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Sequence of the Hexameric Juvenile Hormone-binding Protein from the Hemolymph of *Locusta migratoria**

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The cDNA for the hexameric hemolymph juvenile hormone-binding protein (JHBP) from the migratory locust has been cloned and sequenced. Antiserum raised against purified JHBP was used to identify clones in an expression library. The 4.3-kilobase JHBP mRNA codes for 668 amino acids (74.4 kDa) and contains 2 kilobases of 3′-untranslated region. The derived amino acid sequence reveals that locust JHBP represents a new group within the hexamerin family of arthropod proteins. JHBP appears to be more closely related to arthropod hemocyanins, the believed ancestors of the family, than to the other known insect hexamerins. The mRNA shows a high (89%) bias to codons ending in G or C and the codons ending in A or T are clustered and concentrated toward the 5′ end, suggesting a mosaic gene structure. The recombinant bacterially expressed protein bound [^{3}H]JH III with the same affinity as the protein from hemolymph. A truncated version of JHBP lacking 53 amino acids from the N terminus did not bind JH III. Hybridization analysis of fat body JHBP mRNA in locusts that had been treated with precocene and a JH analog did not give clear evidence for regulation by JH.

Juvenile hormone (JH)[1] regulates many diverse events in the life cycle of an insect (1, 2). Evidence exists for both cell membrane and nuclear receptors involved in the actions of JH, but as yet no protein with proven JH receptor activity has been isolated (2). Before JH interacts with its receptor, biosynthetic enzymes in the corpus allatum and carrier proteins and degradative enzymes in the hemolymph and target cells act to modulate the levels and location of the hormone. Thus, the combined activity of many proteins orchestrates the final effect of JH, and knowledge of the structures and functions of these is essential for an understanding of JH action at the molecular level. Certain JH analogs are effective in insect pest management (3), and knowledge of the ancillary proteins may also be instrumental in the further development of such agents.

JH-binding proteins (JHBP) or carriers in the hemolymph are responsible for the transport of JH to its target tissues (4). JHBP can limit the loss of JH by nonspecific effects such as adsorption to hydrophobic surfaces or cleavage by general esterases, while the bound hormone may remain susceptible to specific JH esterases that modulate its hemolymph titers. Three classes of JHBP have been characterized in different insect groups. Low molecular weight JHBP, found in the Lepidoptera, are the best studied type, and include the 31-kDa JHBP from the hornworm, *Manduca sexta*, which has been cloned and sequenced (5, 6) and shows no clear homology to other known hormone-binding proteins. The high molecular weight carriers of various insects (for example, cockroaches and beetles) are lipophorins, composed of 240- and 80-kDa subunits and up to 40% of lipid (4, 7). In the locust, *Locusta migratoria*, however, the major hemolymph JHBP has been identified as a high molecular mass protein distinct from lipophorin, having a native M_r of 566 kDa, six 77-kDa subunits, and 15% lipid (8–10). It has a high affinity for 10*R*-JH III (K_d = 1–2 nM), and 6 binding sites/hexameric molecule (8, 11). The hemolymph JHBP of the grasshopper, *Melanoplus sanguinipes*, has recently been described as a similar hexamer (12). These findings strongly suggest that the JHBPs of these members of the order Orthoptera belong to the protein superfamily known as hexamerins (13, 14).

The hexamerins are arthropod proteins composed of six 70–80-kDa subunits and include the oxygen-carrying hemocyanins of crustaceans and arachnids, as well as the insect hexamerins, the roles of which include providing amino acid reserves (storage proteins) and binding small molecules such as biliverdin (13, 15). The insect hexamerins that have been sequenced are mainly from the advanced (endopterygote) orders, Lepidoptera and Diptera, and have been placed in four groups (14): 1) methionine-rich, containing approximately 10 mol % methionine; 2) lepidopteran arylphorins which have a high content of aromatic amino acids, especially tyrosine; 3) dipteran arylphorins; and 4) so-called JH-supressible proteins of certain Lepidoptera. On the basis of new sequence data, an additional grouping comprising JH-suppressible arylphorins from the Colorado potato beetle (Coleoptera) and a cockroach (Dictyoptera) has recently been proposed (16). To enhance our understanding of hexamerin evolution and function, additional sequences of hexamerins from the more primitive exopterygote insect orders, and especially proteins with specific physiological roles, are needed. This paper contributes to these goals through the cloning and sequencing of cDNA for locust hexameric JHBP.

MATERIALS AND METHODS

Hormones and Chemicals—10*R*-[^{3}H]JH III (45–60 Ci/mmol) synthesized biologically (17) was given by Dr. S. S. Tobe. Racemic unlabeled JH III and ethoxyprecocene were purchased from Sigma. [^{3}H]EFDA (10*R*,11*S*-epoxyfarnesyldiazoacetate) was given by Dr G. Prestwich. Pyriproxyfen was a gift from the Sumitomo Chemical Co. (Osaka, Japan).

Locusts (*L. migratoria*) were reared and some were treated with ethoxyprecocene to destroy the corpora allata as described previously

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) U74469.

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[1] The abbreviations used are: JH, juvenile hormone; apoLp-III, apolipophorin III; EFDA, 10*R*,11*S*-epoxyfarnesyldiazoacetate; HAP, hydroxylapatite; JHBP, juvenile hormone-binding protein; PAGE, polyacrylamide gel electrophoresis; PEG, polyethylene glycol; rJHBP, recombinant JHBP; kb, kilobase(s).

