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Gene expression and specificity in the mature zone of the lobster olfactory organ

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Stepanyan, Ruben, Kristen Day, Jason Urban, Debra H. Hardin, Ranjit S. Shetty, Charles D. Derby, Barry W. Ache, and Timothy S. McClintock. Gene expression and specificity in the mature zone of the lobster olfactory organ. *Physiol Genomics* 25: 224–233, 2006; doi:10.1152/physiolgenomics.00276.2005.—The lobster olfactory organ is an important model for investigating many aspects of the olfactory system. To facilitate study of the molecular basis of olfaction in lobsters, we made a subtracted cDNA library from the mature zone of the olfactory organ of *Homarus americanus*, the American lobster. Sequencing of the 5′-end of 5,184 cDNA clones produced 2,389 distinct high-quality sequences consisting of 1,944 singlets and 445 contigs. Matches to known sequences corresponded with the types of cells present in the olfactory organ, including specific markers of olfactory sensory neurons, auxiliary cells, secretory cells of the aesthetasc tegumental gland, and epithelial cells. The wealth of neuronal mRNAs represented among the sequences reflected the preponderance of neurons in the tissue. The sequences identified candidate genes responsible for known functions and suggested new functions not previously recognized in the olfactory organ. A cDNA microarray was designed and tested by assessing mRNA abundance differences between two of the lobster's major chemosensory structures: the mature zone of the olfactory organ and the dactyl of the walking legs, a taste organ. The 115 differences detected again emphasized the abundance of neurons in the olfactory organ, especially a cluster of mRNAs encoding cytoskeletal-associated proteins and cell adhesion molecules such as 14-3-3 ζ , actins, tubulins, troponin, Fax, Yel077cp, suppressor of profilin 2, and gelsolin.

microarray; bioinformatics; crustacea; neurogenesis

LOBSTERS ARE IMPORTANT MODELS for studying olfaction. Lobster olfactory sensory neurons are large compared with most other animal species. This advantage is one reason that they were the first olfactory sensory neurons investigated by patch-clamp electrophysiology, and, more broadly, the first neurons of any type used for patch clamping in situ in tissue sections (2). This approach led to numerous discoveries such as hyperpolarizing receptor potentials, modulation of sensory neuron sensitivity by neuroactive compounds, presynaptic inhibition of olfactory sensory neurons, dual olfactory transduction pathways, and regulation of olfactory transduction by phosphatidylinositols (1, 49–51, 76, 83, 84). Equally important to the success of the

lobster as a model is an understanding of the chemistry and behavioral significance of odors in crustaceans to the extent where specific behaviors can be evoked by synthetic odors consisting of mixtures of amino acids and nucleotides (13, 14). The development of associative conditioning tests for lobsters provided further behavioral measures that could be directly correlated with physiology, thereby allowing pioneering studies into the perception of odor mixtures (for reviews, see Refs. 19 and 20). The means to investigate chemistry, behavior, and physiology in a single organism has made the lobster a significant model for the study of the neural coding of odor quality.

Lobsters also share with other animals the ability to continuously replace the olfactory sensory neurons, even as adults. The lobster's olfactory epithelium contains multipotent progenitor cells that give rise to new olfactory sensory neurons associated with replacement of aged, damaged, or lost tissue (35, 70, 71). This is an unusual property given the static and irreplaceable nature of most other types of neurons. Lobsters have a unique anatomic separation of the site of neurogenesis from the location of mature sensory neurons. The olfactory organ has a proximal-to-distal age gradient, with nascent neurons born at the proximal end, a transitional zone presumably containing immature neurons, a mature zone containing odor-responsive neurons, and a senescence zone at the distal tip that will be lost at the next molt (37, 70) (Fig. 1). This separation by age and developmental stage has significant advantages for the investigation of mechanisms of adult neurogenesis (35, 36, 71).

Another area in which the anatomy of the lobster olfactory organ provides advantages by segregation of function is the packaging of the sensory neuron's outer dendrites, equivalent to olfactory cilia of vertebrates, into long hairlike setae (33). The constraint of having an external skeleton presumably forced arthropods to develop specialized cuticular setae to house sensory structures. Isolated olfactory aesthetasc setae are nearly pure preparations of the outer dendrites. This allows easy harvesting of the cellular compartment where odor detection takes place, facilitating investigation of processes such as odorant binding, olfactory transduction, and odorant clearance (11, 30, 32, 57, 58, 74, 75, 82).

