EST library sequencing of genes expressed during early limb regeneration in the fiddler crab and transcriptional responses to ecdysteroid exposure in limb bud explants

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Synopsis We have constructed directional and randomly primed cDNA libraries from mRNAs isolated during progressive stages of fiddler crab (*Uca pugilator*) limb regeneration. Data from these libraries are being assembled into an on-line database (http://www.genome.ou.edu/crab.html) that is both BLAST and keyword searchable; the data set is also available through GenBank. The first characterized library was made from mRNA isolated 4 days post-autotomy, when the first sign of morphological differentiation, cuticle secretion, is observed. Analysis of 1698 cDNA clones led to assignment of 473 contigs and 417 singlets, for a total of 890 sequences. Of these, ∼86% showed no assignments to characterized genes on database searching, while 14% could be assigned to a known ortholog in the COG (Clusters of Orthologous Groups) database. BLAST searches to specific protein domains in the Gene Ontology database led to assignments for ∼40% of the assembled sequences. Sequence similarity searches of other crustacean EST databases produced hits to 13–30% of the *Uca* query sequences. The ESTs include several genes that may be potentially ecdysteroid-responsive, such as homologs to chaperone proteins and cuticle protein genes, as well as homologs to arthropod proteins involved in retinoid/terpenoid metabolism. We have tested 3 potential candidate genes for their ability to be induced by ecdysteroid in limb bud explants; an arthropodial cuticle protein gene, and the nuclear receptor genes *EcR* and *RXR*. A subset of early blastemal limb buds (8 days post autotomy) show a positive response to ecdysteroid by 1–1.5 h, followed by a decrease in transcript abundance at longer periods of sustained incubation. Later stage buds (12 days post autotomy-late premolt) show decreases in steady-state mRNA levels by 1.5 h, or are completely refractory to ecdysteroid exposure.

Introduction

Ecdysteroid hormones have been demonstrated to play a major role in the regulation of gene expression in arthropods (Thummel 2002; Truman and Riddiford 2002; Yin and Thummel 2005). In insects, changes in circulating ecdysteroid titers are correlated to major developmental transitions, the expression of genes involved in reproductive competency, growth, and programmed cell death. How a "common" endocrine signal might lead to differential tissue responses is unclear, and might occur at multiple levels in either primary response or downstream signal transduction pathways.

Crustaceans represent a relatively unexplored model for investigating ecdysteroid function at the molecular level. In adults, reproduction, development of embryos retained by the female, growth, and a specialized type of growth (regeneration of limbs) can be coordinately regulated. These varied physiological functions are at least partially under ecdysteroid control (Hopkins 1988a,b, 1989, 1993; Subramoniam 2000). As a first step in the identification of crustacean tissues competent to respond to ecdysteroid signaling, we have cloned from the fiddler crab, *Uca pugilator*, homologs (*UpEcR* and *UpRXR*) of the functional insect ecdysone receptor (Durica and Hopkins 1996; Chung, Durica, Clifton, and others 1998; Durica and others 1999, 2002). These transcription factors, members of the nuclear receptor (NR) superfamily, have been implicated in positive and negative gene regulation in insects (Kozlova and Thummel 2000; Truman and Riddiford 2002). Probes derived from the *UpEcR* and *UpRXR*

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genes have identified putative ecdysteroid target tissues throughout the crustacean molt cycle, including the regenerating limb, the ovary, and the hypodermis (Chung, Durica, and others 1998; Durica and others 2002). Postzygotic expression has also been observed during embryonic development (Durica and others in preparation). Analysis of clones recovered from cDNA libraries and RT-PCR cloning have also demonstrated heterogeneity in receptor isoforms and variation in receptor mRNA expression (Durica and others 2002). Expression of receptor isoforms *in vitro* and in cell culture indicates differences in isoform dimerization, DNA-binding, and transactivation properties (Wu and others 2004). These differences may, in turn, lead to implementation of different genetic programs in the developing limb primordium (blastema), the ovary, and other tissues throughout the molt cycle.

To continue to explore this hypothesis, we are interested in a systematic identification of crustacean primary response genes under ecdysteroid control. To begin identification of putative response genes expressed in developing limbs, we have developed expressed sequence tag (EST) libraries from stages of the limb regeneration cycle subject to hormonal effects on gene expression. We report here the characterization of a library derived from early blastemal limb regeneration. This library was constructed from mRNA isolated 4 days post-autotomy, at a time when the first sign of morphological differentiation, cuticle secretion, is observed (Hopkins and Durica 1995). Included in this database are several genes that may be potentially ecdysteroid-responsive, such as homologs to chaperone proteins and cuticle protein genes, as well as homologs to arthropod enzymes involved in retinoid/terpenoid metabolism (for example, farnesoic acid *O*-methyl transferase; juvenile hormone esterase). A homolog to a cytoplasmic retinoic acid binding protein has also been identified. A web site for this database has been established (http://www.genome.ou.edu/crab.html) providing both search capabilities and sequence information via anonymous file transfer.

Using sequence information derived from cDNA cloning, we have also designed primers to investigate 3 potential ecdysteroid primary response candidate genes; the 2 genes encoding the heterodimer partners of the ecdysteroid receptor, *UpEcR* and *UpRXR*, and a homolog to an arthropodial cuticle protein recovered from the blastemal cDNA library, denoted *Up378C*. Using quantitative reverse transcription polymerase chain reaction (Q-RTPCR) and ribonuclease protection assays (RPA), we have examined whether these genes are subject to changes in transcript abundance following ecdysteroid exposure. During early blastemal development (8 days post-autotomy, abbreviated A+8), short intervals of 20-hydroxyecdysone (20E) exposure increase *378*, *EcR* and *RXR* gene transcript levels in a subset of individual animal explants, while explants from other individuals, indistinguishable in terms of staging, are refractory. Exposure of A+8/A+12 blastemal tissues to 20E for periods >2 h leads to decreases in transcript levels. Bud explants taken from animals in late proecdysial stages appear refractory to 20E exposure; neither Q-RTPCR nor RPA indicate significant changes in transcript abundance. These results suggest major differences in the competency of tissues to respond to ecdysteroid, even when levels of the hormone may be high in the circulating hemolymph.

Methods

Animals and explant dissections

Male *U. pugilator* were purchased from Gulf Specimen, Panacea, Florida and acclimated to the laboratory as previously described (Hopkins 1982; Hopkins and Durica 1995). From an intermolt population, 7 limbs, including the large cheliped, were induced to autotomize by pinching with a forceps distal to the coxa. Autotomized animals were placed in shoeboxes filled with artificial sea water, and fed every 2 days on oats and cat chow. For early blastemal explants, buds were harvested at 8 and 12 days following autotomy. For animals in late proecdysis, limbs were harvested on the basis of *R* value and the decrease in growth rate seen just prior to ecdysis (Hopkins 1993).

For hormone induction experiments, 3 walking limb buds from a particular side of the animal were removed using a sterile needle, after washing the coxae with 70% ethanol. These limb buds were placed in 200 μl incubation buffer. Incubation buffer contained *Uca* saline (286 mM NaCl, 42 mM Na_2SO_4, 16 mM $CaCl_2$, 11 mM KCl, 76 mM Tris, pH 7.8) supplemented with 0.01 g glucose, 10 μl antibiotic/antifungal mix (Sigma A7292), 0.1 mg phenol red and 1 ml 100 mM HEPES, pH 7.0 per 9 ml of saline. For hormone inductions, the amount of 20-hydroxyecdysone (20E; Sigma H5142) suspended in methanol required for a final concentration of 5×10^{-7} M (Q-RTPCR experiments) or 1×10^{-6} M (RPA experiments) was evaporated to dryness then re-suspended in DMSO, which was taken to 1% final concentration in incubation buffer. Hormone concentrations in this concentration range are routinely used for explant and tissue culture studies in insect systems (Ashburner 1972; Karim and Thummel 1992; Siaussat and others 2004). For controls with no hormone, the 3 contralateral limb buds were removed and incubated in 200 μl incubation buffer containing 1% DMSO carrier. The

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3 limb buds taken for treatment were randomized with respect to axis. We also tested for transcriptional changes correlated to tissue culture but independent of hormone treatment. In control experiments comparing limb-bud tissue where RNA was isolated immediately, relative to explants kept for 1.5 h just in incubation medium, *Up378C* and *UpEcR* transcript levels overlapped with controls, but the *UpRXR* transcripts were variable, fluctuating between 40 and 300% of control levels, depending on the animal. The experimental and control comparisons for hormone treatments were therefore always based on explants isolated from the same animal.