(18). This treatment has been shown to radically reduce hemolymph JH (19) and prevent expression of the JH-dependent vitellogenin genes (18, 20, 21). The JH analog, pyriproxyfen, was applied as 10 μg topically in acetone, a dose that is effective in inducing vitellogenin synthesis (22). The hydroxylapatite JH-binding assay, Scatchard analysis, and polyacrylamide gel electrophoresis (PAGE) were performed as described in detail elsewhere (11). Standard procedures for molecular biology were carried out according to Ausubel *et al.* (23) or the manufacturers' instructions for specific kits.

Purification of JHBP—Hemolymph (about 5 ml) was collected by neck puncture from adult male locusts into cold buffer (50 mM imidazole, pH 6.6, 0.1 mM phenylmethylsulfonyl fluoride) containing a few crystals of phenylthiourea, and was centrifuged at 1,000 $\times$ *g* for 20 min to remove cells. Imidazole buffer was added to make 7-fold dilution relative to the original hemolymph volume, and polyethylene glycol 20,000 (PEG) was added to make 5% (w/v) concentration. After 1 h on ice, the sample was centrifuged at 10,000 $\times$ *g* for 30 min; the supernatant was then adjusted to 10% PEG, held 1 h on ice and spun again. The pellet was dissolved in column buffer (20 mM Tris-HCl, pH 8.0, 25 mM KCl, 1 mM dithiothreitol, 10% glycerol) and applied to a 10-ml heparin-agarose column (Life Technologies, Inc.). After extensive washing with column buffer, JHBP was eluted with a 25–500 mM KCl gradient. Fractions containing JHBP, identified by nondenaturing PAGE, binding of [^{3}H]JH III in the hydroxylapatite assay, and reactivity with JHBP-specific antisera (11), were pooled and concentrated by ultrafiltration. At this stage, the preparation showed a single band in heavily loaded SDS-PAGE (not shown), but two bands in nondenaturing PAGE (Fig. 1). Additional purification was therefore attempted by preparative isoelectric focusing in a Rotofor apparatus (Bio-Rad) in a pH 3–10 ampholyte gradient at 12 W for 3 h, and by chromatography on a 270 ml of Sephacryl S-300 (Pharmacia) column in column buffer (as above) with 100 mM KCl, but the product after these steps still showed a doublet pattern in nondenaturing PAGE. The yield was about 250 μg of protein/ml hemolymph after heparin-agarose chromatography, or 100 μg/ml after the two additional steps.

N-terminal sequencing was performed by the Queen's University Protein/DNA Chemistry Core Facility, using a protein sample prepared by the extended procedure (isoelectric focusing and gel exclusion chromatography). Antiserum to this same protein sample was produced in male New Zealand White rabbits.

Cloning of JHBP—A cDNA library, made from adult female fat body mRNA using a ZAP-cDNA synthesis kit (Stratagene), was screened for expression from recombinant clones with the *pico*Blue Immunoscreening Kit (Stratagene). Briefly, the expression of a short β-galactosidase coding sequence and the cDNA insert were induced with isopropyl-β-D-thio-galactoside and clones were screened using polyclonal JHBP antiserum (1:10,000). Recombinant Bluescript plasmids were obtained by *in vivo* excision of the λ clones. Plasmids grown in XL-1 Blue bacteria were purified with a Wizard mini-plasmid preparation kit (Promega), analyzed for restriction sites, and used for sequencing.

For Northern blot analysis, poly(A)$^+$ RNA was purified by a microextraction kit (Pharmacia), electrophoresed in formaldehyde gels, transferred to membranes (GeneScreen *Plus*), hybridized with probe at 42 °C for 24 h, and subjected to audioradiography. Labeled probes for JHBP mRNA were prepared with 5 μCi of [α-^{32}P]dCTP in a polymerase chain reaction using sequencing primers 1 and reverse 5 (see Fig. 2, legend), *Taq* polymerase (Promega), and 20 cycles of amplification (2 min each at 94° for denaturation, 50 °C for annealing and 72 °C for extension). A control probe with sequences of locust apolipophorin III (apoLp-III; Ref. 24) was labeled using an OligoLabeling Kit (Pharmacia). Probes were purified from unincorporated nucleotides by Sephadex G-50 spin columns. For quantification, bands were cut from the GeneScreen membrane and counted by liquid scintillation. JHBP mRNA levels were expressed relative to apoLp-III mRNA, which provides a JH-independent control since locust hemolymph lipophorin and fat body transcription of the *apoLp-III* gene have been shown to be unaffected by JH (21, 25).

Sequence Analysis—Sequence entry, editing, and analysis were carried out using programs from the IBI Sequence Analysis System and Data base Manager in Cyborg Environment (International Biotechnologies, New Haven, CT). Homology searches of amino acid sequences were done using the BLAST algorithm and the nonredundant protein data base at the National Centers of Biotechnology Institute (Bethesda, MD) (26). The Phylogenetic Inference Package (PHYLIP) from Dr. J. Felsenstein (University of Washington, Seattle) was used to calculate protein distances and generate phylogenetic trees. To produce a consensus tree, sequences were bootstrapped (100 replicates) using the SEQBOOT program, followed by the PROTDIST program (default values, Dayhoff PAM matrix), NEIGHBOR (default values), and then CONSENSE.

