Structure, expression and gene sequence of a juvenile hormone esterase-related protein from metamorphosing larvae of *Trichoplusia ni*

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A carboxylesterase with an encoded molecular size of 61 kDa and a high sequence similarity to juvenile hormone esterase (JHE) has been cloned from cDNA prepared from final instar larvae of *Trichoplusia ni*. The absence of a recognizable encoded signal peptide suggests that the enzyme, JHER (for JHE-related) may not be secreted, in contrast to JHE. When the amino acid sequence of JHE, JHER and other esterases were mapped onto the secondary and tertiary structure determined crystallographically for acetylcholinesterase, certain structural features for the substrate binding/catalytic site were identified as common only to JHE and JHER. However, several differences between JHE and JHER were identified in residues at the binding/catalytic site, suggesting that although the two enzymes prefer similar

natural substrates, these substrates are not identical. JHER is present as a single-copy gene, transcribed during the feeding stage of the final stage of the final larval stadium, but not after metamorphic commitment to the pupal developmental programme. The gene transcribes a single-size message of 2.0 kb. The genes for JHER and JHE appear to be physically juxtaposed in the *T. ni* genome. The 5' flanking sequence to the JHER gene possesses some sequences in common with the JHE gene, but is also missing some regulatory elements previously identified in the JHE gene. Sequences conserved between the promoters for the two genes were identified that were different from previously reported regulatory elements of eukaryotic transcription factors.

INTRODUCTION

Carboxylesterases have been the subject of much biochemical research, in terms of their catalytic mechanism, molecular evolution and regulation of expression. The critical residues participating in catalysis appear to be a serine, histidine and an acidic residue [1,2]. Other residues adjacent to the critical serine are highly conserved, resulting in a canonical GX₂SAG motif that appears to have evolved independently from the similar motif at the catalytic serine of proteases [3]. Much less is known about the contribution of other residues to substrate specificity.

The evolution of esterases into subfamilies with differing specificity towards various substrates has also been studied. The existence of various subgroups of carboxylesterases is now recognized [4], but the evolutionary relationships among groups of esterases are not well understood [3]. Juvenile hormone esterase (JHE) is a carboxylesterase (EC 3.1.1.1) that has attracted great interest with regard to its critical role in regulating larval to adult transition in insects and other arthropods. While steroid hormones drive the timing of the moulting process, JHE is the only protein thus far experimentally verified as being essential for normal metamorphic commitment of tissues, and inhibition of the enzyme results in larval moulting to a supernumerary larval instar, rather than a metamorphic moult to the pupa [5]. JHE is encoded in the genome of *Trichoplusia ni* as a single copy gene [6]. The reported isoforms of the enzyme [7] all occur as secreted, serum-carried proteins, and the JHE activity reported from tissues is biochemically indistinguishable from that of the serum forms [8,9].

As a protein, the JHE sequence is distinct from other reported esterases, and its evolution and relationship to other esterase groups are unclear [10–12]. While the specific natural target of many esterases is unknown, it has been firmly established that the invertebrate juvenile hormone (JH) is the natural, endogenous substrate of the enzyme during metamorphosis to the pupa

[13,14]. In fact, JHE is extremely specific for JH. However, essentially nothing is known about the molecular basis for this specificity. An understanding of the catalytic site is of considerable practical interest in the design of selective enzyme inhibitors as insect control agents [15,16].

The molecular regulation of esterase expression is also not well understood. There is evidence that variation in the regulation of two esterases in *Drosophila* is mediated by a mechanism involving changes in *cis*-acting regulatory elements and not through alteration in *trans*-acting factors [4]. Some of these regulatory sequences impart tissue specificity to the expression of adult insect esterases [17]. In mammals, promoter regions necessary for expression of canine prostate arginine esterase have been identified, but the transcriptional regulators binding to these sequences are unknown [18]. Recently, the promoter sequence for JHE has been reported and shown to mediate JH-induced transcription of the JHE gene [6], but specific sequences which mediate JH action have not been identified. A powerful method for locating possible regulatory promoter elements is comparative analysis of the promoters of related genes and the identification of conserved or distinguishing sequences [19]. As yet, no promoters related to the JHE gene promoter have been reported to enable such a comparative analysis.