RNA isolations

For transcript abundance studies, tissue was removed from incubation buffer, and total RNA isolated as described by the supplier using either a RNeasy™ kit (Qiagen) with a DNAse step incorporated into the column isolation, or the RNAqueous™-4PCR kit (Ambion), followed by a separate DNAse digestion step. RNA concentrations were determined by fluorometric assay using a RiboGreen™ RNA quantification kit (Molecular Probes) and the RNA was aliquoted and stored in water at −80°C. During Q-RTPCR, no reverse transcriptase-added controls indicated that the on-column DNAse treatment in the Qiagen protocol occasionally left residual amounts of DNA in the sample. Although this contamination could be detected very late in the amplification cycle, it did not interfere with analysis.

Quantification of mRNA transcripts

RPA experiments were performed on 10 μg of total RNA and quantified using electronic autoradiography as described (Chung, Durica, and others 1998; Durica and others 2002). For Q-RTPCR, primers for the *UpEcR, UpRXR, Up378C,* and *Up*18S ribosomal RNA genes were designed using Primer Express software (Applied Biosystems Inc. [ABI]) and synthesized by a commercial supplier (Integrated DNA Technologies). Control validation experiments indicated that these primers were appropriate for relative quantification studies. Randomly primed reverse-transcribed RNA was synthesized using a high capacity cDNA archive kit (ABI) and the reactions stored at −20°C. PCR reactions on an estimated 20 ng of reverse-transcribed template per reaction were run according to the supplier's instructions, using SYBR green detection (SYBR Green PCR Master Mix, ABI). Individual PCRs were run in duplicate or triplicate, with at least 2 replicate animals per treatment condition. Data were acquired on an ABI 7500 Sequence Detection System and normalized against 18S rRNA using the integrated software package. Dissociation experiments were performed to verify specific amplification. In both RPA and Q-RTPCR experiments, *UpRXR* and *UpEcR* probes and primers recognized conserved regions of the molecule and did not distinguish between isoforms of these NRs (Durica and others 2002; Wu and others 2004).

cDNA libraries

Protocols for the construction of the late proecdysial limb-bud library have been previously described (Chung, Durica, Clifton, and others 1998; Durica and others 2002). For construction of the early blastemal library, 50 animals were multiply autotomized, leaving the feeding claw, contralateral first walking leg, and ipsilateral fourth walking leg intact. After 4 days, blastemal tissue was harvested from under the autotomized cheliped and coxal stumps of walking legs, total RNA extracted (Trizol, Life Sciences) and mRNA isolated using affinity spin chromatography (Qiagen). A bacteriophage cDNA library was constructed using the lambda Uni-ZapXR vector system (Stratagene) which allows directional cloning. The number of independent clones in the primary library was $\sim 1.05 \times 10^4$ isolates. Most clone inserts range from ∼0.9 to 1.9 kb, consistent with the size fractionation of cDNAs used for library construction.

Mass excision of the primary lambda-based cDNA library was done using XL1-Blue MRF′ cells and the ExAssist helper phage according to the manufacturer's protocols (Stratagene). Colonies of phagemids were then obtained by infecting SOLR cells with the f1 lysates, incubating at 37°C for 20 min and plating on LB plates containing ampicillin. Colonies were picked robotically and replicates stored in duplicate 384-well microtiter plates at −80°C as glycerol stocks.

DNA sequencing, annotation, and database searching

Automated cycle sequencing was performed on ABI DNA sequencers at the Advanced Center for Genome Technology (AGCT). Each sequence was analyzed using the editing program Phred (Ewing and Green 1998). Additional scripts then evaluated each EST sequence for a series of criteria. These include (1) determining and marking the high quality endpoints; (2) removing any low quality EST sequences; (3) marking the vector left endpoint; (4) marking and removing ESTs which are completely vector; (5) checking for the vector right endpoint; (6) marking sequences that go to this endpoint, and (7) checking if the correct tag sequence is present. If the correct tag sequence was not present and/or if the poly(T) tail was not present

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on the 3′-end sequenced EST, the program removed these files from further consideration. The protocol also removed insert sequences <50 bp. The Phrap sequence assembly program was used for the assembly of EST sequences into extended consensus sequences. These consensus sequences make up the assembled database, which was examined for GenBank sequence similarity hits via the tBlastX search engine (Zhang and Maden 1997). Annotated EST sequences were deposited in the dbEST of the National Library of Medicine (NCBI) and are also available on the ACGT web site at http://www.genome.ou.edu/crab.html. Crustacean EST database information for the crabs *Carcinus maenas* (D. Towle, this volume) and *Callinectes sapidus* (T. Shafer, this volume), the lobster *Homarus americanus* (T. McClintock, this volume), the penaeid shrimp *Marsupenaeus japonicus* (K. Yamano, unpublished), *Litopenaeus vannamei* (A. Alcivar-Warren, unpublished) and *Penaeus monodon* (A. Tassanakajon, unpublished), and the branchiopod *Daphnia magna* (Watanabe and others 2005) were recovered from NCBI. Sequence similarity comparisons were performed using tBLASTx, and visualization of degrees of library similarity was performed with the PyMood™ software package (M. Marvienko, Allometra). Assignment criteria used the BLOSUM62 matrix and an expect value of 10^{-8}. Assignments to the Clusters of Orthologous Groups (COG) and Genome Ontology (GO) databases were made by the ExtractCOG (F. Najar, unpublished) and Blast2Go (Conesa and others 2005) programs at an expect value of 10^{-5}.

Results

Construction and sequencing of an early blastemal cDNA library

In *Uca pugliator*, damage to a limb causes autotomy, a reflexive loss of the injured limb at the junction between the coxa and the basioischium (Hopkins 1993). The limb will usually regenerate during the subsequent molt cycle. Invasion of granulocytes and blastocytes will lead to scab formation at the wound site, and the movement of migrant cells under the scab will form a hypodermal layer of cells that both proliferate and undergo changes in shape to a more columnar form (Emmel 1910). Histological examination of early limb-bud morphogenesis in *U. pugilator* indicates that of the first overt morphological features associated with blastemal organization, a particular feature is the secretion of a cuticle at ~4 days post-autotomy (Hopkins 1993; Hopkins and Durica 1995; Hopkins 2001). We reasoned that transcripts recovered at this stage of regeneration should therefore represent genes involved in these morphogenetic, proliferative, and synthetic processes. A directionally cloned, oligo-dT primed library was constructed in pBluescriptII SK vectors, representing $\sim10^4$ individual isolates, with average insert sizes ranging between 0.9 and 1.5 kb. Although subtractive techniques may have resulted in greater representation of rare blastemal transcripts, the amount of tissue that could be obtained via dissection was limited, and no normalization of transcript representation was used for A+4 library construction (see Methods).

A total of 1698 individual clones were sequenced, leading to 2309 individual high quality reads after clipping and trimming. These reads were assembled into 473 separate contigs and 417 singlets, for a total of 890 non-overlapping sequences.

EST representation in the blastemal library

A total of 2030 ESTs have been screened using tBLASTx and compared with the COG of proteins database (Tatsusov and others 2003). A summary of assignments is given in Figure 1. Approximately 86% of *Uca* A+4 EST sequences cannot be assigned to an ortholog; ~1% can be assigned to a gene of unknown function, and the remainder are approximately equally distributed (3–5%) in the “information storage and processing”, “cellular processes”, and “metabolism” categories. In the information storage and processing category, the overwhelming majority of assignments (88%) are to the conserved genes involved in translation, ribosomal structure, and biogenesis. The genes assigned to cellular processes are divided approximately equally among genes involved in cell division and chromosome partitioning (26%), post-translational modification and protein turnover/chaperones (37%), and inorganic ion transport and metabolism (35%). In the metabolism category, the majority of orthologs (47%) have been assigned to the energy production and conversion subgroup, with assignments in the amino acid-transport and carbohydrate-transport and metabolism subgroup (~16%) showing approximately twice the number of assignments to the lipid metabolism, coenzyme metabolism, and nucleotide transport and metabolism hits (6–8%). The *Uca* EST database was also screened against the Gene Ontology (GO) database (not shown; available on web site). In the GO annotations, sequences can be multiply assigned on the basis of 3 parameters; molecular function, cellular process, or cellular component. tBLASTx searches of this database produced a significantly greater number of biological functional assignments than for the COG database; ~40% of the domains identified among the assembled sequences could be assigned a GO annotation.