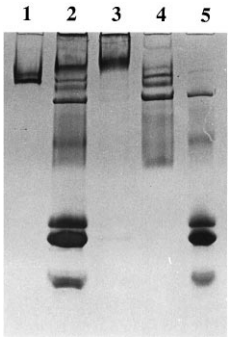

FIG. 1. **Proteins from adult male locust hemolymph present in stages of JHBP purification, visualized by Coomassie Blue staining after nondenaturing PAGE (4–15% acrylamide gradient).** *Lane 1*, purified JHBP after heparin-agarose chromatography (5 μg); *2*, crude hemolymph (25 μg); *3*, 5% PEG pellet (10 μg); *4*, 10% PEG pellet (10 μg); *5*, 10% PEG supernatant (10 μg).

Most of the sequences used for comparison were obtained from Beintema *et al.* (14), and original references may be found in their paper. Following their practice, proteins are named in capital letters with an abbreviated genus name followed by a functional designation (see Fig. 4, legend).

Recombinant JHBP—Overnight cultures of bacteria containing recombinant plasmids were spun down and pellets were suspended in extraction buffer (50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride) containing 0.1 mg/ml lysozyme and kept at room temperature for 1 h. The extract was spun in a microcentrifuge for 15 min and the supernatant was used for JH binding assays.

RESULTS

Purification of JHBP—Male hemolymph was used in order to avoid the abundant vitellogenin present in females, and JHBP was purified as described under "Materials and Methods." Fig. 1 illustrates the fractionation of locust hemolymph proteins by PEG precipitation. A bright yellow precipitate from 5% PEG solutions was almost solely lipophorin (*lane 3*). The hexamerins, including JHBP and at least two storage proteins, one of which dissociated partially to subunits (27), precipitated mainly with 10% PEG (*lane 4*), while the low molecular weight proteins remained soluble (*lane 5*). JHBP obtained after PEG precipitation and heparin-agarose chromatography showed a doublet, but no other bands, on Coomassie Blue-stained nondenaturing PAGE (Fig. 1, *lane 1*). Both components of the doublet are believed to be JHBP since two additional steps of purification (see "Materials and Methods") resulted in no perceptible change in the electrophoretic picture, and the preparation yielded an unambiguous 41-amino acid N-terminal sequence. All Western blots showed the doublet regardless of the tissue source of the protein (hemolymph, oocytes, testes, male accessory glands, fat body; data not shown). When JHBP was labeled with [^{3}H]EFDA, a JH III photoaffinity analog, and subjected to nondenaturing electrophoresis, long exposures indicated a similarly-placed second minor band (data not shown). It remains possible, however, that the two electrophoretic components represent different proteins sharing very similar physicochemical properties, one of which is N-terminally blocked.

Cloning of JHBP cDNA—The JHBP antiserum was used to screen approximately 10^5 plaques from an adult female locust fat body cDNA expression library and six positives were picked. Three of these and a negative control were purified, and the recombinant Bluescript plasmids were recovered by *in vivo* excision. Restriction enzyme analysis showed that two of the positives were identical, with an insert of approximately 4.6 kb, and the third had an insert of about 2.5 kb. The bacterially