A new structure recently described in the lobster olfactory organ is a secretory gland, called the aesthetasc tegumental gland (60, 68). These glands are closely associated with aesthetasc setae and have ducts whose pores open at the base of these setae. While its precise roles are not known, it may be broadly functionally analogous with secretory glands in the

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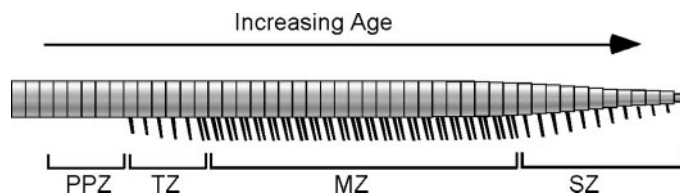


Fig. 1. Depiction of the age gradient organization of the lobster olfactory organ. The olfactory organ is the distal end of the lateral flagellum of the first antenna, or antennule. This structure consists of repeating annuli, each of which bears a similar pattern of several types of setae (16, 70). Olfactory sensory neurons are associated with aesthetasc setae, which are shown extending from the ventral face of the annuli. New aesthetascs and their olfactory sensory neurons are born in the proximal proliferation zone (PPZ) and to a lesser degree in the transition zone (TZ), mature in the TZ, function as odorant detectors in the mature zone (MZ), and senesce in the senescence zone (SZ) before being lost at the next molt. The number of aesthetasc setae is the visible indicator of the different zones and guided the dissection and collection of PPZ, TZ, and MZ for this project.

mammalian olfactory epithelium (26) by modifying and maintaining the environment around the dendrites of olfactory sensory neurons.

The wealth of physiological and biochemical data generated from the lobster olfactory organ has made it an attractive system to investigate underlying molecular events. Candidate gene approaches and small-scale expression profiling efforts have identified components of olfactory transduction pathways, several ion channels, specific markers of the major cell types in the organ, a surprising wealth of proteases and their inhibitors (including some associated with the aesthetasc tegumental gland), and a marker of reactive epithelial cells that appears to be critical for regeneration of the organ (39, 40, 46, 52, 54, 68, 69, 79–82). However, much more could be rapidly learned about how this organ functions if we understood more about the genes it expresses. To this end, we generated expressed sequence tags (ESTs) from >5,100 cDNAs from the mature zone of the olfactory organ of the American lobster, *Homarus americanus*. The annotation of these sequences provided a broad perspective on the molecular functions and biochemical pathways used in the organ. A DNA microarray generated from the cDNAs provided a means to identify individual mRNAs enriched in the olfactory organ.

MATERIALS AND METHODS

Lobsters and tissue collection. Adult lobsters (*H. americanus*) were obtained from Falmouth Fish Market (Falmouth, MA). Live lobsters were maintained in Instant Ocean (Aquarium Systems; Mentor, OH) at 6°C for no more than 2 wk before use for tissue harvest. Lobsters that were lethargic or had visible exobiont growth on their exoskeletons were discarded. Tissues were dissected from lobsters chilled on ice, flash frozen in liquid nitrogen or dry ice, and stored at –80°C. The lobster olfactory organ is located at the distal end of the lateral flagellum of the antennule. The three different regions of the olfactory organ used in this project (the mature zone, proximal proliferation zone, and transition zone) are depicted in Fig. 1. We also collected other tissues from lobsters: leg dactyl, which is the distal-most segment of the four pairs of walking legs (pereopods 2–5); pereopod without dactyl; medial flagellum of the antennule; second antenna; eye (the eye proper and the eyestalk with its protocerebral ganglia); and tail (abdominal) muscle.

Subtracted cDNA library. TriReagent was added to the frozen tissue samples, the mixture was immediately homogenized with a Polytron, total RNA was isolated, and poly A⁺ RNA was then purified

on oligo-dT cellulose columns (Molecular Resource Center; Cincinnati, OH). cDNA was made by oligo-dT priming with the Superscript Choice System Kit (Invitrogen; Carlsbad, CA). To maximize the number of clones that had an open reading frame (ORF) at or near the 5'-end, we selected for cloning the cDNA size fraction of ~1–4 kb, which contained the peak of the frequency distribution of cDNA sizes (~2 kb), instead of trying to maximize the number of longer full-length cDNAs in the library. cDNAs were cloned into the *NorI* and *SalI* sites of pBluescript to generate a directional library. We randomly selected 384 clones and had them sequenced (Agencourt Bioscience; Beverly, MA). To generate a set of 5,184 clones with minimal redundancy, we screened 96,000 colonies against a complex radiolabeled probe using standard methods (59). The probe was a mixture of two random-primed labeling reactions (Random Prime Labeling Kit, Roche Molecular Biochemicals; Indianapolis, IN); one using the 384 sequenced clones as a template and the other using the complex cDNA mixture originally used to make the cDNA library. This complex probe should label any sequences we had already obtained and many abundant cDNAs, i.e., those representing mRNAs more frequent than 1/1,000. We picked 5,184 negative colonies that also contained a cDNA insert (blue-white selection) and grew them on Luria broth (LB)-ampicillin plates. Clones were then prepared for sequencing by growth in LB liquid cultures with ampicillin in 96-well plates, followed by plasmid purification according to a protocol supplied by Agencourt Bioscience. A one-pass sequence from the 5'-end of each clone was obtained.