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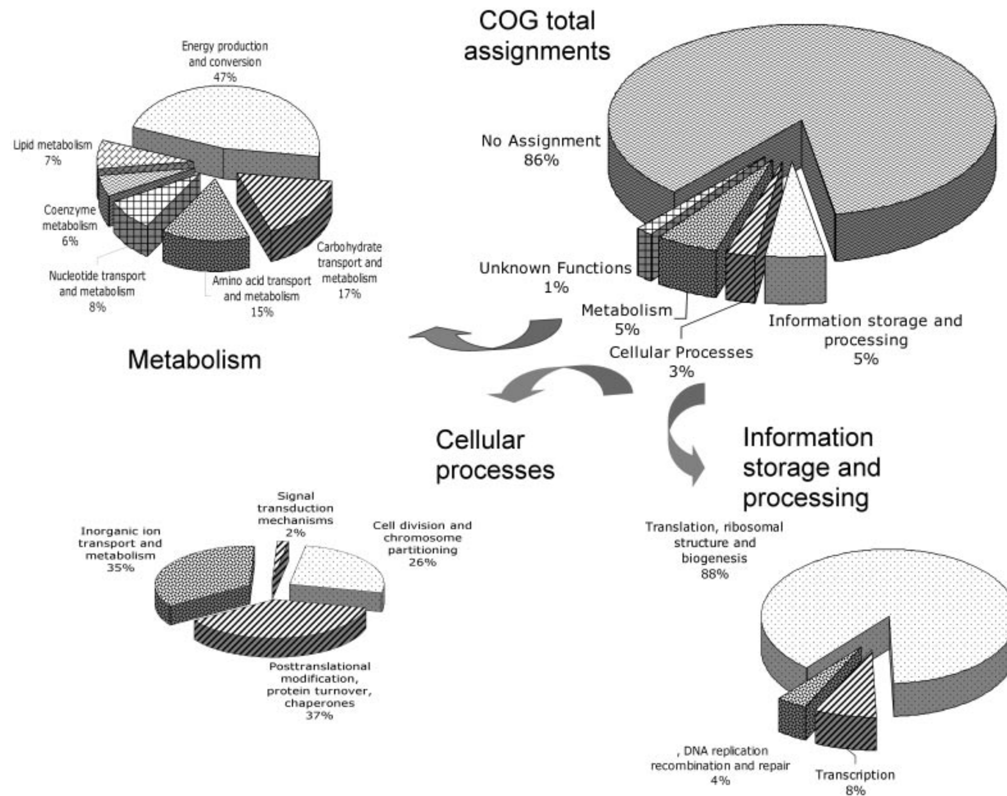

Fig. 1 Graphical representation of COG Assignments for *Uca* A+4 Blastemal cDNA Library. A total of 2030 ESTs from the *Uca* database were screened using the ExtractGo program (F. Najar) and assignments to COG database categories represented in the form of a pie chart. COG total assignments are found in the pie chart on the extreme right. The 3 major individual categories are further broken down into information storage and processing (bottom right), cellular processes (bottom left), and metabolism (top left). Specific assignments to each category can be found on the *Uca* database web site.

Table 1 Summary of COG search hits between *Uca* A+4 EST library and selected crustacean EST databases

COG category	*Callinectes* (13% of *Uca* ESTs)	*Carcinus* (18% of *Uca* ESTs)	*Homarus* (14% of *Uca* ESTs)	*Penaeus* (13% of *Uca* ESTs)	*Marsupenaeus* (18% of *Uca* ESTs)	*Litopenaeus* (23% of *Uca* ESTs)	*Daphnia* (19% of *Uca* ESTs)
% No hits	30	72	58	35	59	61	48
% Information storage and processing	21	13	10	34	19	20	24
% Cellular processes	9	6	10	12	8	7	10
% Metabolism	18	8	12	15	13	11	16
% Unknown functions	2	1	4	1	1	1	2

A web site for the *Uca* A+4 blastemal EST database has been established (http://www.genome.ou.edu/crab.html) providing both search capabilities and sequence information via anonymous file transfer. Information can be recovered from the database either through a keyword search or using the BLAST algorithm. A summary of COG and GO assignments for the *Uca* ESTs (COG) and assembled sequences (GO) is also available in table form and graphic form on the web site.

Comparisons with other crustacean EST databases

We have used tBLASTx and the *Uca* A+4 blastemal EST database to query the NCBI database for COG hits to sequences in other crustacean EST libraries. Comparisons have been made with libraries representing the crabs *Callinectes sapidus* (10 543 sequences), *Carcinus maenas* (9586 sequences), the lobster *Homarus americanus* (4067 sequences), the shrimp species *Penaeus monodon* (3057 sequences),

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Litopenaeus vannamei (8857 sequences), *Marsupenaeus japonicus* (3199 sequences), and the branchiopod, *Daphnia magna* (11 106 sequences). A summary of these data are given in Table 1. For the 3 pleocyematan (crab and lobster) decapods, between 14 and 30% of the *Uca* ESTs show significant hits, depending on the library. Of these sequences between 50 and 72% of the ESTs cannot be assigned to an ortholog in the COG database. Similar results were obtained for searches against the penaeid shrimp databases; 13–23% of the *Uca* ESTs show hits, with 35–68% of these sequences not assignable to a COG ortholog. For all decapods, ~20% of the assignments represent genes involved in information storage and processing, 10% represent genes involved in cellular processes, and 15% represent genes involved in metabolism. An additional comparison was made to a library constructed from a non-decapod crustacean, the branchiopod *Daphnia magna*, which contained the largest number of ESTS of the sequences assembled for analysis. Both hits to the *Uca* EST library and COG assignments were similar to the other searches; 19% of the *Uca* EST library produced hits against the *Daphnia* database, and 48% of these were to unknown orthologs. The distribution of hits into known categories was also similar to other searches: 24% information storage and processing, 10% cellular processes, and 16% metabolism.

As a means of further investigating similarities between sequences shared between crustacean libraries, tBLASTx comparisons were visualized using the program PyMood (Allometra). This program presents a colorimetric and spatial representation of degrees of sequence similarity between a query database (that is *Uca* blastemal ESTs) and up to 3 individual target databases. A 2-dimensional representation comparing the *Uca* database to the *Callinectes*, *Carcinus*, and *Homarus* databases is shown in Figure 2A, where each square represents a specific EST. In this comparison, the intensity of the color is an index of the degree of match to a particular EST in the query database; black indicates no similarity, while an intense color indicates high similarity. Combinations of hues (for example, equivalent red + green similarities = yellow) indicate hits to more than a target library, and an intense white color indicates a high degree of sequence similarity between all libraries and the query EST. In Figure 2A, ~65% of the *Uca* sequences do not show a match to the other 3 crustacean libraries. As indicated earlier, the greatest overall degree of similarity is seen with the *Callinectes* library (~30%), represented in blue. In Figure 2B, a comparison matrix is shown after the substitution of the *Daphnia* EST database for the *Homarus* database

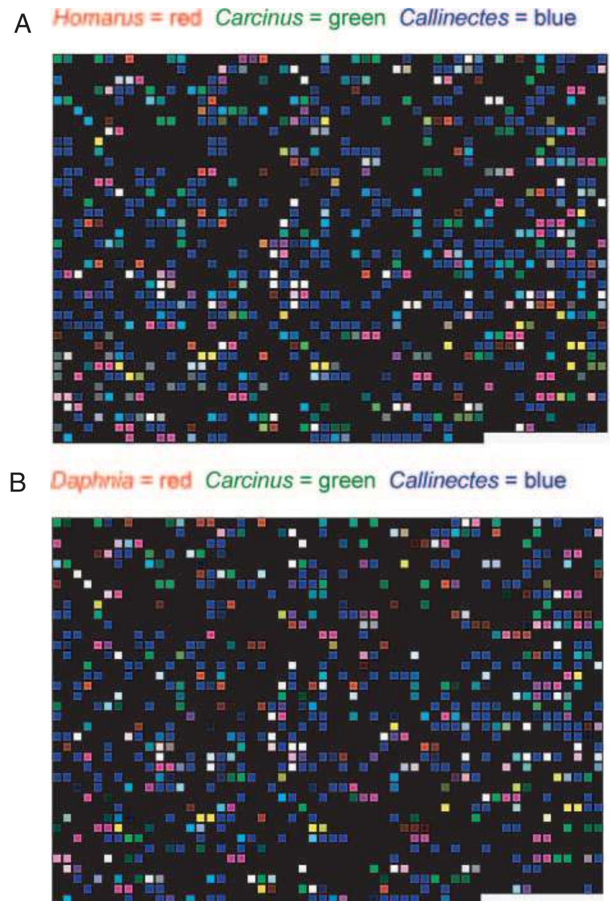

Fig. 2 Representations (2-dimensional) of EST database similarities between different crustacean EST libraries. The diagrams represent the output from the PyMood program (Allometra), comparing 3 different crustacean EST libraries to the *Uca* A+4 Blastemal EST database. Each of 2030 individual *Uca* ESTs is represented by a square; the color of the individual squares designates degrees of sequence similarity of the *Uca* query sequence to a single or more of the crustacean databases (see Results). (**A**) Comparison of *Uca* EST library to *Homarus* (red), *Carcinus* (green), and *Callinectes* (blue) EST libraries. (**B**) Comparison of *Uca* EST library to *Daphnia* (red), *Carcinus* (green), and *Callinectes* (blue) EST libraries.