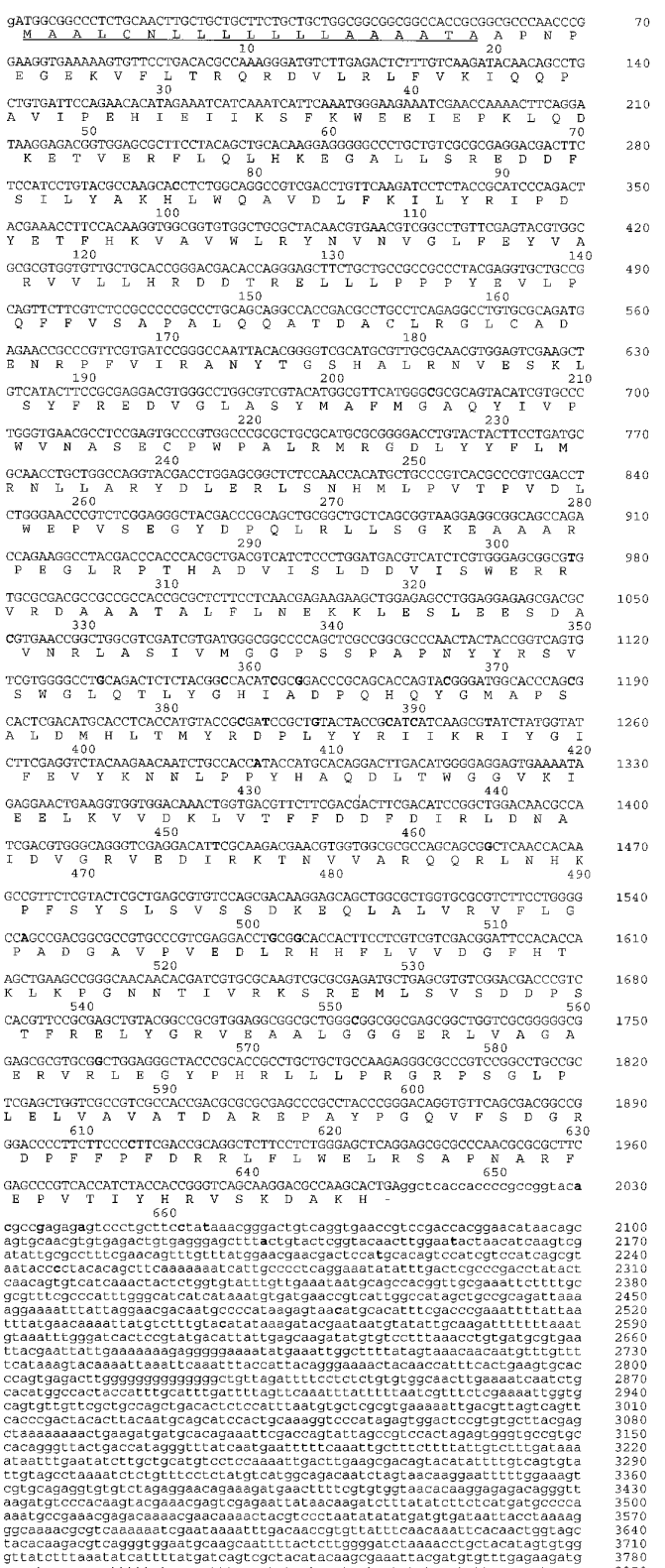

FIG. 2. **Nucleotide sequence of jhbp and predicted amino acid sequence.** Numbering of nucleotides is at the right and numbering of amino acids is below the sequence. Nucleotides that are different in jhbp-s are in *bold*. The putative signal peptide and a consensus polyadenylation signal are *underlined*. Methods: sequencing was done by the Queen's University Core Facility, using successive primers and an Applied Biosystems 373A automatic DNA sequencer. Vector-specific primers were used to obtain terminal sequences. Internal primers were 18 nucleotides long, starting at the following numbers: 1) 276; 2) 625; 3) 879; 4) 1256; 5) 1603; 6) 1649; 7) 1956; 8) 2297; 9) 2648; 10) 3018; 11) 3401. For sequencing in the opposite direction the corresponding reverse complement primers were used.

expressed proteins were tested for binding of JH III. Both of the clones with large inserts produced protein that bound 10*R*-[^{3}H]JH III specifically (competed by unlabeled JH III), whereas proteins expressed from the small-insert clone and the negative control did not show any binding (see Fig. 5). DNA sequencing was therefore initiated on a clone with a 4.6-kb insert, designated jhbp, using a series of consecutive primers. The clone with the 2.5-kb insert, designated jhbp-s, was also partially sequenced to identify its product.

Nucleotide Sequence of jhbp—The complete sequence of jhbp (Fig. 2) contains 4016 nucleotides, with an open reading frame of 2004 nucleotides, coding for a protein of 668 amino acids, followed by a stop codon and a 3′-untranslated region of 2008 nucleotides. A consensus polyadenylation signal sequence occurs 22 nucleotides upstream of the poly(A) tail. At the 5′ end, a single nucleotide (G) precedes an ATG codon, which is followed by a sequence coding for a hydrophobic-rich putative signal peptide of 19 amino acids. The next 41 amino acids are identical with the sequence obtained from the purified JHBP. The reactivity of the recombinant protein to JHBP antiserum, specific binding of JH III and identical N-terminal sequence to the JHBP protein purified from hemolymph, indicate that the jhbp clone represents the sequence of JHBP mRNA.

Sequencing into the vector DNA revealed that the jhbp insert is out of frame with the β-galactosidase coding region, so that the expressed protein is not a fusion protein. Since the clone was identified from expressed protein, this suggests that translation of the recombinant JHBP (rJHBP) was initiated from its own methionine. Western blots of proteins separated by SDS-PAGE showed the size of rJHBP to be consistent with expectation from the jhbp sequence (74 kDa), and slightly smaller than the protein from hemolymph (77–80 kDa), even though rJHBP presumably includes a signal peptide. This suggests that the hemolymph JHBP may be glycosylated, and the jhbp sequence contains three potential glycosylation sites at Asn^{196}, Asn^{236}, and Asn^{542}.

A surprising feature of the jhbp sequence is the bias for codons ending in G or C. Of the 668 codons, 592 (88.6%) were found to end in G or C and only 76 (11.4%) end in A or T. This bias makes the coding region G/C-rich (64% G/C), whereas the 3′ non-coding sequences are A/T-rich (38% G/C). The codons ending in A or T are distributed in a non-random fashion so that most of them (40) are next to another A/T codon, and of these 6 are in triplets and 8 are in quadruplets; this clustered distribution is significantly different from that predicted by Monte Carlo analysis, in which only 18 A/T codons would be adjacent to another A/T codon (data not shown). In addition, the A/T codons are concentrated toward the 5′ end of the sequence, so that 41% of the first 70 codons end in A or T, while only 9% of the the remaining 598 codons do so. Codon usage in the *jhbp* gene, therefore, exhibits a non-random pattern with the 5′ end differing from the remainder of the message.

The exceptionally long 3′-untranslated sequence of the JHBP mRNA shows no evidence for insertion of a Lm1-family SINE repeat element, which accompany some locust genes (28), or any other sequence duplication. Northern blots (see Fig. 6) revealed a 4.3-kb message, which corresponds to the size expected from the cDNA clone.