Sequence analysis. Raw sequence data were processed, including the trimming of vector sequence and low-quality regions, using the phred/phrap/cross_match program suite (Ref. 24; <http://www.phrap.org>). Contigs were assembled with stackPACK version 2.2 (53). BLASTN and BLASTX searches of nonredundant databases and BLASTN searches of the dbEST database were done in June 2003 and December 2005 to identify similarity to sequences previously deposited at the National Center for Biotechnology Information. Matches with expect values <0.001 were considered significant. Sequence matches with identities of >80% were inspected to check for sequences that might arise from contaminating organisms such as algae, fungi, bacteria, and sessile marine invertebrates that can colonize the surface of the exoskeleton. No such sequences were found. For all ESTs that lacked a significant match to known sequences, predictions of ORFs were made by inspecting start and stop codon position plots generated in DNASTrider version 1.2 (23). An internal ORF of >250 bp and terminal ORFs of >150 bp were counted as potential ORFs. Predictions of transmembrane domains were made using the Kyte-Doolittle algorithm of DNASTrider and confirmed via the SMART 4.0 database of protein domains (45). Functional categorization for each EST was based on the Gene Ontology terms describing the best BLAST match (34).

Construction and use of a cDNA microarray. We designed a cDNA microarray that consisted of five subarrays to cover all of the cDNA species in our library. We generated two of the subarrays, each with 1,152 clones spotted in duplicate, giving 2,304 spots/subarray. A common set of 96 clones, consisting of positive and negative control sequences, was spotted on each subarray. Each cloned cDNA insert was amplified by PCR and spotted on nylon membranes using an Affymetrix 417 Arrayer according to protocols supplied by Dr. Kevin Becker (6, 73). Arrays were hybridized against ³³P-labeled cDNA produced by oligo-dT priming of reverse transcription. Hybridization was done in quadruplicate at 50°C in 50-ml tubes in a hybridization oven as described in Barrett et al. (6). Washing was done twice in 2× SSC and 0.1% SDS for 10 min and twice in 1× SSC and 0.1% SDS for 15 min at room temperature. Signals from hybridized arrays were quantified using a STORM phosphorimaging device running ImageQuant software (Molecular Dynamics; Sunnyvale, CA). ImageQuant was used to segment each spot and calculate the signal intensity by the volume method for each. The arrays were found to have uniform background intensity, so no background subtraction was necessary.

Signal intensities were normalized by the Z-score method, which measures the distance of each signal from the mean signal on the array relative to the variance of this mean (17). The Z-ratio method, which calculates an average across replicates and compares ratios between treatment groups (tissues in this case), was used to calculate the amount of difference for each cDNA (17). A Z-ratio outside ± 1.96 has been empirically determined to consistently identify real differences. To help eliminate samples with high variation, we also performed a two sample for means Z-test statistic (17, 55) to identify spurious Z-ratios due to mRNAs with high variation, but we found that all cDNAs with Z-ratios beyond ± 1.96 were significant according to the Z-test statistic.

Real-time quantitative RT-PCR. RNA samples were obtained from different lobsters than those used for the microarray experiment. We generated a single pooled sample from each tissue because some of the tissues were insufficiently large to allow reliable RNA isolation from one animal. Reverse transcription was performed on 1 μ g of total RNA using SuperScript II and random hexamers (Invitrogen) in a 50- μ l reaction. Primers were designed using Primer Express software (Applied Biosystems; Foster City, CA) and purchased from Integrated DNA Technologies (Coralville, IA) (Table 1). Amplification was performed on triplicate reactions in an ABI Prism 7700 Sequence Detection System using 1 μ l of the cDNA reaction and the Sybr Green Core Reagent Kit under conditions recommended by the manufacturer (Applied Biosystems). Thermal cycler conditions were 50°C for 2 min and 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Whole olfactory organ cDNA was used as a template for standard curves, which were required to exceed a