In this paper we report a new esterase which is structurally distinguishable from JHE based on certain residues predicted to function at the active site but which is clearly more related to JHE than to any other reported esterase. We also report the sequence of its promoter, and its non-sensitivity to JH, and identification by comparative analysis of possible functional motifs in its promoter and the promoter of classical JHE.

METHODS AND MATERIALS

Insects

T. ni were reared and staged as described previously [20].

Abbreviations used: AChE, acetylcholinesterase; JH, juvenile hormone; JHE, JH esterase; JHER, JHE-related carboxylesterase.

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Figure 1 cDNA and inferred protein sequence for JHER

The arrow indicates the putative methionine translation start site and the associated stop codon is indicated by the asterisks. The sequence shown above the N-terminus of JHER is the N-terminal sequence of human cholinesterase, and the plus symbols indicate similar or identical residues. Consensus glycosylation sites [NX(S/T)] are indicated by the solid triangles. The open diamonds around residues demarked with the solid triangles denote glycosylation sites conserved among JHE and JHER sequences. Open triangles show the locations of the three residues constituting the classical catalytic triad (serine, histidine and glutamate). A solid line connects two cysteine residues conserved in nearly all reported carboxylesterases and which have been shown to participate in a disulphide bridge with each other. Lines connecting methionine and asparagine residues in open boxes denote the positions corresponding to cysteine residues that are conserved in cholinesterases and two *Dictyostelium* esterases and that form a disulphide bridge with each other. The tyrosine residue in an open box attached to a solid line shows the position corresponding to a cysteine conserved in cholinesterases; that line ends at another open box showing the position corresponding to the partner cysteine residue in a disulphide bridge in cholinesterases. The four cysteine residues in an open diamond denote cysteine residues conserved only among JHER and JHE, and not in other carboxylesterases. The solid squares indicate residues corresponding to those of AChE that are either at or near the catalytic site.

Hormone treatments were performed as described by Jones and Manczak [21].

cDNA and genomic library screening

A cDNA library was prepared from mRNA isolated from day 2, final instar larvae [22]. A probe for screening the library for JHE was prepared from a PCR product that was synthesized from library cDNA using two degenerate primers designed from protein fragment sequences (as described by Venkataraman et al. [6]). As described by Jones et al. [12], these protein fragments for JHE were prepared from purified protein that showed the same substrate kinetics as JHE activity in insect serum, and which was used to prepare a monospecific antibody that immunoprecipitates JHE activity from insect serum. The probe made from the PCR product based on these primers identified a cDNA clone for JHE reported elsewhere [6] and also identifies clones for the new esterase, JHE-related carboxylesterase (JHER), reported here. A 5'-located, 400 bp *SpeI/NcoI* restriction fragment for JHER

cDNA was used as a probe to screen a *T. ni* genomic library prepared in EMBL3 cells. All library screening was performed at 65 °C, as described previously [23].

Northern analysis

The cDNA clone for JHER was radiolabelled with [α -³²P]dCTP by the random priming method [24]. Poly(A) RNA from each of the four days of the final larval stadium was extracted by methods described previously [22,23]. mRNA (5 μ g) from each day was fractionated on formaldehyde 1.2% (w/v) agarose gels, transferred to nitrocellulose, and incubated at 65 °C with the radiolabelled probe.

Determination and analysis of DNA sequences

The cDNA and genomic clones were sequenced in both directions using the Sanger method of dideoxy chain terminators [25]. Primary and secondary structure analysis of DNA sequences

and encoded protein sequence were performed using PC Gene software.

RESULTS

Identification of cDNA for JHER

Screening of the cDNA library with a fragment of JHE cDNA as a probe yielded two uniform classes of signals on duplicate filters. The strong signal was invariably associated with homologous clones for JHE. The weaker signal was found to be invariably associated with a cDNA encoding an esterase that was related to, but distinct from, JHE. The complete cDNA sequence for the new esterase, JHER, and its encoded protein are shown in Figure 1. The 1854 base sequence contains a short 5' untranslated region, a 1662 base open reading frame and a 187 base 3' untranslated region. The cDNA encodes a poly(A) tail, and while it does not possess a canonical polyadenylation signal (AATAAA), it possesses several similar sequences in an A/T-rich region just 5' to the encoded poly(A) tail (Figure 1). The identity of this encoded protein as a carboxylesterase is shown by its possession of a sequence similar to the canonical serine catalytic-site motif (GX_SAG; but see text below on cysteine as residue X) and its predicted molecular mass (61 kDa), which is within the range of masses reported for carboxylesterases.

