0013-7227/05/$15.00/0
Printed in U.S.A.

Endocrinology 146(5):2165–2170

doi: 10.1210/en.2004-1514

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

Mark O. Huising and Gert Flik

Department of Animal Physiology (M.O.H., G.F.), Institute for Neuroscience, Radboud University Nijmegen, 6525 ED Nijmegen, The Netherlands; and Department of Cell Biology and Immunology (M.O.H.), Wageningen University, 6709 PG Wageningen, The Netherlands

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

First Published Online February 17, 2005

Abbreviations: BP, Binding protein; DH, diuretic hormone; GPCR, G protein-coupled receptor.

***Endocrinology* is published monthly by The Endocrine Society (http://www.endo-society.org), the foremost professional society serving the endocrine community.**

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 (AATGACAATGAGGAGGTCTGT) 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 (GGATTCTTGAGGTTTCATTAGAA) 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 (GAGCGCGACATAAGTGCAATT). 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 (CCTAGCACCATCCACCATGAA), actin.rv1 (GAAGCAAGAATTGACCCACCAA), 40S.fw1 (CCCAAAAGACGGAAGCCTATG), and 40S.rv1 (AAGAATGCGTCCTCTAATAGAAATGTT). 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.

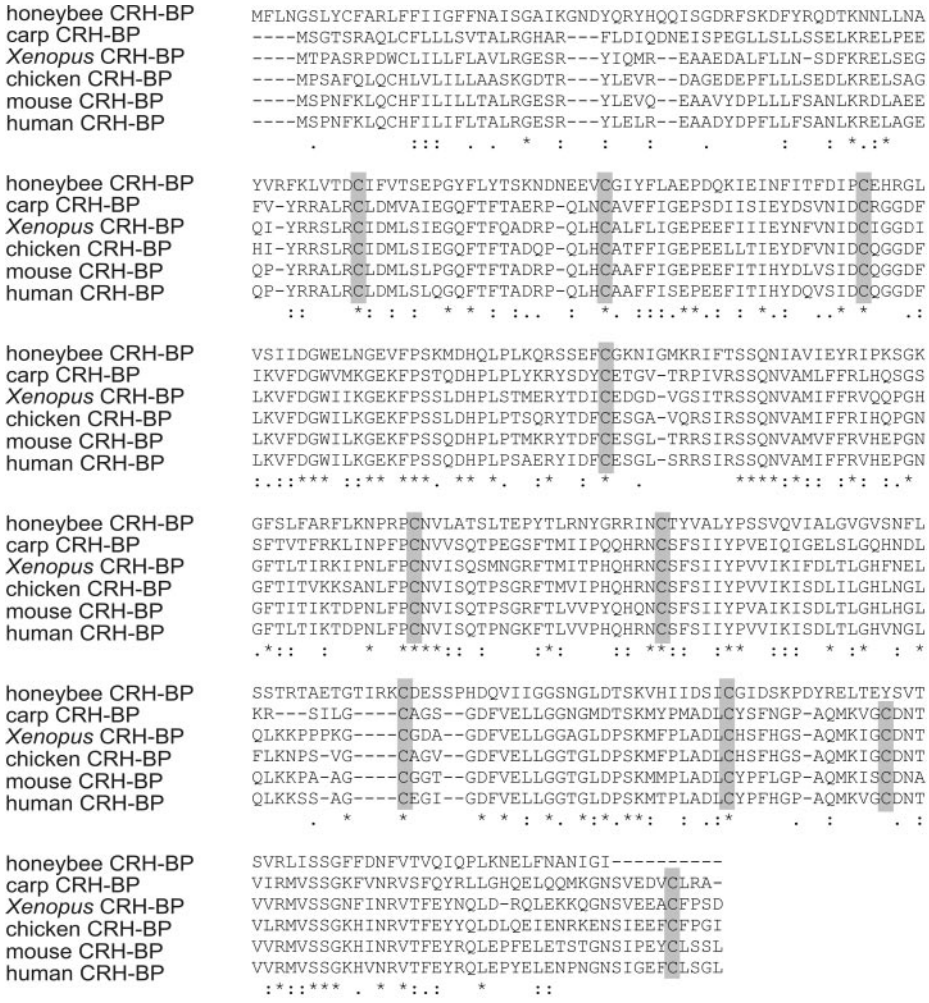

FIG. 1. Amino acid alignment of honeybee CRH-BP with CRH-BP sequences of various vertebrate species. Cysteine residues that are involved in the formation of disulfide bonds are *shaded*. *Asterisks* indicate amino acid identity, whereas *colons* and *dots* indicate decreasing degrees of amino acid similarity. 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.

