P24388	Rat	CRH-BP	8×10^{-29}
Q28557	Sheep	CRH-BP	4×10^{-27}
Q60571	Mouse	CRH-BP	3×10^{-26}
P24387	Human	CRH-BP	1×10^{-25}
Q91653	*Xenopus*	CRH-BP	2×10^{-25}
P22482	*Bacillus pseudofirmus*	ATP synthase γ-chain	1.2
Q9Y6W3	Human	Calpain 7	6.4
P43153	*Clostridium perfringens*	Microbial collagenase precursor	8.1

Discussion

Here we describe the identification of the complete cDNA sequence of CRH-BP from the honeybee. The conservation of unique features such as key cysteine residues and gene structure provide testimony to its *bona fide* orthology with vertebrate CRH-BP sequences. The discovery of CRH-BP in the honeybee substantiates that the CRH system predates vertebrates and is likely to share ancestry with insect DH-I and its receptor. Overall amino acid identity of honeybee CRH-BP with various vertebrate CRH-BP sequences is moderate at around 25–29%, which is not surprising as the evolutionary distance between insects and vertebrates is estimated at between 700 and 993 million years (22–24). Nonetheless, the high similarity in gene structure, stable clustering in phylogenetic analyses, as well as the conservation in presence and spacing of the first eight cysteine residues all point clearly to the unambiguous orthology of vertebrate and insect CRH-BPs. The final two C-terminal cysteine residues are missing from the honeybee CRH-BP sequence. The simultaneous disappearance of this pair of cysteines is in line with the observation that they form an intrachain disulfide bridge (1). Furthermore, the obvious sequence identity between the location of both missing cysteine residues, complemented by the presence of this C-terminal cysteine pair in the predicted CRH-BP sequences of *Drosophila melanogaster* and *Anopheles gambiae*, indicates a loss of these two cysteines in the honeybee after its divergence from both dipteran species. Other than the cysteine residues, several short amino acid stretches are identical in all sequences, which suggests that these residues are structurally imperative or indispensable for ligand binding.

Establishment of orthology for the vertebrate CRH family members with insect DH-I is not straightforward, because the CRH motif is not very stringent and the mature neuropeptides are short (46 amino acids or fewer), which impairs phylogenetic analyses. Furthermore, the genes encoding all four vertebrate CRH family members (CRH, urotensin-I/urocortin-I, urocortin-II, and urocortin-III) possess two exons and are encoded completely by the second exon, whereas the coding region of tobacco hornworm (*Manduca sexta*) DH-I is divided over four exons (25). And although insect diuretic receptors and vertebrate CRH receptors both belong to the class B (secretin-like) family of GPCRs, this family also includes receptors for secretin, vasoactive intestinal peptide, PTH and its related peptide, GHRH, calcitonin, and others (26), which complicates establishment of one-to-one orthology. In contrast, the uniqueness of CRH-BP greatly facilitates establishment of orthology over large evolutionary distances.

CRH-BP takes its name from the modulation of CRH bioactivity, either antagonistically via abrogated CRH signaling (8, 9) or agonistically via extension of protein half-life (6). But CRH-BP also has the potential to bind to and modulate signaling of other members of the CRH family. In fact, several reports indicate that CRH-BP has a similar or higher affinity for urocortin-I and urotensin-I compared with CRH (4, 27). Insect DH-I, with its CRH family motif, is the most likely candidate to bind to CRH-BP. Given the colocalization of CRH and CRH-BP in the pars intermedia of carp, it is obvious to assume that CRH-BP colocalizes with DH-I in the insect corpora cardiaca. Although we were unable to demonstrate so in the honeybee, this is indeed the case in the locust *Schistocerca gregaria* (De Loof, A., and M. O. Huising, unpublished observation). Furthermore, the gene expression pattern of honeybee CRH-BP is consistent with these findings.