Relationship of new JHER to JHE and acetylcholinesterase

Primary structure

The N-terminal sequence of JHER shown in Figure 1 shares

Table 1 Concordance between the structure of regions of *Torpedo californica* AChE from crystallography [28] and the analytical prediction [26,27] of the structure of the corresponding regions of *T. ni* JHER

a denotes an α -helix and B denotes a β -turn structure.

Structural region of <i>T. californica</i> AChE	Predicted similar structure in <i>T. ni</i> JHER
B ₋₂	No
B ₋₁	Yes
B ₁	Yes
a ₄ B ₂	Yes
B ₃	Yes
a ₈	No
B ₄	Yes
a _C	No
B ₅	No
a _D	Yes
B ₆	Yes
a _{E'1}	No
a _{E'2}	Yes
a _{E'3}	No
a _E	No
B ₇	No
a _{1'1}	Yes
a _{1'2}	Yes
a _{1'3}	No
a _F	Yes
B ₈	Yes
a _G	Yes
B ₉	No
B ₁₀	No
a ₁	No

some similarity in sequence with human cholinesterase. JHER does not have a signal peptide recognizable by PC gene software that searches for the canonical signal peptide sequences. Over much of the remainder of its linear amino acid sequence JHER most closely resembles JHE from *T. ni* and *Heliothis virescens* [11]. JHER and JHE differ distinctly from cholinesterases and other carboxylesterases in the conservation and placement of cysteine disulphide bridges. Cholinesterases have three conserved disulphide bridges, placed as shown in Figure 1. All reported esterase sequences examined in the present study, including JHER and JHE, possess the most N-terminal bridge. In contrast, only JHER and JHE lack the second bridge. The third bridge is present only in cholinesterases and two *Dictyostelium* esterases. However, JHER and JHEs conserve four cysteine residues, located at positions corresponding to 365, 427, 523 and 528 in Figure 1, and these four cysteine residues are not conserved at these positions in other reported carboxylesterases.

Also shared between JHER and JHE are three conserved potential glycosylation sites [NX(S/T)], at positions corresponding to 193, 384 and 417. These locations are not potential glycosylation sites in the other reported carboxylesterase sequences.

Secondary structure

The α -helical and β -strand secondary structures of JHER, as inferred by the computational methods of Chou and Fasman [26] and Garnier et al. [27], were analysed and compared. These comparisons were directed by focusing on sequences which correspond to the homologous regions of *Torpedo* acetylcholinesterase (AChE) that were established by crystallographic studies of that enzyme to be of α -helical or β -strand structure [28].

As a first step, the primary sequence of AChE was analysed for predicted secondary structures by these two analytical methods. The number of agreements between the two analytical methods on the theoretical, predicted secondary structure of AChE was determined. These congruent predictions between the two analytical methods were then compared with crystallographic determinations of the actual structure of corresponding regions of AChE. Both analytical methods agreed with each other, and also with the crystallographic determination, for 14 of the 26 regions determined by crystallography to be either α -helix or β -strand. Computational analysis of 23 corresponding α - or β -structure regions in JHER (the JHER C-terminus is short relative to AChE, and lacks the final 3 regions) found that both of the computational methods agreed with each other and with the crystallographic determination of AChE at 13 of the 23 regions (Table 1). Thus, the frequency of matching between the computational congruencies and the actual structure for these 26 regions for AChE (14/26) was essentially the same frequency as the match between the number of computational congruencies on theoretical structure of JHER with the corresponding empirical structure of AChE (13/23). Most of the regions of JHER predicted to have the same secondary structure known for AChE were also regions of AChE for which the analytical predictions matched the known secondary structure. These results suggest that to the extent that secondary structure influences tertiary structure, JHER shares much tertiary structural similarity with AChE. This should be the result of these analytical considerations, since JHER appears to have an esterolytic function just as AChE does.