Table 1. Oligonucleotide primers used for real-time quantitative RT-PCR

	Primers
OET-03 protease	
Forward	5'-CAAGGTTACGGGACCCTCG-3'
Reverse	5'-CACCGGGATTAGTTCGAA-3'
14F03	
Forward	5'-TCTCTGGTGCACAGAAATAGCTG-3'
Reverse	5'-GCTGGATGGCAACTTGCAA-3'
8G07	
Forward	5'-TTGCCAGTTTGGCTCAGTGT-3'
Reverse	5'-TGAGGAGGAACTGCCGATA-3'
Fax	
Forward	5'-AGTTCACCTCGCACCCAGTC-3'
Reverse	5'-GGCGAGCCTCAACCAAGTC-3'
9F01	
Forward	5'-CTTTGTGTTGTCTGTGTGTCAG-3'
Reverse	5'-CAGAAATAATTTGTGCATCAAATG-3'
16E08	
Forward	5'-CGTCTGCACTTACCAACTCTAA-3'
Reverse	5'-CCTAACTGTTGATTGGCTGGAAT-3'
44C08	
Forward	5'-GGGTAGGAAGGTCAGAGGATAATAGTAG-3'
Reverse	5'-GACTCTGTCAAGTTATGCCCTTCT-3'
21A10	
Forward	5'-TGGCACCTCAAGACGCAAG-3'
Reverse	5'-TCCACGAGCTCGCTCATACTC-3'
3G08	
Forward	5'-TGTCTTGCCCTGAGGTAGCC-3'
Reverse	5'-AAAATTAATACATTCCCTCTGGTACGA-3'
1C09	
Forward	5'-TGTTTTCTTAAACCTTTGCAATCA-3'
Reverse	5'-CCTTATCTGTTCAACCACTCTTAGA-3'
18F10	
Forward	5'-TGGAACATAAACCATAGAAGCAGC-3'
Reverse	5'-TCACCTTTGGATGTTGGCTTACT-3'
42G09	
Forward	5'-CCTGTGAGTGTCTCTTTCG-3'
Reverse	5'-GCTGCATGTTTCTGTTGACA-3'

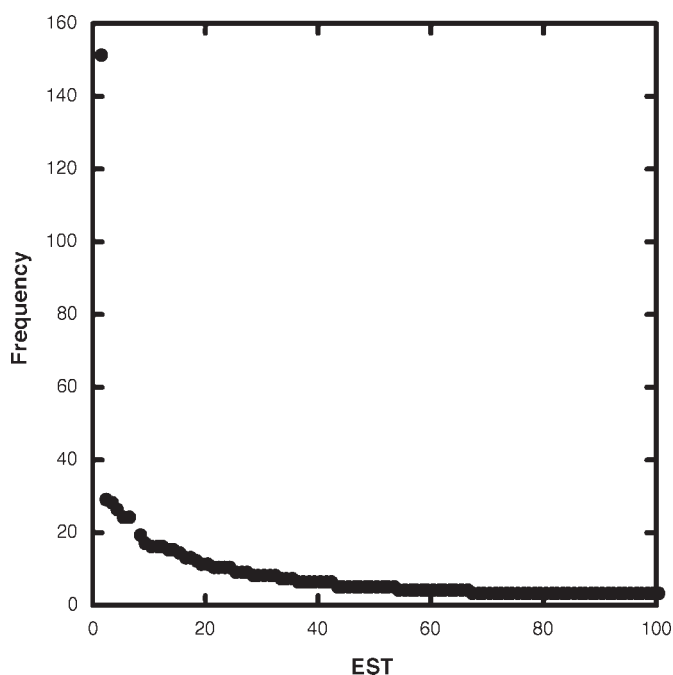


Fig. 2. Frequency distribution of the 100 most abundant of the 5,184 expressed sequence tag (EST) sequences. The ordinate shows the number of identical or overlapping sequences of each type.

criterion correlation coefficient of 0.98 before being accepted for analysis. Melt curves were performed on each sample to verify that each reaction produced a single product. Quantities were normalized to the amount of 18S rRNA in the sample.