Although the pars intercerebralis-corpus cardiacum axis is considered the analog of the vertebrate hypothalamo-pituitary axis in general, the insect corpora cardiaca bear a morphological resemblance to the pituitary gland of fish in particular. Because fish lack a median eminence, their pituitary gland is directly contacted by hypothalamic neurons. Furthermore, the nerve
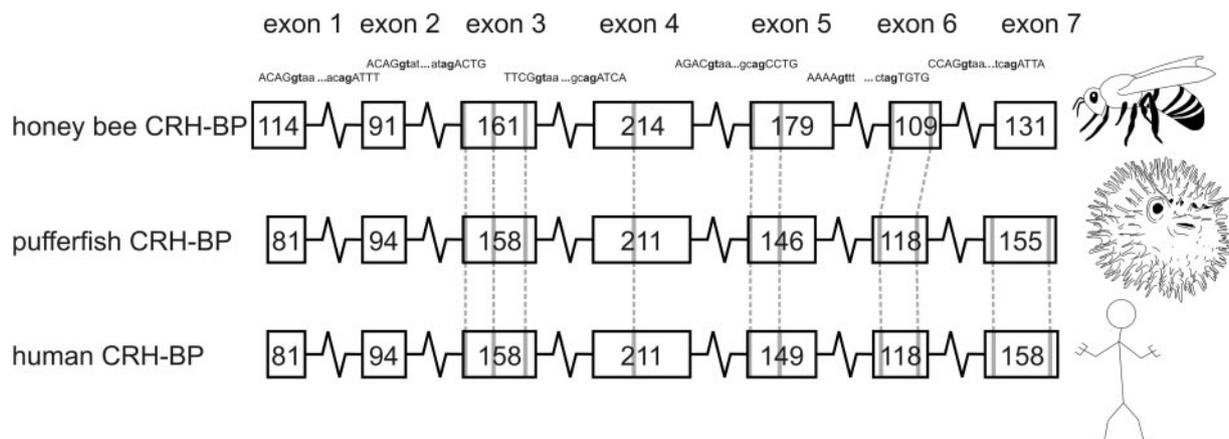

Fig. 2. Comparison of the honeybee, pufferfish, and human CRH-BP genes. *Boxes* represent exons and are drawn to scale. Exon lengths are indicated in nucleotides. The nucleotide residues surrounding each splice site are displayed, and coding residues are represented by *capitals*.

FIG. 3. Neighbor-joining tree of CRH-BP amino acid sequences. Numbers at branch nodes represent the confidence level of 1000 bootstrap replications. Accession numbers are as follows: human (*Homo sapiens*), P24387; mouse (*Mus musculus*), Q60571; rat (*Rattus norvegicus*), P24388; sheep (*Ovis aries*), Q28557; chicken (*Gallus gallus*), BU358572/BU367671; *Xenopus* (*Xenopus laevis*), Q91653; pufferfish (*Takifugu rubripes*), BN000457; carp (*Cyprinus carpio*), CRH-BP1 AJ490880 and CRH-BP2 AJ490881; sea squirt (*Ciona intestinalis*), AABS01000063; honeybee (*Apis mellifera*), AJ780964; malaria mosquito (*Anopheles gambiae*), XP_309147; and fruitfly (*Drosophila melanogaster*), NM_143536.
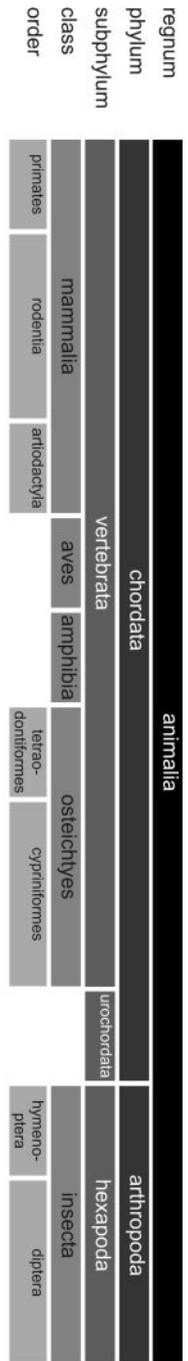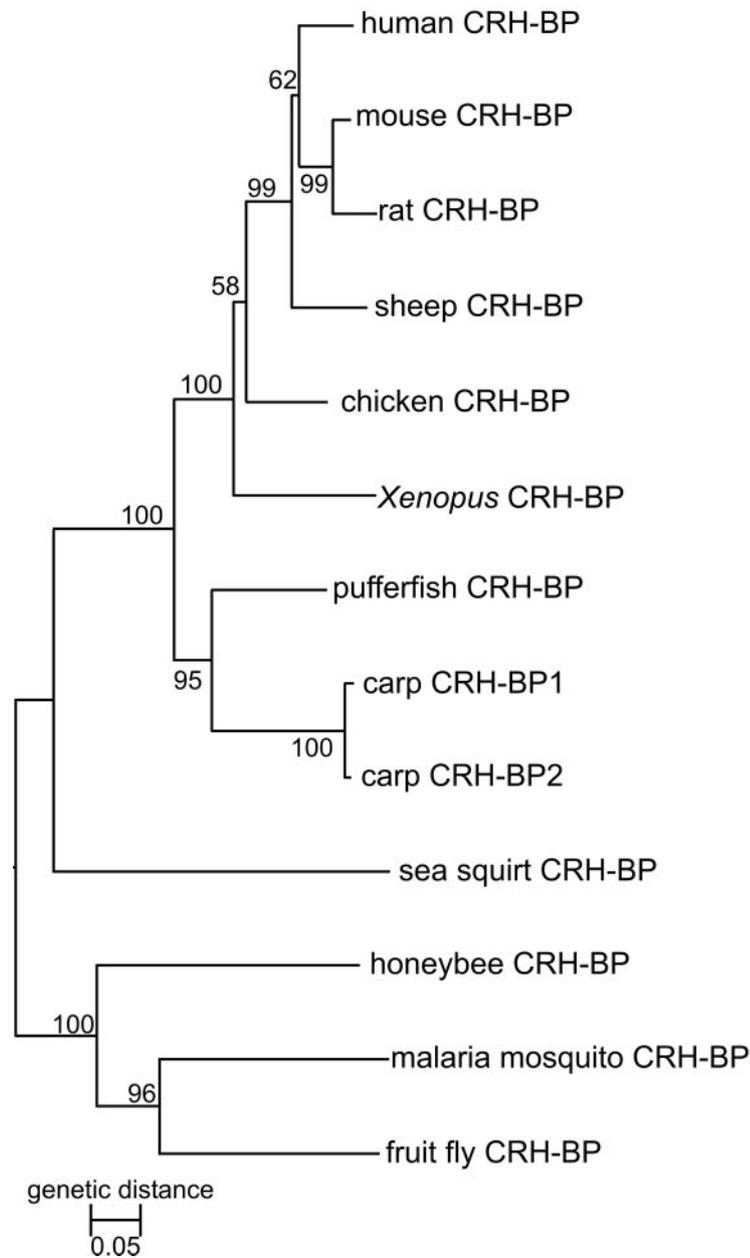
FIG. 4. Amino acid alignment of the mature honeybee DH-I peptide with DH-I peptides of other insects. Residues that conform to the prosite CRH motif (PS00511) are *shaded*. *Asterisks* indicate amino acid identity, whereas *colons* and *dots* indicate decreasing degrees of amino acid similarity. Accession numbers are as follows: honeybee (*Apis mellifera*), AJ876408; housefly (*Musca domestica*), P41537; and Pacific beetle cockroach (*Diploptera punctata*), P82373.