(represented by red in each instance). Inspection shows that these 2 matrices are extremely similar; <1% of the *Uca* sequences show a match to the *Daphnia* database that was not detected in the decapod comparison matrix.

These data can also be visualized in a 3-dimensional array, where the intensity of hits along the *x*, *y*, and *z* axes represents the degree of sequence similarity to a specific library; the faces of the resulting cube represent hits between the query sequence and 2 libraries, and the diagonal within the cube represents hits to all sequences. Figure 3A represents a 3-dimensional representation of hits to the *Callinectes*, *Carcinus*, and

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Daphnia databases relative to the query *Uca* database. On a scale of between 1 and 100, the PyMood program has defined all sequences (white hexagons) with similarities >50 for all 3 libraries, that is, the most conserved sequences in all databases. Represented in this subset of sequences are high quality matches ($e^{-49} - e^{-109}$) to histone H3, ribosomal proteins (S2, L3, L4, L5, L7A, S8, L18), cytoplasmic actin,

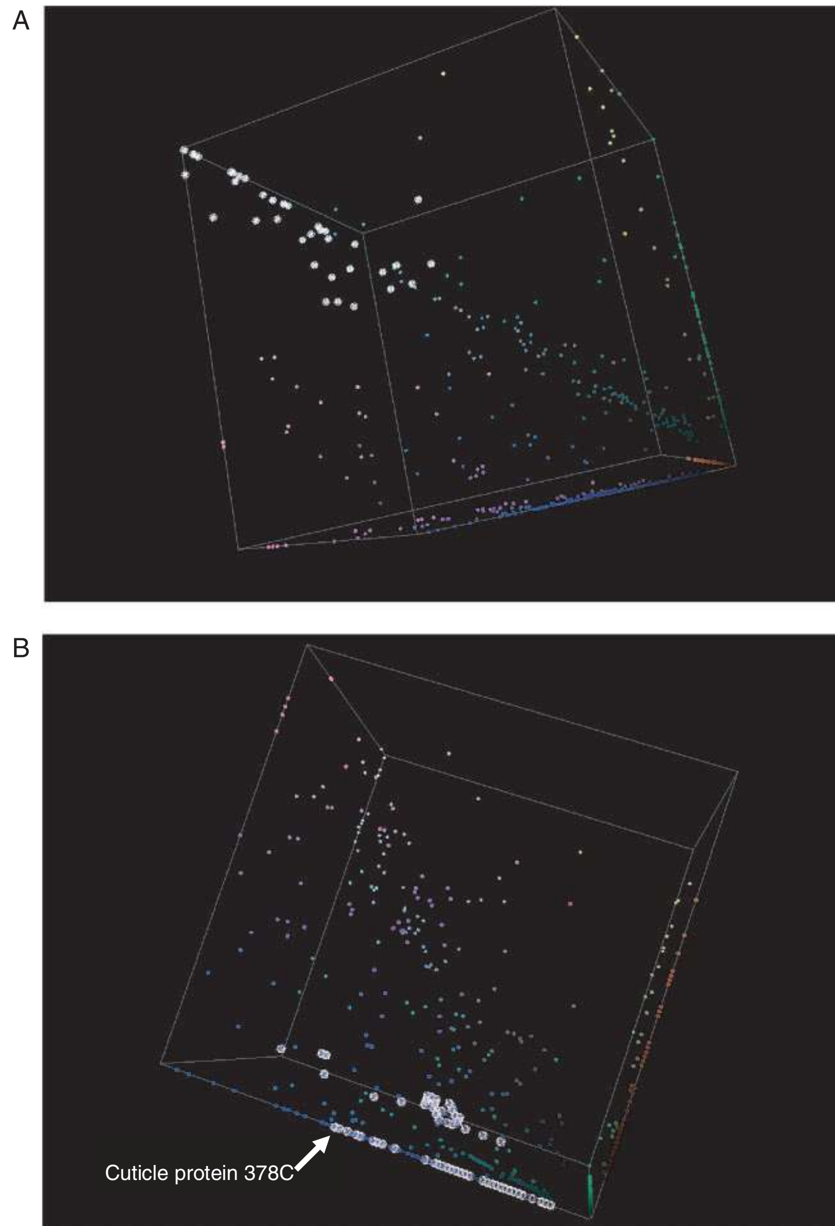

Fig. 3 Representations (3-dimensional) of EST database similarities, indicating hits for selected proteins. The diagrams represent the output from the PyMood program (Allometra) where the degree of sequence similarity between *Uca* query sequences to a single or more of the crustacean databases is indicated by both the intensity of dot color and its distance from the origin. Absence of a *Uca* query hit to any of the libraries is represented at the common intersection of the 3 axes (see Results). (**A**) Hits to most conserved sequences in the 4 libraries, using *Uca* query sequences against *Daphnia* (red), *Carcinus* (green), and *Callinectes* (blue) databases. The white hexagons on the diagonal represent highly conserved proteins constitutively expressed in all libraries. (**B**) Hits to putative cuticle proteins. The white hexagons represent putative cuticle protein sequences that match the *Uca* query sequences and sequences in the blue crab and water flea databases. The circled hexagon represents the *Up378C* cuticle protein contig. (**C**) Hits to putative farnesoic acid *O*-methyl transferase. The white hexagons found on the blue crab/green crab cube face represent hits between the *Uca* query sequence and putative homologs in the blue and green crab databases. (**D**) Hit to putative β-carotene 15,15′-monooxygenase. The white hexagon found on the blue crab axis represents a hit to a putative enzyme involved in conversion of β-carotene to retinal.

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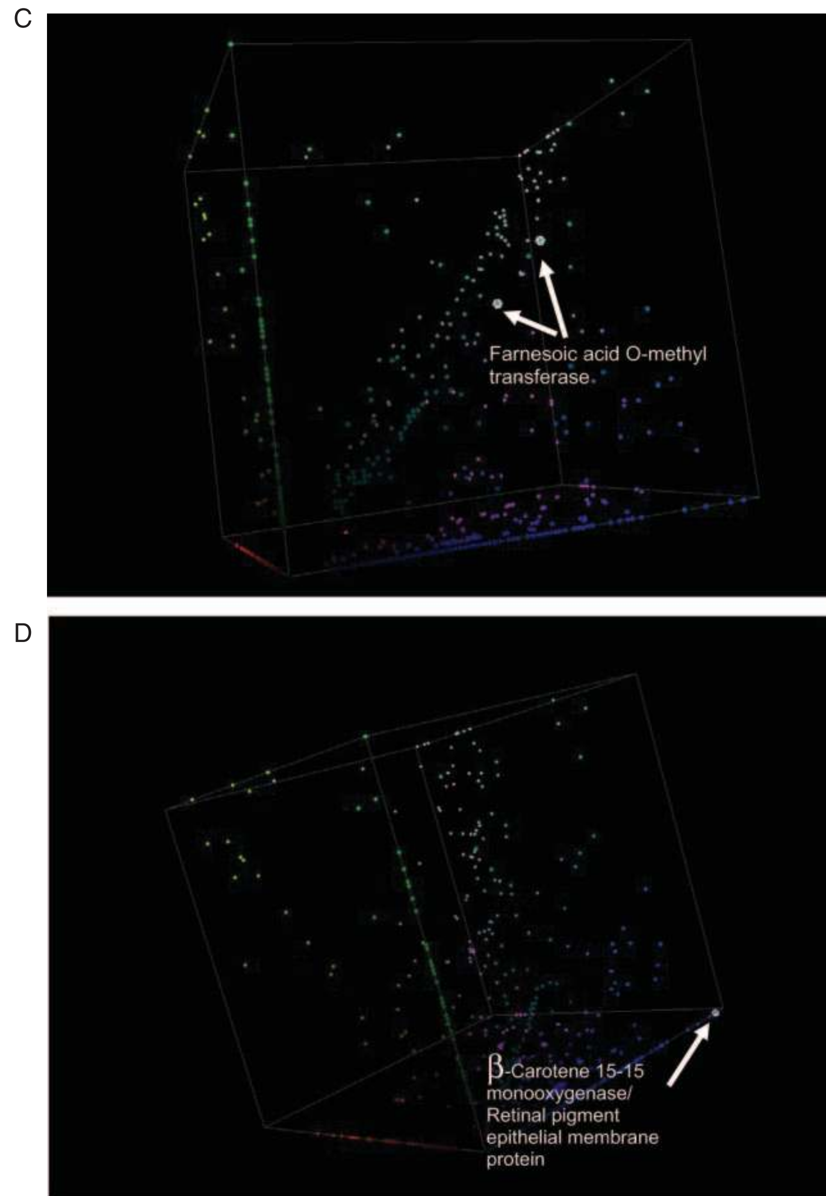

Fig. 3 Continued.

alpha, and beta tubulins, eiF-5A, ferritin, and proteins involved in energy/nucleotide metabolism (glyceraldehyde-3-phosphate dehydrogenase, ADP–ATP translocator protein, ADP ribosylation factor).