RESULTS

Sequence frequency and annotation. To broadly investigate gene expression in the odor-responsive region of the lobster olfactory organ, we generated a subtracted cDNA library from the mature zone of the organ. To maximize the chances that a single sequence from each clone would encounter an ORF, we sequenced the 5'-end of each cDNA clone from the subtracted directional library. Overall, 50% of the sequences were high quality (more than half the bases with a phred score of at least 20), and 74% of the sequences (3,836 sequences) contained at least one high-quality stretch of >100 bp. From these 3,836 sequences, we found 2,389 different sequences consisting of 1,944 singlets and 445 contigs. Frequency distributions of mRNA abundance in cells or tissues typically approximate an exponentially declining function with a long tail of low-frequency mRNAs due to a relatively few mRNAs that are abundant and thousands of mRNAs that occur at 1–15 copies/cell (9, 18). Presumably due to the subtraction of common cDNAs, the abundant cDNA end of the frequency distribution was truncated in the subtracted library (Fig. 2). Only one sequence, a rRNA fragment detected in 3.1% of the clones, could be considered common. The next most common sequence was found in 0.6% of the clones, and only 23 contigs consisted of >10 clones. These results indicate that we successfully increased the representation of low-abundance mRNAs and reduced the frequency of high-abundance mRNAs.

BLAST searches identified significant similarity to known genes for 869 distinct lobster sequences. Of these matches, 412 were to arthropod sequences, primarily *Drosophila*, *Apis*, and

Anopheles species whose genomes have been sequenced, and included 93 sequences from these species that had similarity to no other known sequences. Of the remaining clones, 372 clones had matches to ESTs in dbEST. Two of these matches were to vertebrate ESTs and 370 were to crustacean ESTs, including 332 matches to ESTs from *H. americanus*. The remainder, >1,100 sequences, matched no entries in the databases. The majority of the ESTs and novel sequences lacked an ORF, leading to the conclusion that many derived from untranslated regions of mRNAs or perhaps from unspliced introns and genomic DNA contaminants. However, 208 of them had a potential ORF, and 35 of these ORF fragments were predicted to contain at least one transmembrane domain. The pool of sequences therefore appeared to contain portions of coding regions from ~40% to 45% of the cDNAs, ~1,000 of the 2,389 different sequences. The sequences are resident in dbEST at the National Center for Biotechnology Information (Accession Nos. DV771035–DV775073). They and the BLAST search results may also be retrieved from <http://app.mc.uky.edu/medicine/mcclintocklab/>.

Encoded proteins and the physiology of the olfactory organ. The types of sequences with significant similarity to the ESTs ranged from intermediary metabolism enzymes to transcription factors, receptors, and neuropeptides (Tables 2 and 3). Among these were specific markers previously identified as associated with three cell types located in the mature zone of the olfactory organ: olfactory sensory neurons, auxiliary (glial) cells that ensheath the bundles of dendrites extending from each cluster of olfactory sensory neurons, and secretory cells of the aesthetasc tegumental glands (39, 47, 68, 69). OET-07, an ionotropic glutamate receptor, and OET-10, a tubulin isoform, both of which are specifically expressed in olfactory sensory neurons, were detected. OET-02, a homolog of dopamine β -monoxygenase, and glutamine synthase, which are expressed only in auxiliary cells, were detected. OET-03, a serine protease that is specific to the secretory cells of the aesthetasc tegumental glands, was also detected.

Because olfactory sensory neurons are the dominant type of cell in the mature zone of the olfactory organ, we expected to detect additional mRNAs characteristic of neurons, and this proved to be the case. Gene products typically enriched in neurons that we detected included *N*-cadherin, long-chain fatty acid-CoA ligase, ciboulout, phospholipase C, adenylyl cyclase, calmegin, Down syndrome cell adhesion molecule (Dscam), neuroigin, dorfin, orckinin, synaptotagmin, failed axon connections (Fax), trophinin, glutamate synthase, ionotropic glutamate and GABA receptors, GABA receptor-associated protein, chloride channels, and Na⁺-K⁺-ATPases. Some of these sequences, like those of phospholipase C, adenylyl cyclase, ionotropic receptors, and ion channels, are already known to be expressed by olfactory sensory neurons or are implicated in their physiology (21, 39, 40, 79, 83, 84).

The other major type of cell in the olfactory organ is the epithelial cell. These not only lay down the exoskeleton but also appear to harbor the multipotent progenitor cells that give rise to new aesthetasc sensory units that replace aged, damaged, or lost tissue (35, 70, 71). Our expectation of finding mRNAs characteristic of epithelial cells was rewarded with the detection of chitinase, crustacyanin, DD5, and some antimicrobial proteins (42, 71, 85).