Based on this inference, that some JHER tertiary structure resembles that of AChE, especially that part of the structure conferring esterolytic properties, we next used the structure of

Figure 2 Sequence similarities among JHER and other reported carboxylesterases

The areas chosen for analysis are regions in which AChE contains residues shown by crystallography to be positioned at or near the catalytic site [28]. Abbreviations and references for esterase sequences are as follows: ERACHE, *Torpedo* AChE [50]; DMACHE, *Drosophila melanogaster* AChE [51]; HUCHE, human cholinesterase [52]; HVJHE, *H. virescens* JHE [11]; TNIJHE, *T. ni* JHE [6]; DDD2, *D. discoideum* esterase D2 [53]; DDCP, *D. discoideum* crystal protein esterase [53]; DME6, *D. melanogaster* esterase 6 [54]; EAGSYN, mouse esterase-22 [55]; ESN, mouse esterase N [56]; HMSEI, human monocyte/macrophage serine esterase 1 [57]; RATCEST E1, rabbit carboxylesterase [58,59]; RABCEST 1, rabbit liver microsomal carboxylesterase 1 [60]; RABCEST 2, rabbit carboxylesterase 2 [61]; RATCEST 6.1, rat carboxylesterase 6.1 [62]; RATCOLE 1, rat pancreatic cholesterol esterase [63]. Asterisks indicate residues for which side chains point towards the substrate-binding or catalytic site in ERACHE [28].

Figure 3 Expression of transcript for JHER, probed with cDNAs for JHER and RP49

(a) Northern analysis of expression of transcript for JHER, probed with a cDNA for JHER, during the final, 4-day-long metamorphic larval stadium (L5). The message for JHER is expressed during the 2-day feeding stage, but drops to very low levels after the metamorphic commitment (on day 3) to the pupal developmental programme. D1, D2, D3, D4 indicate the day of the final stadium from which mRNA was extracted. Shown along the right-hand side are molecular size positions as determined by a molecular size ladder run on the same gel. (b) The same blot, probed instead with a cDNA for RP49.

AChE to target residues of JHER that might contribute to substrate specificity and catalytic activity. Crystallographic

Figure 4 Southern blot analysis of the genomic copy number of the gene for JHER

Shown above each lane is the restriction enzyme used to digest the genomic DNA. Shown along the right-hand side are molecular size positions as determined by a molecular size ladder run on the same gel. The results suggest that JHER is represented as a single copy gene. Arrows denote bands that were also signals when the same blot was probed with JHE cDNA fragment [6].

analysis of *Torpedo* AChE identified specific residues that were either at the bottom of the active-site gorge, i.e. near the catalytic residues, or part-way down the gorge [28]. The homologous residues in JHER were examined in the sequences of a number of cholinesterases and other carboxylesterases. As shown in Figure 2, some of these residues were conserved in all esterases, others in some esterases, still others only in cholinesterases, and finally

Figure 5 Sequence of 5' flanking region of JHER gene, and comparison with 5' flanking region of JHE gene

The transcription start site as determined by primer extension analysis (results not shown) is indicated by a bent arrow. The locations of various transcription factor response elements reported in other systems are shown. Abbreviations above DNA sequences refer to various proteins that have been reported to bind to the indicated elements: RAR/TR, retinoic acid receptor/thyroid hormone receptor [33]; HLH, helix-loop-helix protein [31]; CEBP, CCAAT/enhancer-binding protein [49]; CEBPRC, reverse complement of CCAAT/enhancer-binding-protein site; T, octamer-motif-binding protein [30]; PPAR, peroxisome proliferator-activated receptor [32]; FB, lepidopteran fat body element, proposed by Fujiwara and Yamashita [64]. The motifs underlined cross-reference with Figure 7.

some were not conserved at all. The presence of particular residues at or near the catalytic site in a subgroup of esterases would suggest that esterases in that subgroup have specificity towards a common form of moiety on the carboxyl ester substrate. In contrast, the conservation of a specific residue in all esterases would suggest that while the residue may be important for promoting hydrolysis of a carboxyl ester, it does not contribute to the specificity of the enzyme for a particular kind of carboxyl ester substrate.

