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Comparative Biochemistry and Physiology, Part D 1 (2006) 200 – 208
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Expressed sequence tags from normalized cDNA libraries prepared from gill and hypodermal tissues of the blue crab, *Callinectes sapidus*

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Received 17 July 2005; received in revised form 10 October 2005; accepted 11 October 2005
Available online 5 December 2005

Abstract

Expressed sequence tags (ESTs) were produced from two normalized cDNA libraries from the blue crab, *Callinectes sapidus*. The gill library represented pooled RNA from respiratory and transporting gills after acclimation to either high or low salinity. The hypodermis library was from arthrodial and dorsal tissue from both pre- and post-molt crabs. Random clones were single-pass sequenced from the 5′-ends, resulting in 11,761 high quality ESTs averaging 652 bases. All the ESTs were assembled using Paracel Transcript Assembler software, producing 2176 potential transcripts—883 contigs and 1293 singlets. Of these, 1235 (56.7%) were sequenced only from the gill library, while 578 (26.6%) were exclusively hypodermal. There were 363 contigs containing ESTs from both tissues (16.7% of the putative transcripts). All contigs and singlets were compared to the public protein database using BLASTx, and descriptions of the three most similar proteins for each were recorded. Additional annotations included an Interpro analysis of protein domains and a listing of Gene Ontology (GO) categories inferred from similar proteins in GO-annotated databases. All sequences are available on a web page (http://firedev.bear.uncw.edu:8080/shaferlab/). The annotations can be searched, and BLAST alignment of user-inputted sequences against the putative transcripts is possible. In addition, the ESTs have been submitted to GenBank.

Keywords: Blue crab; cDNA; *Callinectes sapidus*; Expressed sequence tag; Gill; Hypodermis; Transcriptome

1. Introduction

Expressed sequence tags (ESTs) are partial sequences of cDNAs typically produced by rapid single-pass sequencing of hundreds or thousands of randomly chosen clones from cDNA libraries. The most highly expressed genes will be represented in the library many times by identical or nearly identical clones. To reduce this redundancy and increase the chances of identifying rare transcripts, the cDNA libraries used in EST projects are often normalized to increase the relative frequency of rare clones (Bonaldo et al., 1996). Once the sequencing is completed, computerized clustering and assembly can be used to order the large collections of ESTs into overlapping contiguous sequences ("contigs") that represent unique transcripts. In this way, an incomplete representation is obtained of the transcriptome of the tissue used to produce the original cDNA. This is possible even without an accompanying genome project. Thus, for organisms with as yet poorly described genetic backgrounds, the ease and relative economy of partial sequencing of cDNA libraries can be a highly productive way to generate a great deal of information about functioning genes (Boguski et al., 1993; Rudd, 2003). The data in such EST projects can be mined directly for rapid gene discovery (Blackshear et al., 2001), as well as lead to the production of microarrays useful for characterizing multi-gene expression patterns (Whitfield et al., 2002).

Callinectes sapidus, the blue crab, is an economically relevant natural resource. According to the US National Marine Fisheries Service (http://www.st.nmfs.noaa.gov/st1/

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doi:10.1016/j.cbd.2005.10.004

commercial/landings/annual_landings.html), commercial landings this decade on the Atlantic and Gulf coasts have been worth over $150 million annually. Yet the fishery has recently experienced serious declines in catch per unit effort in traditional fishing grounds such as the Chesapeake Bay (Maryland Sea Grant, http://www.mdsg.umd.edu/crabs/bbcac/status01.html). More research on the biology of this species is warranted. A large literature exists describing the development, molting, physiology, and behavior of the blue crab because it is readily available and is commercially taken (Kennedy and Cronin, in press). Yet very little is known about its genome. Since this species is not easily mated or raised in captivity, classical genetic approaches are difficult. The haploid genome size of the blue crab is 2.14 pg DNA (Rheinsmith et al., 1974), and as much as 30% of the nuclear DNA may be highly repetitive, low complexity sequences (Wang et al., 1999). Only a few (less than 30) complete mRNA sequences coding for different blue crab proteins have been submitted to the public databases. There are no nuclear genomic sequences in GenBank, though the complete mitochondrial genome has recently been submitted (Place et al., 2005).