Genes transcribed at this particular stage of blastemal development that are linked to tissue-specific processes might be expected to have hits to only a subset of the libraries, or found only in the *Uca* database. Figure 3B shows a clustering of hits (white hexagons) to either the *Callinectes* database alone, or to the *Callinectes* and *Daphnia* databases. These all share sequence similarities to arthropodial cuticle proteins; such a sequence (white circle), designated *378C*, shared greatest similarity to arthropodial protein AM1159 of *Homarus*, and was used for further expression studies.

The database was searched for transcripts that may encode proteins involved in ecdysteroid control or retinoid/terpenoid metabolism. A putative homolog of a juvenile hormone esterase (not shown) was identified in all 3 libraries, and a putative homolog of farnesoic acid *O*-methyl transferase (Fig. 3C) was found in the *Carcinus* and *Callinectes* libraries. A match to an EST from the *Callinectes* library contains a retinol-binding domain found in a putative β-carotene 15,15′-monooxygenase (Fig. 3D). Clustal alignments for these *Uca* proteins are shown in Figures 4A–C; an alignment is also given for a putative cellular

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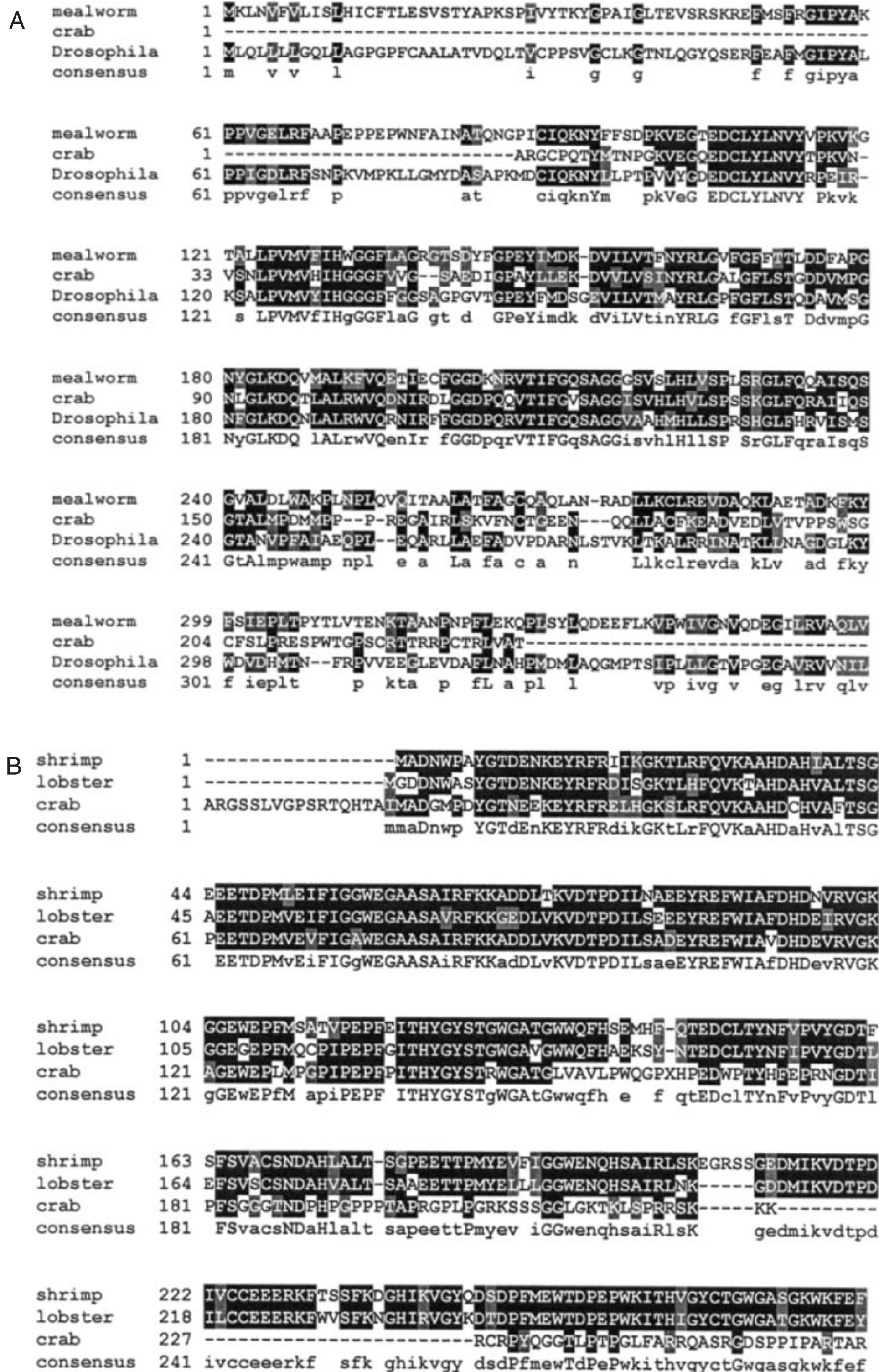

Fig. 4 Clustal analyses of sequence similarities identified by tBLASTx searches. Sequence information retrieved from the Uca A+4 blastemal database for several of the encoded proteins identified in Figure 3 were aligned using Clustal W (Higgins and others 1996) and displayed using BOXSHADE (K. Hoffman and D. Baron). (**A**) Alignment of *Uca* (crab; DW177034.1) sequence to putative juvenile hormone esterases from *Tenebrio* (mealworm; AF448479) and *Drosophila* (AF304352). (**B**) Alignment of *Uca* sequence (DW175044) to putative farnesoic acid *O*-methyl transferases from *Metapenaeus ensis* (shrimp; AF333042), *Homarus americanus* (lobster; U25845), and *Cancer pagurus* (edible crab; AY337487). (**C**) Alignment of *Uca* sequence (DW176347) to putative β-carotene 15,15′-monooxygenases from *Danio* (zebrafish; NM_131798), *Callinectes* (blue crab; CV224867), and mosquito (*Anopheles gambiae*; XP_317319). (**D**) Alignment of *Uca* sequence (A1a14up.r1) to putative cellular retinoic acid binding proteins from *Drosophila* (BT001835) *Manduca* (tobacco hornworm; U75307.1), and mouse (X15481).

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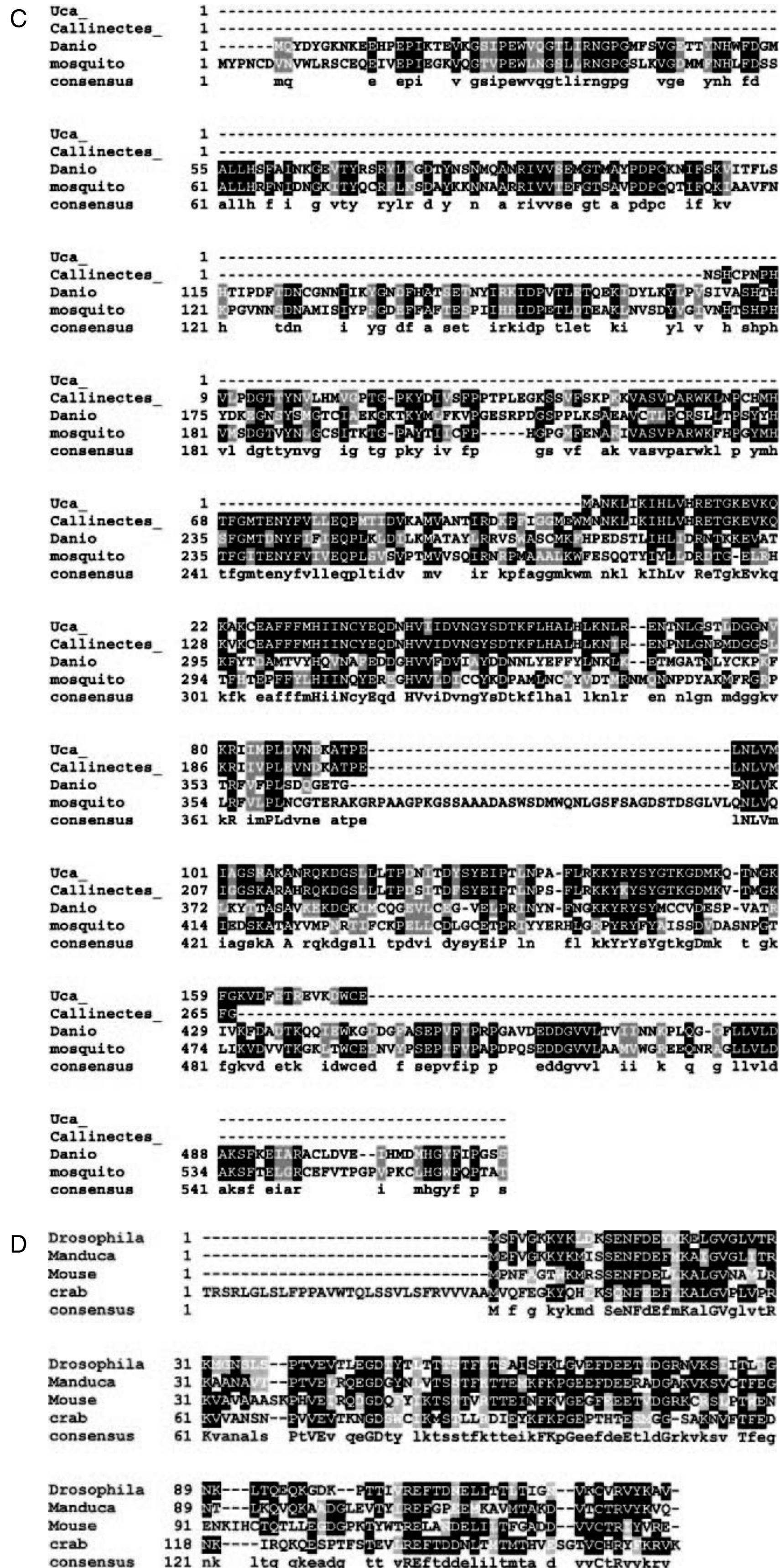