Clone jhbp-s—The clone, jhbp-s, was of interest because its bacterially expressed protein product did not bind [^{3}H]JH III (see below), and it was therefore sequenced. The sequence was found to represent a truncated form of jhbp, missing 215 nu-

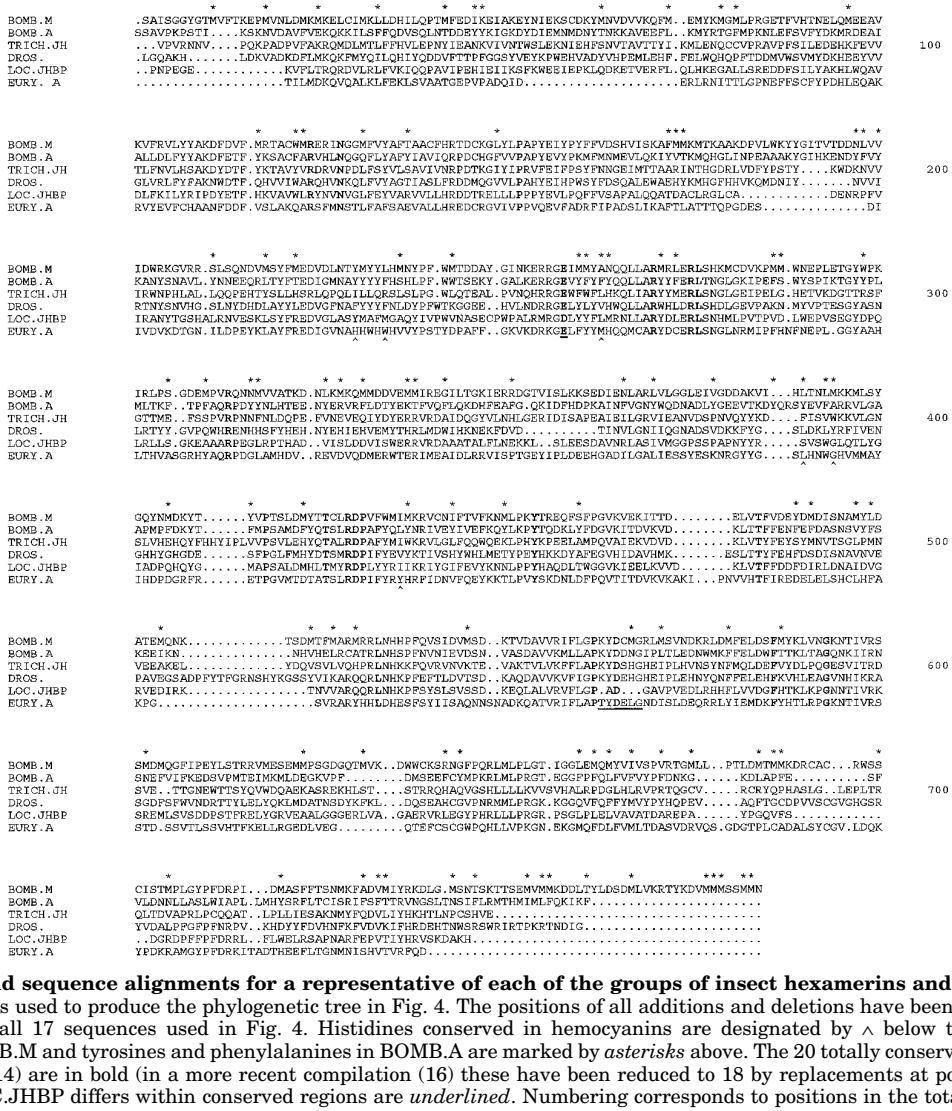

FIG. 3. **Amino acid sequence alignments for a representative of each of the groups of insect hexamerins and a hemocyanin.** The alignment shown was used to produce the phylogenetic tree in Fig. 4. The positions of all additions and deletions have been kept to illustrate the alignment used for all 17 sequences used in Fig. 4. Histidines conserved in hemocyanins are designated by ^ below the sequence. Unique methionines in BOMB.M and tyrosines and phenylalanines in BOMB.A are marked by *asterisks* above. The 20 totally conserved residues identified by Beintema *et al.* (14) are in bold (in a more recent compilation (16) these have been reduced to 18 by replacements at positions 258 and 276). Residues where LOC.JHBP differs within conserved regions are *underlined*. Numbering corresponds to positions in the total diagram and not to a specific sequence. Protein designations are listed under Fig. 4.

cleotides from the 5′ end, presumably as a result of incomplete reverse transcription during cDNA synthesis. At the N terminus, 53 amino acids of the mature protein and the 19-amino acid signal sequence are lacking; as a result of in-phase fusion, these are replaced by 41 amino acids from β-galactosidase. The coding region of jhbp-s was found to differ from jhbp in 25 nucleotides, all of which were synonymous substitutions in the third positions of codons, while a further 12 base changes in the 3′ region of jhbp-s were distributed randomly. jhbp-s may therefore represent an allele of jhbp. The sequence of jhbp-s is also truncated in the 3′ end, and no polyadenylation signal can be found; the data indicate that cDNA transcription initiated from oligo-(dT) primer bound to the A-rich sequence seen at nucleotide 2674 of the jhbp sequence. No species of mRNA that could correspond to jhbp-s was detected.

Amino Acid Sequence of JHBP—From the amino acid sequence predicted from jhbp, mature JHBP was calculated to have a molecular mass of 74.4 kDa and an isoelectric point of pH 6.72. The calculated amino acid composition is similar to those of other hexamerins, except that JHBP is relatively rich in alanine (10 mol % *versus* approximately 4 mol % in others; Ref. 29), and there are fewer methionine (2 mol %) and tyrosine (4 mol %) residues than in the methionine-rich hexamerins and arylphorins, respectively.

Using the BLAST search, locust JHBP was found to have similarity to other members of the hexamerin superfamily but not to any other proteins, including the JHBP of *M. sexta* (5), the JH esterase of *Trichoplusia ni* (30), or other hormone-binding proteins. For comparing hexamerin sequences, the amino acid sequence of JHBP was aligned with the framework constructed by Beintema *et al.* (14) which, based on x-ray crystallographic structures, should be more reliable than computer-derived alignments. Seventeen sequences (see Fig. 4) were used in the alignment, which is illustrated in Fig. 3 by representatives of each major hexamerin group. Of the 18 residues that are conserved in all the other sequenced hexamerins (14, 16), which are mostly charged or polar and presumably important for protein structure (31), all are retained in JHBP. This suggests that JHBP is similar in higher-order structure to the other hexamerins.