Previous studies in our laboratories on two physiologically important epithelial tissues of the blue crab have used a gene-by-gene approach for the study of relevant proteins. A search for transporters and transport-related proteins that might be involved with the ion-transporting function of the gill epithelium has identified and characterized several cDNAs from *C. sapidus* or the closely related green shore crab, *Carcinus maenas*. These include the Na^+/H^+ exchanger (Towle et al., 1997a), the $Na^+/K^+/2Cl^-$ cotransporter (Towle et al., 1997b), the α-subunit of Na^+/K^+-ATPase (Towle et al., 2001), a V-type H^+-ATPase (Weihrauch et al., 2001), a putative ammonium transporter (Weihrauch et al., 2004) and a carbonic anhydrase (Henry et al., 2003).

Studies related to biomineralization in the blue crab have shown that the composition of the proteins secreted into the cuticle by the hypodermis changes dramatically after the molt (Shafer et al., 1995; Coblentz et al., 1998). One protein thought to be an inhibitor of initial mineral formation has been purified and partially sequenced (Tweedie et al., 2004). The sequences of five other hypodermal cDNAs have been published, one coding for a prophenoloxidase activating factor (Buda and Shafer, 2005) and the others coding for small proteins that bind chitin and are differentially expressed in either the calcifying cuticle or the flexible arthrodial membrane (Wynn and Shafer, 2005). This gene-by-gene approach has been successful, but by necessity, it is rather slow. With the production of ESTs and microarrays, gene discovery in relation to both ion transport during osmoregulation and cuticle deposition and mineralization will be greatly enhanced.

This paper reports the first large-scale EST project on the blue crab, *C. sapidus*. It is a partial sequencing and assembly of the most abundant transcripts in the gill and hypodermis. It includes an attempt to annotate the data in ways that will aid the discovery of new functional genes in these tissues. The project will advance our own work to the transcriptome level, especially since these sequences will form the basis for future microarray studies. Moreover, the sequences and the annotations are being made available to others in the crustacean research community.

2. Materials and methods

2.1. Animals and tissues

Pre-molt (D_2) and post-molt (3 h after ecdysis) adult *C. sapidus* were obtained in May at a "shedding" operation in Kill Devil Hills, NC, USA. Hypodermis was dissected from above the cardiac chamber (mid-dorsal hypodermis). This location was chosen because the epithelium is obtained without non-epithelial tissue contamination and is synthesizing cuticle destined to calcify. Hypodermis was also dissected from a small triangular region of the carpus joints of the chelipeds. This hypodermis produces the arthrodial membrane, a portion of the cuticle that does not calcify and yet is similar to mid-dorsal hypodermis in both overall morphology and the timing of deposition (Williams et al., 2003). Adult intermolt male crabs purchased locally in mid-summer in Wilmington, NC, were acclimated at 21 °C to salinity of either 5 ppt or 35 ppt for 3 days before their anterior and posterior gills were dissected. This acclimation period was chosen because a significant change in the expression of the Na^+/K^+-ATPase gene has been seen in as little as 4 days (Lucu and Towle, 2003).

2.2. RNA extraction and cDNA library production

In order to obtain enough RNA for library construction, it was necessary to pool tissue from more than one crab. Mid-dorsal hypodermis was pooled from six pre-molt crabs and from eight post-molt crabs. Seven crabs each from pre-molt and post-molt stages were used to obtain arthrodial hypodermis RNA. The anterior gills and posterior gills of three crabs adapted to each of the two salinities were pooled. RNA was extracted from each of these eight tissue samples using Trizol (Invitrogen) according to the manufacturer's protocol. The concentration of RNA was determined spectrophotometrically. All 260/280 ratios were between 1.8 and 2.1. Quality of the RNA was checked by observing intact rRNA on denaturing RNA gels. The four hypodermis RNA extractions were then combined, as were the four gill RNA extractions.

Two cDNA libraries, one from the combined hypodermal RNA and the other from the combined gill RNA, were constructed and normalized by Invitrogen Corporation. The cloning vector was pCMVSport6.1, the Invitrogen plasmid of choice for normalized libraries. The normalization procedure is proprietary but generally follows the method of Bonaldo et al. (1996) involving directional cloning and subtractive hybridization at two different Cot values,