Table 2. Hierarchical categorization of enzymes and regulators of enzymes (molecular function categories only) detected in the mature zone of the lobster olfactory organ

304 Catalytic activity		
8 Helicase		
1 ATP-dependent COOH-terminal DNA helicase		3 ATP-dependent RNA helicase
108 Hydrolase		
1 α/β -Hydrolase superfamily		3 Chitinase
9 Cysteine-type endopeptidase		4 Nuclease
10 Chymotrypsin		4 Carboxypeptidase
3 Trypsin		7 Protein phosphatase
7 Other serine-type endopeptidases		1 Transglutaminase
3 Serine carboxypeptidase		2 Adenosylhomocysteinase
14 Other endopeptidases		18 ATPase
2 Hexosaminidase		4 GTPase
1 Phosphodiesterase I- β		
9 Isomerase		
3 Peptidylprolyl- <i>cis-trans</i> -isomerase		1 Phosphoglucomutase
1 Glucosamine-6-phosphate deaminase		1 Prostaglandin D synthase
1 Phosphoglucose isomerase		1 Protein disulfide isomerase
22 Ligase		
1 L-Glutamate:ammonia ligase		1 4-Coumarate:CoA
1 Long-chain fatty acid-CoA ligase		2 Ammonia
9 Ubiquitin-protein ligase		1 Tryptophan-tRNA ligase
9 Lyase		
3 Cystathionine γ -lyase		
3 Phosphoenolpyruvate carboxykinase (GTP)		
65 Oxidoreductase		
32 Dehydrogenase		
5 Dopamine		12 NADH
2 Peroxidase		2 Glutamate
3 Glyceraldehyde-3-phosphate		3 Acyl-CoA
86 Transferase		
1 Phospholipase C		
2 Phosphotransferase		
24 Kinase		
10 Protein kinase		2 Nucleotide
3 Amino acid kinase		1 Ketoheokinase
1 Nucleic acid kinase		1 Pyruvate kinase
1 Phosphomevalonate		
1 Phosphatidylcholine:ceramide cholinephosphotransferase		
17 Transcriptase		
16 RNA-directed DNA polymerase		
1 DNA polymerase		
5 Transposase		
25 Enzyme regulator		
1 Ornithine decarboxylase antizyme		2 GTPase regulator
3 Kinase regulator		1 Tryptophan hydroxylase activator
2 Kinase inhibitor		
14 Peptidase inhibitor		
8 Serine-type endopeptidase inhibitor		

Numbers in italics are numbers of expressed sequence tags (EST.) in each category. Categories are nonexclusive. Not all members of the more general categories are listed due to instances of ambiguity in the specific identification of some ESTs.

Microarray test of differential expression between olfactory and taste organs. As a preliminary test of whether the cDNAs we cloned represented mRNAs enriched in the olfactory organ, we generated a cDNA microarray and hybridized it against olfactory organ and dactyls of the walking legs. The walking leg dactyl is arguably the tissue most similar to the olfactory

Table 3. Hierarchical categorization of lobster olfactory organ mature zone sequences identifiable by molecular function or biological process category of the encoded protein

10 Antimicrobial/antibacterial	
6 Antioxidant	
1 Peroxiredoxin	2 Catalase
1 Superoxide dismutase	2 Peroxidase
221 Binding	
25 ATP	3 Peptide hormone
8 GTP	16 Nucleic acid
83 Protein	41 RNA
6 Carbohydrate	36 DNA
41 Metal ion	7 Lipid
15 Zinc	
19 Calcium	
4 Iron	
10 Hsp	
4 Hsp70	1 Hsp27
2 Hsp90	1 Hsp25
1 Hsp60	1 Hsp10
44 Motor activity/structural	
7 Motor activity	8 α -Tubulin
24 Structural	5 β -Tubulin
21 Receptor and other signaling proteins	
9 Receptors	
2 Glutamate receptors (ionotropic)	1 Ecdysteroid receptor
2 Ionotropic GABA receptor	1 Mannose receptor
1 Low-density lipoprotein receptor	1 Integrin
1 Macrophage colony stimulating factor receptor	
12 Other signaling proteins	
1 PWP2 periodic tryptophan protein	1 Reticulon (Rtn11)
2 Neuropeptide (orcokinin)	1 Ephrin B2b
1 G protein- γ 1	1 Adenylyl cyclase
1 Phospholipase C	1 Cdc42
1 Dopamine β -monooxygenase	1 N-cadherin
47 Ribosomal	
43 Ribosomal proteins	3 Ribosomal RNA genes
76 Transporters and channels	
6 Electron transport	3 Chloride channel
9 Hydrogen transport	5 Gap junction
10 Lipid transport	11 Protein carriers
2 Sodium:potassium exchangers	1 Copper ion transport
47 Translation/transcription regulator	
19 Transcription regulation	15 Translation regulation
8 Transcription factor	
149 Miscellaneous	