Fig. 4 Continued.

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retinoic acid (RA) binding protein (Fig. 4D) that shows similarity to insect sequences and to the shrimp *Metapenaeus ensus* (Gunawarende and others 2002).

Hormone induction experiments using limb-bud explants

The *EcR* gene has been shown to respond to ecdysteroid exposure in tissue explants in several insect systems (Karim and Thummel 1992; Siaussat and others 2004). We therefore tested whether continuous 20E exposure in limb-bud explants would modulate steady-state transcript levels of *UpEcR*, its cognate pairing partner *UpRXR*, and *378C*, a cuticle protein gene expressed during the development of the early limb primordium. When RNA samples taken from 8-day post-autotomy (A+8) explants were examined by Q-RTPCR, a subset of the buds exhibited the transcript abundance pattern shown in Figure 5A. In some A+8 explants, exposed to 20E for 1.5 h, transcript abundance increases ~5- to 9-fold for all 3 genes relative to control incubations. At longer incubation times, up to 2.5 h in these experiments, transcript levels show either no induction, or lower levels (<3-fold) of induction. For *UpRXR*, transcript abundance drops off ~4-fold after 2 h of incubation in hormone.

Not all A+8 explant experiments exhibited a 20E response with the kinetics seen in Figure 5A, indicating intrinsic differences between animals. In about half of the A+8 explants, the *UpEcR* and *Up378C* genes appeared refractory to 20E exposure at the 1.5-h time points, while the *UpRXR* transcripts decreased earlier, ~4.5-fold within the 1.5-h time course (Fig. 5B). Incubations for periods of up to 2 h led to either no difference in transcript abundance, or decreases on the order of 2-fold. Incubation of explants taken from animals 12 days post-autotomy (Fig. 5C) showed some similarity to the pattern exhibited by the A+8 explants shown in Figure 5B. In this case, *Up378C* transcript abundance was unchanged from control levels at the 1.5-h time point, while the other samples showed decreases in abundance, showing transcript levels 40–75% of those seen in the contralateral limb controls.

Finally, late limb-bud explants, taken from the transition from rapid limb-bud growth to the plateau in growth seen just before ecdysis (Hopkins 1993) were incubated for 2–3 h in 20E (Fig. 6A). The *Up378C* gene shows no change in transcript abundance until 3 h, when an ~70% increase in abundance is seen over controls. The *UpEcR* and *UpRXR* transcripts levels, however, do not change relative to controls over the course of the experiment. At this stage of limb-bud development, sufficient tissue can be harvested from a single animal to examine hormone-treated explants using RPA. For this experiment, 2 separate animals were incubated for 1 h in a 2-fold higher level of 20E, 1×10^{-6} M, and hybridized to radiolabeled probes that yield protected fragments of 317 nt (*UpRXR*) and 167 nt (*UpEcR*), respectively. The results of this assay are shown in Figure 6B. The steady-state transcript levels for *UpEcR* and *UpRXR*, are refractory to 20E exposure, showing approximately the same levels as the control incubations.

Discussion

Autotomy and early blastemal development: gene representation in the A+4 EST library

Regeneration of walking legs in *U. pugilator* follows tissue injury and is initiated after autotomy, the reflexive loss of the limb (McVean 1984). The first stage of regeneration, called basal growth, involves the formation of a new limb primordium, the blastema; it is an epimorphic process occurring under the wound requiring cell migration, proliferation and differentiation, and leads to the formation of a miniature, fully segmented appendage (Hopkins 1993). Many of the epidermal cells underlying the scab at the wound enlarge and some begin to divide 2–3 days after autonomy (Emmel 1910). These cells make up the new epidermis of the developing limb blastema and by 4 days begin to secrete a thin cuticle below the scab (Hopkins and Durica 1995). The first sign of the developing blastema is a cuticular shell formed beneath the wound. By 5–6 days after autotomy, the shell is populated with cells, and a second wave of cuticle secretion occurs ~7–8 days after autotomy. As the cuticle is secreted it forms infoldings that begin to divide the blastema into segments. The source of the cells that repopulate and differentiate into new limb tissue is unclear. There are reports that muscle and other tissues arise from local epidermal cells, while others suggest that muscle tissue arises from immigrant blastocytes (Adiyodi 1972; Hoarau 1973; Mittenthal 1981). The origin of immigrant cells is also unclear. Some of these researchers suggest that immigrant cells are dedifferentiated cells from various sources and others suggest that they are non-differentiated set-aside cells. Recently, interspecific transplantation experiments (*Cancer gracilis/productus*) have been performed in an attempt to determine whether differentiated limb tissue could contribute to the newly forming limb, as well as to evaluate the degree of competence of those cells in programming limb formation (Kao and Chang 1999). Assessing differences in appendage morphology and coloration, these studies suggested that donor tissues could be incorporated into host coxal stumps, form either claw or limb tissue regenerates,

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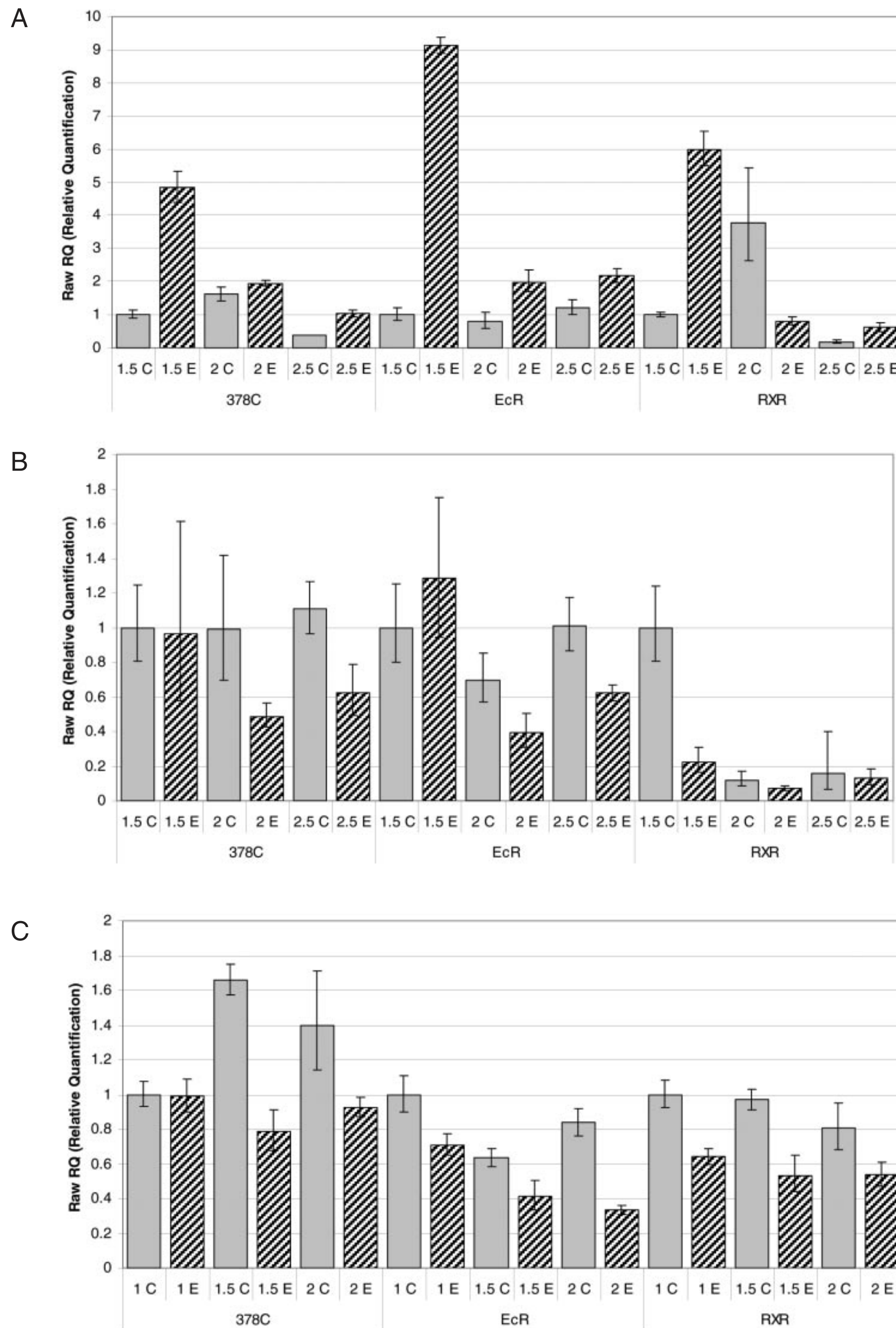