From the aligned hexamerin sequences, a phylogenetic tree was constructed and protein distances were determined, as described under "Materials and Methods." The consensus tree (Fig. 4) has similar topology to that constructed by Beintema *et al.* (14, 16), with the insect hexamerins classed into several groups (see Introduction), distinct from the hemocyanins. Comparison of protein distances showed that locust JHBP is not closely related to any other protein within the set, and represents a new group in the hexamerin superfamily. The JHBP sequence, however, is more similar to the arthropod hemocya-

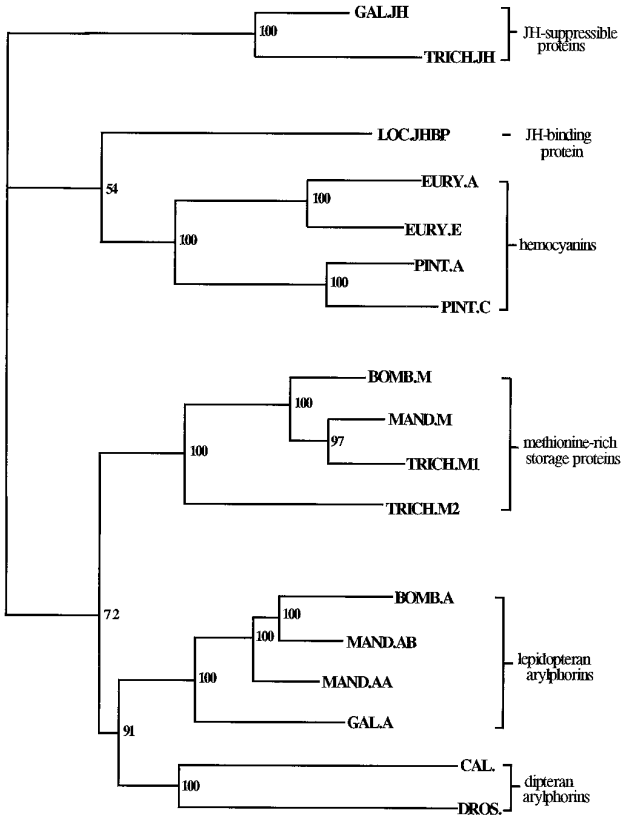

FIG. 4. **Consensus tree of insect hexamerin and hemocyanin sequences constructed by programs in the Phylogenetic Inference Package as described under "Materials and Methods."** *Numbers* represent the percentage of trials (100 replicates) that produced the nodes shown. *Horizontal line* lengths are proportional to the distances between proteins. Abbreviated names of arthropods and proteins: *BOMB*, *Bombyx mori*; silkworm (*A*, arylphorin; *M*, methionine-rich); *DROS*, *Drosophila melanogaster*, fruit fly (arylphorin); *CAL*, *Calliphora erythrocephala*, blowfly (arylphorin); *EURY*, *Eurypelma californicum*, tarantula (*A*, hemocyanin subunit a; *E*, subunit e); *GAL*, *Galleria mellonella*, wax moth (*A*, arylphorin; *JH*, JH-suppressible); *LOC*, *L. migratoria*, locust (JHBP); *MAND*, *M. sexta*, tobacco hornworm (*AA*, arylphorin α; *AB*, arylphorin β; *M*, methionine-rich); *PINT*, *Panulirus interruptus*, spiny lobster (*A*, hemocyanin subunit a; *C*, subunit c); *TRICH*, *Trichoplusia ni*, cabbage looper (*JH*, JH-suppressible; *M1*, basic JH-suppressible protein 1; *M2*, basic JH-suppressible protein 2).