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

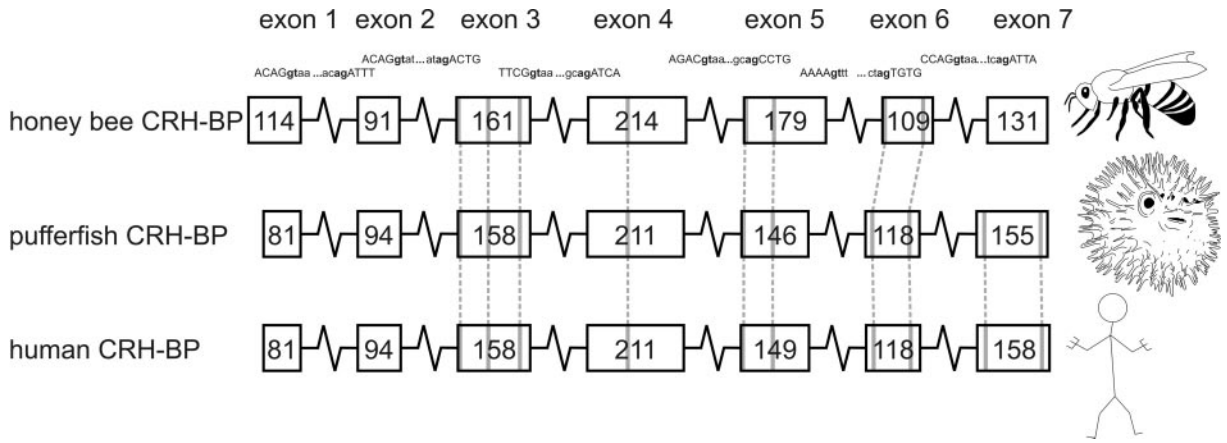

FIG. 2. Comparison of the honeybee, pufferfish, and human CRH-BP genes. *Boxes* represent exons and are drawn to scale. Exon lengths are indicated in nucleotides. The nucleotide residues surrounding each splice site are displayed, and coding residues are represented by *capitals*.