Along this line of analysis, a tryptophan present in the active site of AChE, corresponding to phenylalanine at position 149 of JHER, is conserved in all three cholinesterases examined, but is not conserved in any of the other esterases (Figure 2, panel 5). This situation suggests that residues at that position are important for imparting substrate specificity. Significantly, JHE and JHER do not share a conserved residue at this position, suggesting that their natural substrates are not identical. However, at another position at which cholinesterases uniquely possess a tyrosine

(corresponding to position 371 in JHER), JHE and JHER share an arginine residue (found in only one other esterase). This result suggests that while the substrates for JHE and JHER are not the same, there is a structural feature common to their substrates not found in most other substrates of the esterases sequenced so far. This prediction is supported by the unique glutamine residue found in *T. ni* JHE and JHER at the position immediately N-terminal to the critical catalytic serine residue (Figure 2, panel 1). Further, only *T. ni* JHE and JHER lack the two residues at the positions 4 and 5 residues immediately C-terminal to the catalytic serine (Figure 2, panel 1).

The unique occurrence of cysteine in JHER at the position immediately C-terminal to the catalytic serine is particularly intriguing. In most other esterases this residue is alanine and possesses a much smaller side chain. The only reported esterase with a large side-chain residue (serine) at that position is mouse esterase N, which hydrolyses an unidentified substrate. The large side chain of cysteine in JHER must to some extent contribute to

Figure 6 Southern blot hybridization showing the juxtaposition of the JHER and JHE genes

(a) Restriction digest of genomic clones for JHER and JHE, probed with 1 kb 5' flanking sequence of JHE gene. (b) Same restriction digest probed with 1 kb 5' flanking sequence of JHE gene. Each blot shows strong hybridization of the probe with its own parent gene, and also specific hybridization of the JHER probe to a fragment from JHE. Restriction enzyme abbreviations: S, *SalI*; E, *EcoRI*; B, *BamHI*; H, *HindIII*; P, *PstI*.

		JHER	JHE
1	CATTGT/ATAA	-902	-580
2	CATCAAA/CT	-893	-566
3	CCAAAGC/AG	-1098	-740
4	AAGGATT	-1063	-865
5	TATTCT/AGT	-873	-788
6	ATGTGAT	-1237	-1114
7	TTGATAT	-681	-352
8	TGTATGT/AA	-252	-141
9	TATCGGC/TT	-234	-60
10	GTTGGAT	-229	-43

Figure 7 Sequence motifs found in common in the 5' flanking region of the genes for both JHER and JHE

Numbers indicate the upstream position of the 5'-most base of the motif relative to the position of the transcription start site suggested in Figure 5.

the architecture of the active site. In this regard, when the unique histidine residue that immediately precedes the catalytic serine in *Drosophila* esterase E6 is changed to a conventional alanine, the substrate specificity of the mutant enzyme for acetylcholine substrate increases significantly [28a].

Expression of JHER transcript

Northern analysis determined that the transcript for JHER is present on the first day of the final larval stadium, and markedly increases in abundance during the second day (Figure 3). The size of the 2.0 kb transcript detected by northern analysis is consistent with the 1854 base cDNA for the message. After the larval cells commit for metamorphosis at the end of the second day, the abundance of the transcript decreases markedly, and remains undetectable on the fourth and final day of the final stadium. Additional experiments with exogenous treatment of day 3 larvae with a powerful JH analogue, fenoxycarb, showed that the transcript is not induced to appear by such treatment (results not shown), although the JHE transcript is strongly induced by such treatment [6,13].

Genomic copy number and 5' flanking structure

The results of Southern analysis of restricted genomic DNA using a 3' fragment of cDNA as a probe yielded usually either one or two bands for each digest, which within the resolution of the technique is consistent with the proposal that JHER is represented in the genome as a single copy gene (Figure 4). The close similarity in sequence between the cDNA for JHE and JHER prompted us to consider the physical locations of the two respective genes. Therefore, the same Southern blot that was used as a target for the JHER probe (Figure 4) was stripped of the hybridizing signals and probed with a sequence from the JHE cDNA. The results showed that several of the signals for the JHE probe comigrated exactly with the signals obtained for the JHER probe (Figure 4) [6].

A genomic library was screened with a fragment of JHER cDNA and a clone was isolated containing the 5' flanking sequences for JHER (Figure 5). Given the results shown in Figure 4, we wanted to determine whether the respective genomic clones for JHER (Figure 5) and for JHE (reported by Venkaraman et al. [6]) contained promoter sequences of the other esterase gene. The respective genomic clones in λ EMBL vector were individually restricted and probed sequentially with the 5' flanking sequence for JHER and then JHE. As shown in Figure 6 the probe for JHER not only hybridized strongly to its cognate promoter but also hybridized to a specific restriction fragment of the genomic clone of JHE, although more weakly. An alternative consideration is that the 5' flanking region of the JHER gene contains a third esterase gene also related to JHE and JHER.