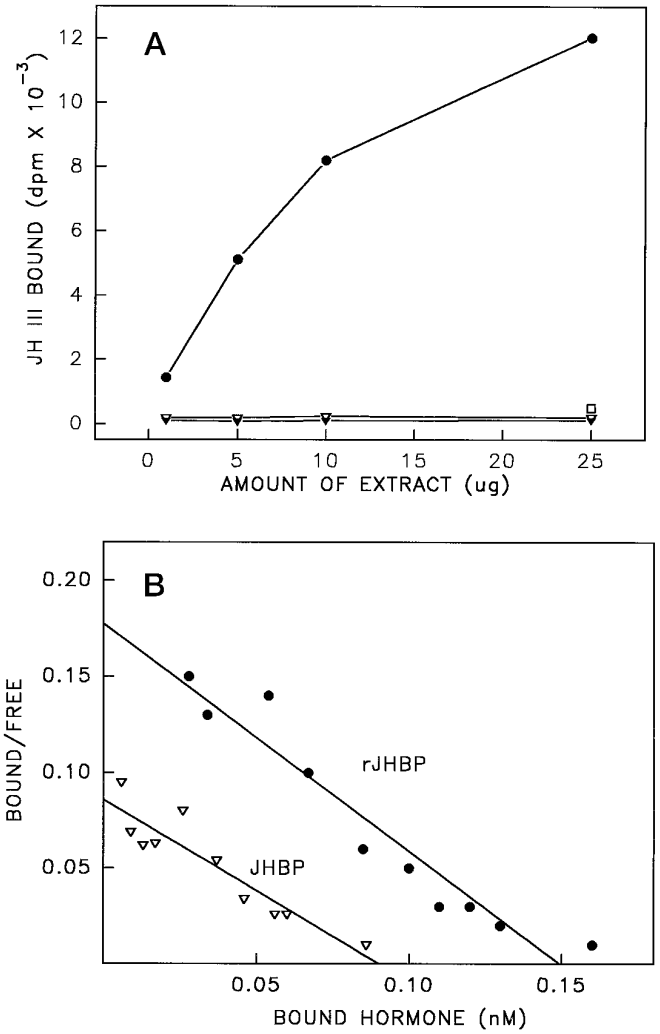

FIG. 5. **JH-binding of recombinant proteins.** *A*, binding of 10*R*-[^{3}H]JH III by extracts (25 μg of protein) from bacteria containing plasmids jhbp (●), jhbp-s (▽), or control plasmid from an immunonegative plaque (▼). Also shown is the binding measured in jhbp extracts in the presence of 100 × excess cold racemic JH III (□). *B*, Scatchard analysis of JH III binding by rJHBP (●) and locust hemolymph JHBP (▽). JH binding by the hydroxylapatite assay and Scatchard analysis were done as described previously (11).

nins than to the other insect hexamerins. When the hemocyanin sequences were removed, JHBP grouped nearest to the lepidopteran JH-suppressible proteins. Recently, the dimeric pro-phenoloxidases from three insect species have been shown to be homologous with arthropod hemocyanins (32–34), but an examination of sequence relationships (not shown) found that locust JHBP is closer to the hemocyanins than to the prophenoloxidases.

Characterization of Recombinant Proteins—In bacterial extracts, only those expressing the rJHBP protein showed any binding of 10*R*-[^{3}H]JH III (Fig. 5*A*). Addition of 100-fold excess of unlabeled JH III reduced the binding to background, indicating that it is specific. Western blots showed that rJHBP-S was present at levels about 5 × less than rJHBP (data not shown), but this difference was insufficient to account for the lack of detectable JH III binding, and fractionation and concentration of rJHBP-S extracts by PEG precipitation still failed to produce any measurable JH III binding. When rJHBP and rJHBP-S extracts were mixed, no evidence was found for an inhibitor in the latter, and there was no strong JH esterase activity (assayed by the method of Share and Roe (35); data not shown). All the evidence indicated that the rJHBP-S protein is unable to bind JH III.

Scatchard analysis of the rJHBP in crude extracts (Fig. 5*B*) found that the affinity for 10*R*-JH III of the recombinant protein (K_d = 0.9 nM, n = 2) is the same as that of the insect-produced protein (K_d = 1.0 nM, n = 2). The binding measured in the extracts was stable for at least 18 h at 4 °C, and under the binding conditions there was no measurable JH esterase activity in the bacterial extract or the hemolymph.

Effect of JH on JHBP mRNA Levels—Northern blot analysis of fat body poly(A)$^+$ RNA from male and female adult locusts showed a major mRNA species, hybridizing to jhbp, of about 4.3 kb (Fig. 6). The bands near 2.5 kb are likely due to nonspecific hybridization with rRNA. In fat body extracts from male precocene-treated (JH-deprived) locusts, the amount of JHBP mRNA, relative to apoLp-III mRNA, was approximately 70% of that found in normal day 10 locusts (n = 3, 2 fat bodies per extract). Two days after application of the JH analog, pyriproxyfen, to precocene-treated animals the level was 76% of normal, not a significant increase over precocene-treated controls. Females, in response to endogenous JH or applied JH analogs, produce massive amounts of vitellogenin mRNA (up to 50% of the total poly(A)$^+$ RNA; Ref. 18), and this effect resulted in a large decrease in the JHBP signal (Fig. 6, *lanes 4* and *6*),

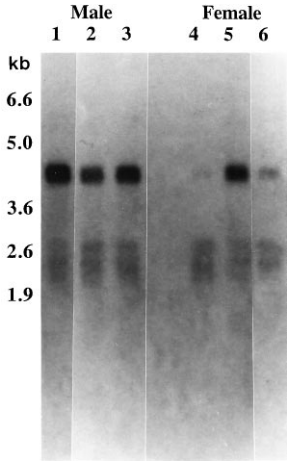

FIG. 6. **Northern analysis of JHBP mRNA.** Poly(A)$^+$ RNA from adult locust fat body from normal males (*Lane 1*), precocene-treated males (*Lane 2*), precocene-treated males treated with 10 μg of pyriproxyfen 48 h previously (*Lane 3*), normal vitellogenic females (*Lane 4*), precocene-treated females (*Lane 5*), and precocene-treated females treated with 10 μg of pyriproxyfen 48 h previously (*Lane 6*) were analyzed as described under "Materials and Methods." Each lane contains 1 μg of RNA isolated from two pooled fat bodies. Positions of RNA size markers (Pharmacia) are indicated.

as well as the control apoLp-III mRNA (not shown), masking any effect on JHBP expression. The presence of JHBP message in precocene-treated adult females, however, shows that it is not dependent on JH.

DISCUSSION

The hexameric JHBP from locusts, the JH-binding properties and tissue distribution of which have previously been studied (8–11), has now been cloned as cDNA and sequenced. This represents the first sequence for a non-lipophorin high molecular weight JH-binding protein, and also one of the first hexamerin sequences from the relatively primitive exopterygote group of insects.