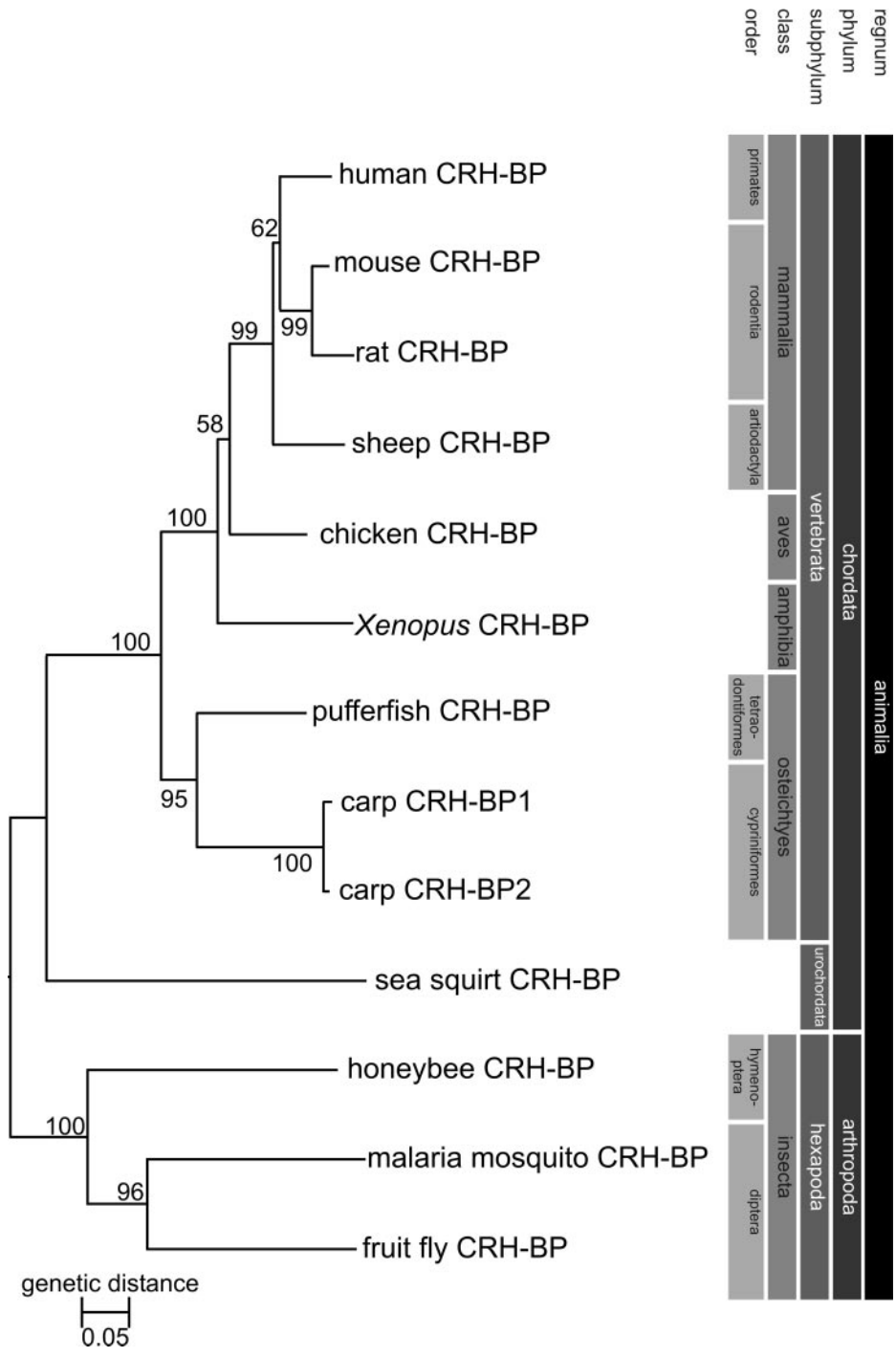

FIG. 3. Neighbor-joining tree of CRH-BP amino acid sequences. Numbers at branch nodes represent the confidence level of 1000 bootstrap replications. Accession numbers are as follows: human (*Homo sapiens*), P24387; mouse (*Mus musculus*), Q60571; rat (*Rattus norvegicus*), P24388; sheep (*Ovis aries*), 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.

```
honeybee DH-I     --RIGSLSIVNSMDVLRQRVLLELARRKALQDQAQIDANRRLLETI
house fly DH-I    --NKPSLSIVNPLDVLRQRLLLEIARRQMKENTRQVELNRAILKNV
cockroach DH-I    TGTGPSLSIVNPLDVLRQRLLLEIARRRMRQTQNMIQANRDFLESI
                  ******.:******:***:***:  :    :: ** :*:.:
```

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

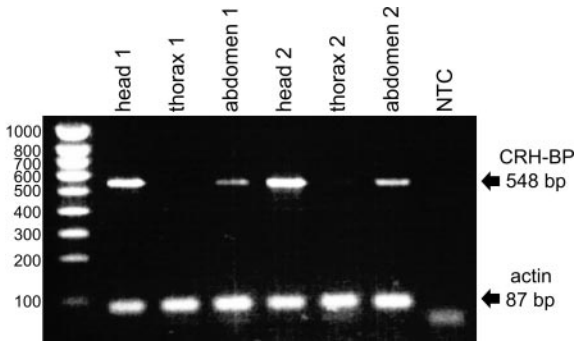

FIG. 5. Expression of CRH-BP mRNA in the head, thorax, and abdomen of two individual female worker bees. Expression of CRH-BP (35 cycles) and actin (30 cycles) was assessed by two-step RT-PCR. Reactions were carried out in separate vials, and corresponding reactions (housekeeping gene and gene of interest) 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.