Numbers in italics are numbers of ESTs in each category. Categories are nonexclusive. Not all members of the more general categories are listed due to instances of ambiguity in the specific identification of some ESTs. Hsp, heat shock protein.

organ. It is a major taste organ in the lobster, and although certainly not the only site for contact chemoreception, it has a high concentration of chemosensitive setae (4). It detects many of the same types of chemicals as the olfactory organ, and, like the olfactory organ, it lacks muscle tissue. Both tissues contain mechanosensory neurons and an epithelium that secretes cuticle. In addition, we (39) have previously detected mRNAs specifically shared only by the walking leg dactyl and olfactory organ. As the tissue most similar to the olfactory organ, it should present the greatest challenge for discriminating differences in gene expression, short of measuring changes within a single tissue.

Examples of the microarray hybridization results are depicted in Fig. 3, A and B. The bias of the library toward its

source, the mature zone of the olfactory organ, was evident in the abundance of spots with stronger hybridization to the olfactory organ probe (green) relative to spots more strongly hybridizing with the walking leg dactyl probe (red). Quantitative analyses confirmed the impression given by the microarray images. Z-ratio calculations identified 92 cDNAs enriched in the mature zone but only 23 cDNAs enriched in the dactyl (Fig.

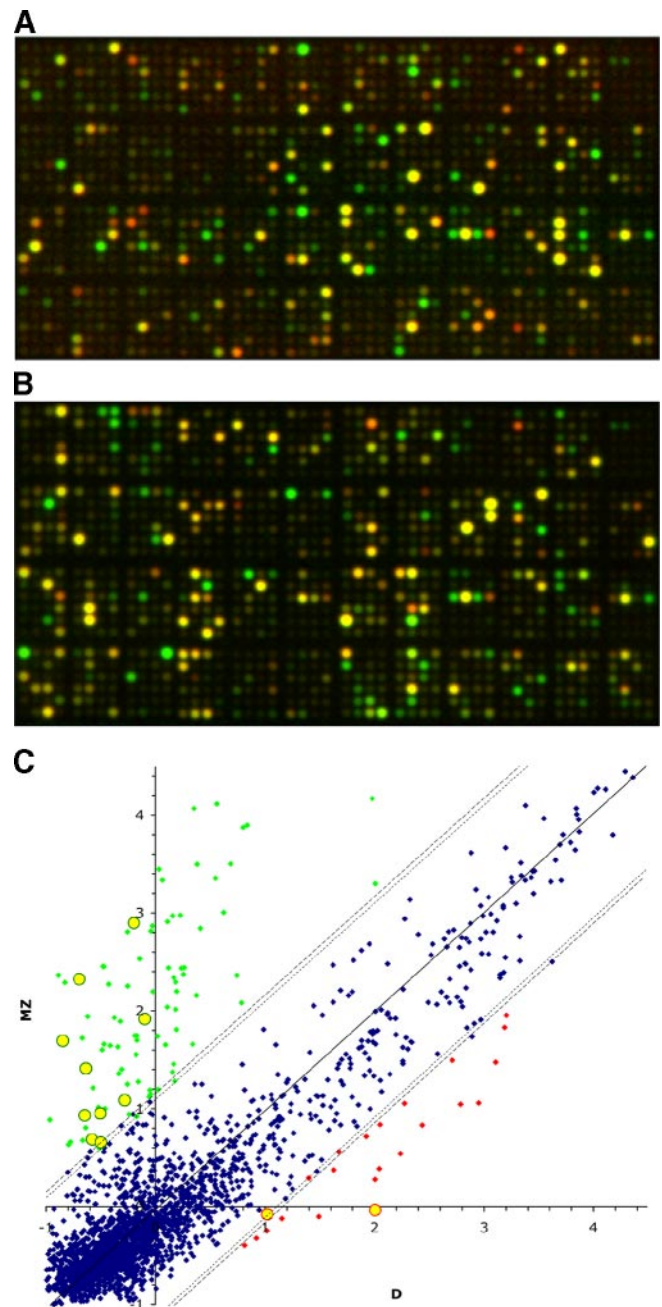


Fig. 3. Detection of mRNAs differentially expressed between the MZ of the olfactory organ and the leg dactyl (D). A and B: microarray hybridization results. MZ (green) and D (red) signals superimposed for the two microarray subsets tested. Only the left half of each array is shown. The right halves duplicate the left. C: Z-ratio distributions for the microarray data. Solid line, equality between the two samples. Dotted lines, Z-ratios of ± 1.96 . Each cDNA is represented by a spot. Green and red, mRNAs predicted to be more abundant in MZ and D, respectively. Yellow symbols, mRNAs selected for independent testing by quantitative RT-PCR.