Comparison of the flanking sequences from JHER with the corresponding 5' flanking sequence for JHE reported elsewhere (Figure 5) shows that these genes share some established transcription elements. Both contain the canonical TATA box and elements reported as binding sites of eukaryotic transcription factors, such as a GATA protein-binding sequence [29], octamer-motif binding proteins [30], helix-loop-helix proteins [31], a peroxisome proliferator-activated receptor [32] and a retinoic acid receptor [33]. Both also contain sequences reported as potential regulatory elements in insect genes, such as two

sequences reported for *Drosophila* hexamerins [34] and a sequence reported for hexamerins from the insect of the present study, *T. ni* [35].

Close comparison of the 5' flanking sequences for the JHE and JHER genes also detected a number of similar sequences not previously reported as binding sites of transcription factors. Attention was directed towards those sequences in the two promoters which were similar and were either similarly positioned with respect to the TATA box or were positioned near an element previously reported as a binding site for a transcription factor. Ten such elements were identified (Figure 7).

DISCUSSION

Structure of JHER

Analysis of the protein sequences in reported carboxylesterase cDNAs suggests the evolution of several distinct groups of serine esterases. Distinctive groups are the JHEs, cholinesterases, *Drosophila* esterase 6 group, two *Dictyostelium* esterases, cholesterol esterase and a number of reported liver esterases (the latter two groups being more similar in sequence to each other than to the other groups). The primary structure of JHER clearly places it in the family of serine carboxylesterases. Further, it possesses the canonical catalytic residues of serine, histidine and aspartate. Among esterases, its sequence is most closely related to JHE, although the extreme N-terminus and occasional small motifs throughout the encoded sequence are more similar to cholinesterases than to JHE. However, at the level of the primary sequence at the catalytic site, the motif VAAH found in *T. ni* JHE and JHER does not closely resemble cholinesterases, but rather is the same as or similar to the two *Dictyostelium discoideum* esterases, crystal protein esterase and esterase D2 respectively.

Function of JHER

JHER appears to have all of the critical catalytic residues and is thus likely to have catalytic (esterolytic) function. Thus, its function is unlikely to be analogous to glutactin or neurotactin, which resemble esterases but have lost catalytic function in lieu of a binding or structural role [36,37]. Also, at least glutactin has significant additional primary structure at the C-terminal domain, while JHER does not. JHER does not have an obvious signal peptide (and analytical PC gene software does not detect such a peptide), and the primary structure does not suggest that the protein is membrane-bound. It may be that this esterase may function intracellularly. In comparison, JHE has a major function as a circulating protein. While at certain stages JHE is sequestered in the fat body, an increase in the JH titre rapidly induces the extracellular release of the enzyme [8,38].

As described in the results, analysis of the computationally predicted structure of JHER and JHE, when mapped onto the known AChE structure, suggests that JHER and JHE have similar but not identical natural substrates. The catalytic site of JHER has a cysteine residue, with its large side chain, immediately C-terminal to the catalytic serine, while most other described esterases, including JHE, have alanine at this position. Such a structural difference suggests that it is unlikely that the JHER enzyme functions as a JH-specific esterase (JH is a methyl ester), especially in view of the high substrate specificity of the 'conventional' JHE [14]. However, Granger has found an as yet uncharacterized new form of JH, which when exposed to a brain esterase (also as yet uncharacterized) yields as a product JH III

acid, the conventional JH metabolite (N. Granger, personal communication). Thus, there may be as yet unidentified ester moieties for JH that are cleaved by specialized esterases related to the known JHE. Further, the occurrence of the motif VAAH at the catalytic site of *T. ni* JHE and JHER and a *Dictyostelium* esterase only suggests that there is a unique aspect of structure of the substrate of these three enzymes. As yet, no chemical structure similar to JH has been reported from *Dictyostelium*. A possible function of the esterase is suggested by the increase in its abundance during the feeding stage of the final stadium, which in profile resembles the feeding stage pattern of expression of circulating JHE [21]. During this developmental period the titre of the substrate for that enzyme (JH) further declines to nearly undetectable levels. By analogy with JHE, the substrate of JHER may decline in titre during days 1 and 2 as the titre of JHER increases. In addition, it may be important for the titre of the substrate to decline before the normal metamorphic commitment made late on day 2 by cells in response to a surge of the steroid hormone ecdysterone. Future substrate specificity experiments on over-expressed JHER enzyme are clearly warranted.