Acknowledgments

We gratefully thank Mr. R. ten Kleij of the Dutch beekeeping expertise center 'Het Bijenhuis', for supplying us with honeybees and advice. We also thank Prof. A. De Loof and Mr. M. Timmermans for useful comments about the manuscript.

Received November 23, 2004. Accepted February 7, 2005.

Address all correspondence and requests for reprints to: Dr. Gert Flik, Department of Animal Physiology, Institute for Neuroscience, Radboud University Nijmegen, Toernooiveld 1, 6525 ED Nijmegen, The Netherlands. E-mail: G.Flik@science.ru.nl.

The honeybee CRH-BP and DH-I sequences have been submitted to the European Molecular Biology Laboratory database under accession numbers AJ780964 and AJ876408.

References

1. **Fischer WH, Behan DP, Park M, Potter E, Lowry PJ, Vale W** 1994 Assignment of disulfide bonds in corticotropin-releasing factor-binding protein. J Biol Chem 269:4313–4316
2. **Linton EA, Wolfe CD, Behan DP, Lowry PJ** 1988 A specific carrier substance for human corticotrophin releasing factor in late gestational maternal plasma which could mask the ACTH-releasing activity. Clin Endocrinol (Oxf) 28:315–324
3. **McLean M, Smith R** 2001 Corticotrophin-releasing hormone and human parturition. Reproduction 121:493–501
4. **Sutton SW, Behan DP, Lahrichi SL, Kaiser R, Corrigan A, Lowry P, Potter E, Perrin MH, Rivier J, Vale WW** 1995 Ligand requirements of the human corticotropin-releasing factor-binding protein. Endocrinology 136:1097–1102
5. **Vaughan J, Donaldson C, Bittencourt J, Perrin MH, Lewis K, Sutton S, Chan R, Turnbull AV, Lovejoy D, Rivier C, Rivier J, Sawchenko PE, Vale W** 1995 Urocortin, a mammalian neuropeptide related to fish urotensin I and to corticotropin-releasing factor. Nature 378:287–292
6. **Kemp CF, Woods RJ, Lowry PJ** 1998 The corticotrophin-releasing factor-binding protein: an act of several parts. Peptides 19:1119–1128
7. **Woods RJ, Grossman A, Saphier P, Kennedy K, Ur E, Behan D, Potter E, Vale W, Lowry PJ** 1994 Association of human corticotropin-releasing hormone to its binding protein in blood may trigger clearance of the complex. J Clin Endocrinol Metab 78:73–76
8. **Cortright DN, Nicoletti A, Seasholtz AF** 1995 Molecular and biochemical characterization of the mouse brain corticotropin-releasing hormone-binding protein. Mol Cell Endocrinol 111:147–157
9. **Potter E, Behan DP, Fischer WH, Linton EA, Lowry PJ, Vale WW** 1991 Cloning and characterization of the cDNAs for human and rat corticotropin releasing factor-binding proteins. Nature 349:423–426
10. **Behan DP, Heinrichs SC, Troncoso JC, Liu XJ, Kawas CH, Ling N, De Souza EB** 1995 Displacement of corticotropin releasing factor from its binding protein as a possible treatment for Alzheimer's disease. Nature 378:284–287
11. **Heinrichs SC, Lapsansky J, Behan DP, Chan RK, Sawchenko PE, Lorang M, Ling N, Vale WW, De Souza EB** 1996 Corticotropin-releasing factor-binding protein ligand inhibitor blunts excessive weight gain in genetically obese Zucker rats and rats during nicotine withdrawal. Proc Natl Acad Sci USA 93:15475–15480
12. **Heinrichs SC, Li DL, Iyengar S** 2001 Corticotropin-releasing factor (CRF) or CRF binding-protein ligand inhibitor administration suppresses food intake in mice and elevates body temperature in rats. Brain Res 900:177–185
13. **Claes SJ** 2004 Corticotropin-releasing hormone (CRH) in psychiatry: from stress to psychopathology. Ann Med 36:50–61