Fig. 5 Relative quantification of *Up378C*, *UpEcR*, and *UpRXR* steady-state transcript levels from hormone-induced and control explants taken from early (basal bud) stages of limb-bud regeneration. Limb-bud explants were incubated with DMSO carrier (C) or 5×10^{-7} M 20E (E) for the indicated time periods (for example 1.5 C represents a control explant incubated for 1.5 h in DMSO). Steady-state transcript concentrations were determined by Q-RTPCR and normalized to 18S ribosomal RNA transcript levels as described in Materials and Methods; for each of the genes, histograms depict ratio of transcript abundance relative to the first control time point; gray bars, carrier control explants, hatched bars, and hormone-treated explants. (**A**) Relative quantification of *Up378C*, *UpEcR*, and *UpRXR* transcript levels from single animal limb buds taken 8 days post-autotomy; $n = 4$ for each time point. (**B**) Relative quantification of *Up378C*, *UpEcR*, and *UpRXR* transcript levels from limb buds taken 8 days post-autotomy; composite data for 2 separate experiments from single animals; $n = 4$ for each time point. (**C**) Relative quantification of *Up378C*, *UpEcR*, and *UpRXR* transcript levels from single animal limb buds taken 12 days post-autotomy; $n = 3$ for each time point.

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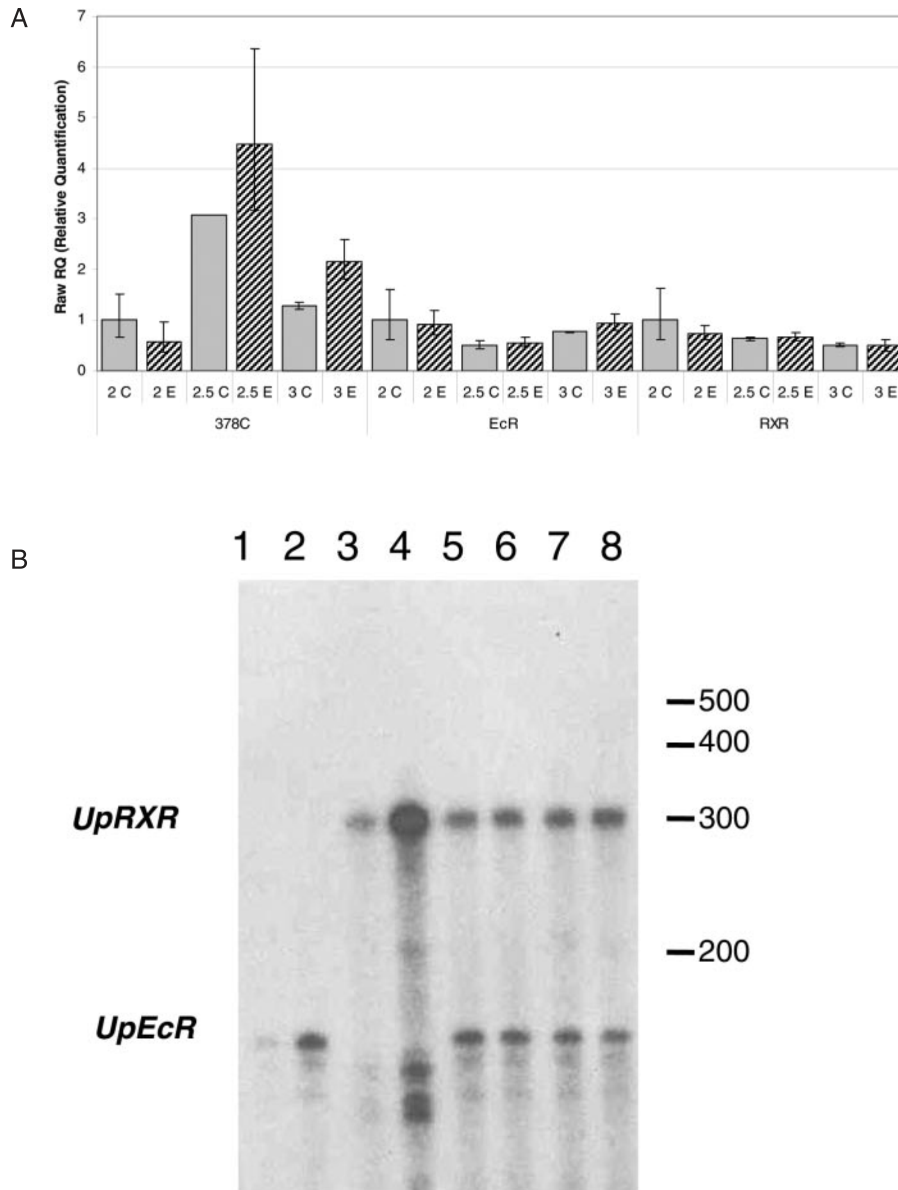

Fig. 6 Relative quantification of *Up378C*, *UpEcR*, and *UpRXR* steady-state transcript levels from hormone-induced and control explants taken from late (premolt) stages of limb-bud regeneration. (**A**) Q-RTPCR experiment performed as described in Figure 5. Relative quantification of *Up378C*, *UpEcR*, and *UpRXR* transcript levels from single animal limb buds taken from late premolt ($D_0 - D_{1\text{–}4}$ transition); $n = 2$ for each time point. (**B**) Ribonuclease protection assays (RPA) on late premolt explants. Single animal explants were incubated in 1×10^{-6} M 20E or carrier for 1 h and processed for total RNA extraction and RPA as described in Material and Methods. Lanes 1 and 2: 1 and 5 pg, respectively, of sense-strand *UpEcR* RNA, synthesized from a plasmid template and serving as a size and standardization marker for protected *UpEcR* fragment. Lanes 3 and 4: 1 and 5 pg, respectively, of sense-strand *UpRXR* RNA, synthesized from a plasmid template and serving as a marker for protected *UpRXR* fragment. Lanes 5 and 6: protected fragments in hormone-induced experimental (lane 5) and DMSO carrier control (lane 6) RNA samples isolated from late premolt limb-bud explants. Lanes 7 and 8: protected fragments in hormone-induced experimental (lane 6) and DMSO carrier control (lane 7) RNA samples isolated from limb-bud explants from a second animal at a comparable late premolt stage.

and perhaps serve as a second organizing focus, as some of the transplants generated bifurcated limbs.

The second stage of limb regeneration represents an hypertrophy of existing structures and is called proecdysial growth, occurring when the animals are preparing for molt (Hopkins and others 1999; Hopkins 2001). A pulse of ecdysteroid, followed by a drop in titer, is observed at the transition into the proecdysial stage (Hopkins 1989). A rapid increase in limb-bud mass occurs, due to protein synthesis

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and water uptake; the increase in protein synthesis correlates with the ecdysteroid pulse (Hopkins 1992, 2001). The rate of proecdysial limb-bud growth plateaus shortly before ecdysis, which is accompanied by another pulse of ecdysteroid just prior to molt and the extension of the new limb with the shedding of the old exoskeleton. The process of regeneration is coordinately controlled with the molt cycle; multiple autotomy accelerates the molt cycle, while removal of a single or more primary limb regenerates will trigger a delay in development so that secondary limbs regenerate (Mykles 2001; Yu and others 2002).