JHBP is the major JH carrier in the hemolymph of locusts and may play a similar role inside the hormone target cell (11). From the hemolymph concentration of JHBP (10) and its affinity for JH III (11), it can be calculated that essentially all (>99.9%) of the JH III in the hemolymph will be bound. Therefore, the recognition of JH III by target cells is expected to be in the form of the JH III-JHBP complex, and JHBP is an important component of the JH signal (2). Knowing the sequence of JHBP is prerequisite for identification of domains or amino acid residues that are involved in its interaction with hormone, or with other proteins such as membrane receptors. The ability of rJHBP to bind JH III with the same affinity as the insect-produced protein suggests that post-translational modification does not greatly affect JH binding. Post-translational modification may be important, however, for the recognition of JHBP by other proteins, and this might be tested by comparison of unmodified protein from bacteria with protein from hemolymph. One characteristic that may have implications for the activity of JHBP is its binding to heparin-agarose, a property unique to JHBP among the hexamerins in locust hemolymph (Fig. 1). Heparin has been implicated in the binding of proteins to membrane receptors in vertebrates (36), and it is possible that JHBP may also interact with membrane receptors using heparin or similar molecules.

As a step toward identifying amino acid residues of JHBP involved in JH binding, we tried using the JH III photoaffinity analog, [^{3}H]EFDA, and specific covalent attachment of the analog to peptide fragments from CNBr cleavage of JHBP was obtained (data not shown), but we were unable to identify these fragments. The six histidine residues that are conserved in all hemocyanins and required for oxygen binding are not found in JHBP (Fig. 3), showing that it does not use an adaptation of the oxygen-binding mechanism. In the methionine-rich hexamerin, BOMB.M, and the arylphorin, BOMB.A, the positions of the "storage amino acids" (methionine and the aromatics, respectively) have been mapped (Fig. 3, *asterisks*), since these might highlight regions that are not structurally important and are free to diverge and accommodate new functions, but these amino acids are distributed throughout the sequence, and especially mutable sites could not be identified.

From comparison with other proteins, the C terminus of JHBP might be expected to bind JH. X-ray studies on fatty acid and retinol-binding proteins, whose ligands are similar to JH, show a domain with a high degree of β-sheet structure that is responsible for ligand binding (37). In the hemocyanins, the C-terminal region exhibits a β-barrel (14, 38), which could be the precursor to a JH-binding site. A sequence difference unique to JHBP among the hexamerins is the deletion at positions 563–568 (Fig. 3), in a region toward the C terminus close to the main β-barrel of hemocyanins. The changes in this region in JHBP might alter the topology to allow JH to bind.

Functional assays, on the other hand, suggest the N terminus as a JH-binding site because the truncated form of JHBP, rJHBP-S, does not bind JH III. The loss of 53 amino acids from the N terminus may have removed residues involved in JH binding. It is also possible, however, that the inclusion of a 41-amino acid β-galactosidase sequence in rJHBP-S may cause an altered conformation which is responsible for the loss of activity.

The jhbp nucleotide sequence is unusual in the composition and distribution of codons, with a strong bias for codons ending in G or C. Codon biases were compiled for five other sequenced locust genes, apolipophorin III (24), fatty acid-binding protein (39), arrestin (40), 19K protein (41), Jhp21 (=21K protein; Ref. 42), and tropomyosin (43), and it was found that all had a high proportion of G/C-ending codons (range 63–90%), while a compilation for 10 hexamerin genes from other arthropods showed a range from 40 to 87%, and an overall correlation with species. Thus, a bias for G/C codons appears to be characteristic of locust genes. The fact that the high G/C bias in locust jhbp is limited to the coding region suggests that it may reflect a role in mRNA activity. Also notable is the clustering of codons ending in A or T near the 5′ end of the sequence, indicating that this region differs in some way from the remainder of the gene. This might be related to the generation of a JH-binding site at the N terminus. Biases in insect hexamerin genes have previously been noted for the codons of phenylalanine, arginine, and tyrosine (31, 44), but not for the entire sequences.

JHBP is present in the hemolymph of both sexes and shows only slight changes in abundance during the fourth and fifth larval instars and early adult life (10). Its 4.3-kb mRNA is the largest mRNA yet identified for any hexamerin, with 2 kb of 3′-untranslated sequence, and it is possible that sequences at the 3′ end of the message, through recognition by specific proteins, may be responsible for maintenance of JHBP levels during times when variations occur in factors such as hormones or nutrition (45). Our preliminary Northern blot analyses failed to show consistent effects of JH deprivation and JH analog treatment on JHBP mRNA levels in male and female fat bodies. Previously, Couillaud and co-workers (46) reported that allatectomy of female locusts was followed by decreased hemolymph JH-binding sites, and that fat body JHBP mRNA was decreased by allatectomy but not restored by treatment with

pyriproxyfen.[2] Therefore, JHBP mRNA levels appear to be regulated in complex manner, not directly controlled by JH.

Amino acid sequence comparisons indicate that JHBP represents a new group within the hexamerin superfamily. The placement of JHBP near the hemocyanins is further evidence that the insect hexamerins had their origin in the hemocyanins. The lack of homology of locust JHBP with the low molecular weight JHBPs of Lepidoptera indicates that the lepidopteran JHBPs must have evolved independently of the orthopteran JHBPs. Although no sequence is available for the JH-binding lipophorins, their molecular composition also indicates a course of evolution distinct from those of the other JHBPs. Further understanding of the function, evolution, and relationships of the different JHBPs depends upon the determination of additional sequences.

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Sequence of the Hexameric Juvenile Hormone-binding Protein from the Hemolymph of *Locusta migratoria*

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