14. **Valverde RA, Seasholtz AF, Cortright DN, Denver RJ** 2001 Biochemical characterization and expression analysis of the *Xenopus laevis* corticotropin-releasing hormone binding protein. Mol Cell Endocrinol 173:29–40
15. **Huising MO, Metz JR, van Schooten C, Taverne-Thiele AJ, Hermsen T, Verburg-van Kemenade BM, Flik G** 2004 Structural characterisation of a cyprinid (*Cyprinus carpio* L.) CRH, CRH-BP and CRH-R1, and the role of these proteins in the acute stress response. J Mol Endocrinol 32:627–648
16. **Kataoka H, Troetschler RG, Li JP, Kramer SJ, Carney RL, Schooley DA** 1989 Isolation and identification of a diuretic hormone from the tobacco hornworm, *Manduca sexta.* Proc Natl Acad Sci USA 86:2976–2980
17. **Schoofs L, Veelaert D, Vanden Broeck J, De Loof A** 1997 Peptides in the locusts, *Locusta migratoria* and *Schistocerca gregaria*. Peptides 18:145–156
18. **Reagan JD** 1994 Expression cloning of an insect diuretic hormone receptor. A member of the calcitonin/secretin receptor family. J Biol Chem 269:9–12
19. **Chang CL, Hsu SY** 2004 Ancient evolution of stress-regulating peptides in vertebrates. Peptides 25:1681–1688
20. **Lovejoy DA, Balment RJ** 1999 Evolution and physiology of the corticotropin-releasing factor (CRF) family of neuropeptides in vertebrates. Gen Comp Endocrinol 115:1–22
21. **Kumar S, Tamura K, Nei M** 2004 MEGA3: integrated software for molecular evolutionary genetics analysis and sequence alignment. Brief Bioinform 5:150–163
22. **Nikoh N, Iwabe N, Kuma K, Ohno M, Sugiyama T, Watanabe Y, Yasui K, Shi-cui Z, Hori K, Shimura Y, Miyata T** 1997 An estimate of divergence time of Parazoa and Eumetazoa and that of Cephalochordata and Vertebrata by aldolase and triose phosphate isomerase clocks. J Mol Evol 45:97–106
23. **Wang DY, Kumar S, Hedges SB** 1999 Divergence time estimates for the early history of animal phyla and the origin of plants, animals and fungi. Proc R Soc Lond B Biol Sci 266:163–171
24. **Gu X** 1998 Early metazoan divergence was about 830 million years ago. J Mol Evol 47:369–371
25. **Digan ME, Roberts DN, Enderlin FE, Woodworth AR, Kramer SJ** 1992 Characterization of the precursor for *Manduca sexta* diuretic hormone Mas-DH. Proc Natl Acad Sci USA 89:11074–11078
26. **Hauger RL, Grigoriadis DE, Dallman MF, Plotsky PM, Vale WW, Dautzenberg FM** 2003 International Union of Pharmacology. XXXVI. Current status of the nomenclature for receptors for corticotropin-releasing factor and their ligands. Pharmacol Rev 55:21–26
27. **Baigent SM, Lowry PJ** 2000 Urocortin is the principal ligand for the corticotrophin-releasing factor binding protein in the ovine brain with no evidence for a sauvagine-like peptide. J Mol Endocrinol 24:53–63
28. **Lu W, Dow L, Gumusgoz S, Brierley MJ, Warne JM, McCrohan CR, Balment RJ, Riccardi D** 2004 Co-expression of corticotropin-releasing hormone and urotensin I precursor genes in the caudal neurosecretory system of the euryhaline flounder (*Platichthys flesus*): a possible shared role in peripheral regulation. Endocrinology 145:5786–5797
29. **Lederis K, Fryer JN, Yulis CR** 1985 The fish neuropeptide urotensin I: its physiology and pharmacology. Peptides 6(Suppl 3):353–361
30. **Coast GM** 2001 Diuresis in the housefly (*Musca domestica*) and its control by neuropeptides. Peptides 22:153–160