Regulation of JHER

Developmental regulation

The transcript for the protein becomes very highly expressed just before metamorphic commitment towards the pupal developmental program and away from the larval program (Figure 3). In this respect the expression of JHER appears similar to that of certain other reported genes that are highly expressed before, but not after, metamorphic commitment, such as arylphorin [23]. Arylphorin is highly transcribed during the feeding stage before the metamorphic commitment that is controlled by ecdysteroids. It is the increase in ecdysteroid titre that terminates the feeding stage and recommitments the larval tissues towards the pupal developmental programme [39,40]. This increase in ecdysteroids also causes a sharp decline in transcription of arylphorin and certain other genes [41], and the transcription and abundance of the arylphorin mRNA at this time closely resembles the pattern observed in the present study for the JHER.

Hormonal implications

In contrast to the pattern of expression of JHE, JHER does not show a peak of expression during the prepupal stage. The prepupal peak in JHE expression is induced by a prepupal increase in the endogenous JH titre [13,40,42], and can be enhanced by exogenous application of JH [13,43]. The lack of inductive effect observed here of JH on expression of the JHER transcript indicates that its gene is not sensitive to 'conventional JH' and that perhaps its own JH-like substrate is not present during the prepupal stage.

Gene for JHER

The results shown in Figure 6 strongly suggest that the JHE and JHER genes are physically juxtaposed, and that a gene duplication event has probably occurred, as reported for several carboxylesterase genes from *Drosophila* [3]. So far as we are aware, this is the first reported evidence of gene duplication and divergence for a gene encoding a JH-inducible larval protein. In fact, the only other documented case of duplication of a JH-inducible gene is vitellogenin [44], but in that case both genes are still inducible by JH, while in the present case only JHE, and not JHER, is JH-inducible.

Promoter regulatory elements

The presence of a single copy of the JHER gene simplifies future studies on transcriptional regulation of the enzyme. Analysis of its promoter sequence showed the presence of a number of motifs identified in other insect genes. Close comparison of the promoter sequence with the promoter of JHE may be informative with regard to the location of possible JH regulatory elements, i.e. sequences found in the JH-sensitive JHE promoter but missing from the promoter of the JHER gene should be tested as possible JH regulatory elements. One such sequence is a DR1-like element at position -1118 of the JHE promoter (Figures 5 and 7), which was not found in JHER promoter (which was sequenced to position -1450). This DR1-like element overlaps with a second sequence that is conserved between JHE and JHER (element 6, see text below).

Of interest is the occurrence of two elements that are closely juxtaposed in the same order and orientation in both promoters (Figures 5 and 7). One of the two elements, element 1, resembles an element reported from the promoter of *Drosophila* larval serum proteins, which are regulated in part by ecdysteroids. The second of the two elements, element 2, does not seem to correspond to any reported regulatory element of which we are aware. It is an emergent theme in eukaryotic gene regulation that the activity of hormone receptors or other transcription factors is modulated by other factors that either bind close by or have overlapping regulatory elements and thus physically compete for a binding site on the DNA [45–48]. In the JHER gene, element 2 overlaps with a regulatory element reported for C/EBP [49]. In the JHE gene, element 2 is situated only several bases away, on each side, from regulatory elements reported for *Drosophila* larval serum proteins. These circumstances prompt further investigations of elements 1 and 2 as possible regulatory elements affecting transcription of the JHER gene.

A second situation was found in which two conserved elements were closely juxtaposed in both genes. Much closer to the TATA box than elements 1 and 2 are elements 9 and 10. These elements are also in the same order in the two genes, and overlap slightly in the JHER promoter, while they are separated by 9 bases in the JHE promoter. A little upstream from the pair of elements, in both genes, is a third element, element 8 (Figure 5). The conservation of these sequences and their relative positions also suggests that attention be paid in the future to these sequences as possible regulatory elements.

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