Although the specification of a new limb primordium following limb loss assumes as yet uncharacterized signaling pathways, the deposition of a flexible cuticle during basal growth, and the relationship of regeneration to the molt cycle, suggest a link to ecdysteroid signaling mechanisms. The 2 components of the ecdysteroid receptor, encoded by the *UpEcR* and *UpRXR* genes, are expressed in limb buds throughout regeneration (Chung, Durica, Clifton, and others 1998; Chung, Durica, and others 1998; Durica and others 2002; Wu 2003; Wu and others 2004). There is also considerable circumstantial evidence for retinoid/terpenoid signaling in crustacean developmental processes. In many vertebrate tissues RA (Oro and others 1992; Means and Gudas 1995; Brockes 1997) induces differentiation by controlling the production of morphogenic signals (Noji and others 1991; Helms and others 1996) and exogenous RA has profound, disruptive effects on the regeneration of vertebrate limbs (Brockes 1997; Maden 2000). We have previously demonstrated that all-trans RA can affect the regeneration of limbs in *Uca* (Hopkins and Durica 1995). When *Uca* is placed in an emulsified preparation of 0.05 mM RA immediately after multiple autotomy, subsequent regeneration of some limbs is significantly inhibited and the organization of the limb blastema is disrupted. The sesquiterpenoid methyl farnesoate (MF), an unepoxidated form of juvenile hormone, JHIII, has also been implicated in a number of hormonal activities mimicking the effects of JH in insects (Laufer and Biggers 2001; Borst and others 2001). MF has been implicated in the stimulation of ecdysteroid synthesis of crustaceans (Borst and others 1987; Tamone and Chang 1993). It is believed to enhance ovarian maturation (Laufer and others 1998; Jo and others 1999) and exogenous MF administration has been reported to result in the formation of larval intermediates and retard larval development (Abdu and others 1998). Due to its chemical similarities to molecules known to function as NR ligands, it is possible that MF could interact with UpRXR or an as yet uncharacterized crustacean NR. We hypothesize that retinoid mediated disruptions of normal signaling pathways, perhaps involving UpEcR/UpRXR interactions, might compromise normal blastemal development.

We constructed a cDNA library, representing $\sim 10^4$ individual isolates, from blastemal limb-bud tissue collected 4 days following autotomy. Analysis of $\sim$1700 cDNA clones led to the assignment of 473 contigs and 417 singlets, for a total of 890 sequences. Approximately 86% of ESTs in this database could not be assigned to an ortholog in the Cluster of Orthologous Groups (COG) database, at an expect criterion of 10^{-5}. Analysis of these unclassified sequences relative to sequencing with $5'$ or $3'$ specific primers showed approximately equal numbers in each category, suggesting that the "no hit" clones may contain a large number of new genes (see below). Biological assignments for the $\sim$14% of ESTs assigned to a COG designator are broken down into information storage and processing, cellular processes, or metabolism categories, and a detailed list of assignments is available on a web site where this database can be searched by keyword and BLAST, and sequence information retrieved by anonymous FTP.

BLAST comparisons with the GO database were also performed at the same expect criterion, in this instance using the assembled dataset. This search yielded a significantly higher number of assignments; $\sim$40% of the assembled sequences had a domain structure assignable within the GO vocabulary. This difference in assignment may be a function of database updating and perhaps the weighting of the COG database to ortholog assignments relative to assignments based on domain structure, which may be paralogous. As with the COG assignments, GO assignments are available from the library web site.

Recently, a number of crustacean tissue-specific EST databases have been constructed (Watanabe and others 2005; T. Shafer, unpublished data; McClintock and others 2006; Towle and Smith 2006), designed to examine differences in gene expression resulting from environmental perturbations (for example osmotic or thermal stress, viral infections) and/or transcriptional responses due to tissue specialization and changes in physiological function (olfactory organ, gill, hypodermis). BLAST comparisons to several of these databases from a variety of crustacean species identifies a subset of common COG assignments, roughly distributed among the COG categories as 20% information storage and processing, 10% cellular processes, and 15% metabolism. Approximately 60% of genes present in the *Uca* database that are shared with other crustacean databases, therefore, are still unassigned to a specific eukaryotic ortholog.

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Nevertheless, there are clear differences between the libraries in terms of BLAST hits to the *Uca* database. The *Homarus* database, which represents an olfactory-organ-specific library contains hits to only 14% of *Uca* ESTs, while the *Callinectes* database, derived from hypodermal tissue contains hits to 30% of *Uca* ESTs. Although this comparison is influenced by the number of ESTs present in the target databases, it also undoubtedly reflects similarities in gene-expression profiles for subsets of genes not related to "housekeeping" functions. For example, this is clearly the case with regard to the arthropodial membrane proteins common to both the *Uca* and *Callinectes* libraries. This class of proteins is found at limb joints and represents cuticle that does not harden; this would be expected for cuticle associated with the growing bud, which must expand greatly as the limb undergoes hypertrophy.

Among the sequences shared between libraries that did not produce an ortholog assignment in the COG databases are several that share domains with enzymes or lipid-binding molecules linked to retinoid and sesquiterpenoid metabolic pathways. We hypothesize that several of these molecules may be involved in retinoid/terpenoid metabolism. These include significant hits to a putative juvenile hormone esterase, a farnesoic acid O-methyl transferase, an RPE65 domain protein most similar to a β-carotene 15,15′-monooxygenase, which catalyzes retinal production, and a cellular RA binding protein. There are also significant hits to proteins that may play a role in ecdysteroid responses, such as homologs to heat shock proteins hsp21 (Dubrovsky and others 1996, 2001), hsp90 (Arbeitman and Hogness 2000), and cuticle protein genes (Hiruma and others 1997; Noji and others 2003).

Effects of ecdsyteroid exposure on *UpEcR*, *UpRXR*, and *378C* transcript levels

The coordination of molt cycle-related events with limb-bud regeneration, the expression of genes linked to ecdysteroid signaling in the limb-bud, and an increase in the rate of protein synthesis correlating with an ecdysteroid pulse in premolt limbs, prompted us to investigate whether limb-bud explants could be used to directly test for changes in gene transcript levels in response to ecdysteroid exposure. These experiments tested buds from different growth stages, exposed to relatively low levels of exogenous hormone (5×10^{-7} M 20E) for periods of up to 3 h. Transcript levels for 3 ecdysteroid-responsive candidate genes, the genes encoding the 2 components of the ecdysteroid receptor *UpEcR* and *UpRXR*, and a cuticle protein gene expressed early in limb-bud regeneration, *378C*, were quantified by Q-RTPCR. For all 3 genes, a prominent increase in steady-state transcript abundance (>5-fold) was observed only in early (A+8) blastemal buds, with the most pronounced effects of induction seen at the shortest time period (1.5 h) of exposure. Furthermore, only a subset (about half) of the animals tested showed an increase in mRNA abundance at the early time point; some animals appeared refractory, and decreases in transcript levels were seen at longer (2–2.5 h) exposure times. These results suggest that all 3 genes may be primary ecdysteroid response genes under appropriate conditions, only a subset of the early buds may be competent to respond to an ecdysteroid signal, and that longer periods of exposure lead to repressed transcript levels for all 3 genes. This interpretation is supported by experiments conducted at the A+12 stage of limb-bud regeneration. For A+12 staged buds, transcript levels for all 3 genes decreased over the experimental time course. Finally, at the latest period monitored, buds taken from the late premolt stage, both *UpEcR* and *UpRXR* transcript levels remained static over a 3-h exposure, and a <2-fold increase in *378C* transcripts was observed after 3 h. The observation that *UpEcR* and *UpRXR* genes are refractory to ecdysteroid stimulation in late premolt buds is further supported by RPA experiments, where sufficient amounts of RNA can be isolated from individual animals to perform this assay. Experiments conducted using buds incubated for 1 h at 1×10^{-6} M 20E indicated no change in transcript abundance relative to controls.

To summarize, the transcriptional profiles of these 3 candidate genes in this limb-bud explant system are complex. These results suggest that intrinsic factors other than hormone concentration influence steady-state transcript levels, and that these factors vary relative to time of incubation and tissue state.

Although the formation of the crustacean limb blastema and its differentiation into an organized limb are just beginning to be studied at the molecular level, it is clear from histological and anatomical investigations that the process is distinct from vertebrate limb morphogenesis and insect imaginal development, making it an attractive model for examining cell recruitment and differentiation in the reprogramming of invertebrate limb development. These experiments have begun to examine the degree of transcript heterogeneity found at this stage of blastemal development and will serve as an index to monitor changes in transcription in preceding and later stages. Information on the primary amino acid sequences derived from this and other limb-bud cDNA libraries will allow for the construction of DNA microarrays

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and multiplex screening to monitor for changes in gene expression in a much larger population of genes than previously possible in this organism. This has great potential for providing information on downstream targets in signal transduction pathways. Finally, comparisons of the crustacean sequences to other genes in the GenBank dbEST should help to define genes used in novel developmental/physiological contexts and may provide clues to their biochemical function.

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