terminals in the pituitary pars intermedia of fish contain such an abundance of several neuropeptides, including CRH and CRH-BP (15), that it is considered a neurohemal site, analogous to the insect corpora cardiaca. A second neurohemal organ in fish, the caudal neurosecretory system, also releases CRH as well as urotensin-I (28). Interestingly, the latter peptide has a
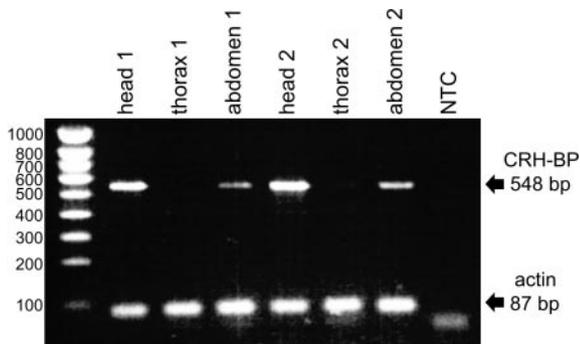
FIG. 5. Expression of CRH-BP mRNA in the head, thorax, and abdomen of two individual female worker bees. Expression of CRH-BP (35 cycles) and actin (30 cycles) was assessed by two-step RT-PCR. Reactions were carried out in separate vials, and corresponding reactions (housekeeping gene and gene of interest) were loaded in the same slots of a 1.5% agarose gel. NTC, nontemplate control. The slight primer-dimer formation in the NTC lane is fully attributable to the CRH-BP primers.

role in osmoregulation (29), analogous to insect DH-I that acts distally on the Malpighian tubules within the insect abdomen to promote active cation transport, thereby increasing primary urine production (30).

In summary, we have demonstrated that CRH-BP is well conserved and is clearly identifiable in insect species. It follows that CRH-BP has been present since the common ancestor to insects and vertebrates. More importantly, the unequivocal orthology of insect and vertebrate CRH-BPs adds substantial weight to the supposition that the vertebrate CRH system and the insect DH system stem from a common ancestor.

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