Table 4. Summary of mRNAs predicted by microarray analysis to be differentially expressed between leg dactyl and the mature zone of the olfactory organ

Z Ratio	Similarity
Enriched in olfactory organ	
6.0	NADH dehydrogenase subunit 2
5.2	OET-3, chymotrypsin-like serine protease
5.2	Fourteen-Three-Three family, Ftt-2 (14-3-3ζ)
5.2	Similar to DnaJ (Hsp40) homolog, subfamily A, member 2
4.7	Chain G, D-glyceraldehyde-3-phosphate dehydrogenase
4.7	β-Actin
3.9	Ferritin 1
3.3	Trophinin (Maged3)
3.1	40S ribosomal protein S2
3.0	Phosphoinositide-specific phospholipase C
2.9	Ionotropic glutamate receptor
2.8	Cytochrome <i>b</i>
2.6	Suppressor of profilin 2
2.0	α ₁ -Tubulin
6.7	Prophenoloxidase activating factor III
4.3	UBX domain containing 2
4.0	Anti-lipoplysaccharide factor
3.9	Hypothetical ORF, Ye1077cp
3.9	NADH dehydrogenase (ubiquinone) 1β-subcomplex
3.8	β ₂ -Tubulin
3.6	Fax
3.5	Hypothetical protein XP_211670
3.4	Phosphoglucose isomerase-2
3.4	Ubiquitin-conjugating enzyme E2H
2.9	Cytochrome <i>c</i> oxidase subunit 1
2.7	Cytoplasmic gelsolin
2.6	Required for invasion and pseudohyphae formation
2.6	PPIC-type PPIASE domain-containing protein (13.3 kDa)
2.4	Tigger transposable element-derived 3
2.3	RIKEN cDNA 2810439F02
2.2	Ubiquitin-specific protease 7
#	61 Novel sequences
Enriched in leg dactyl	
-2.1	DEAD (Asp-Glu-Ala-Asp) box polypeptide 52
-2.1	Eukaryotic initiation factor 3 p66 subunit
-2.3	Ribosomal protein L19
-2.7	Glucosamine-6-phosphate isomerase
-2.7	Serine protease inhibitor
-2.8	Mucin 2 precursor
-2.0	Capillary morphogenesis protein-1
#	16 novel sequences

Fax, failed axon connections.

3C). The identities of the mRNAs are shown in Table 4, and microarray data are available at Gene Expression Omnibus (Accession No. BSE3785). To test the ability of the array to identify real differences, we used quantitative RT-PCR (qRT-PCR) to measure the relative abundance for 12 mRNAs predicted to differ by the microarray results. We purposefully selected mRNAs that had smaller signals on the microarrays because they should represent low-abundance mRNAs that are most likely to harbor false positives. The results confirmed differences for 10 of the 12 mRNAs (Table 5), including the OET-03 protease previously shown to be specific to the olfactory organ because it is expressed only in aesthetasc tegumental glands (68). These results argue that the vast majority of the 115 mRNAs judged different by the microarray were indeed differentially expressed. A few of the differences predicted among low-abundance mRNAs will prove to be false positives, however. For example, the mRNA represented by *clone 18F10* did not differ as predicted, and the mRNA for *clone 42G09* was not enriched in the dactyl but was enriched in the mature zone instead.

Although preliminary, our qRT-PCR data contained some interesting patterns across the 10 tissues tested. None of the mRNAs were enriched in transition zone relative to the mature zone of the olfactory organ, and only two of the mRNAs were as abundant in the proximal proliferation zone as in the mature zone. These results are consistent with the developmental differences between these easily separated portions of the olfactory organ. None of the mRNA abundance patterns showed consistent enrichment among the tissues with a high density of excitable cells: the mature zone of the olfactory organ, brain, tail muscle, and eye plus eyestalk [which includes both the retina and portions of the forebrain (i.e., the protocebrum)]. However, three of the mRNAs (14F03, 8G07, and Fax) were at least twofold higher in the olfactory organ mature zone than in any other tissue. While not as highly specific as that of OET-03 protease, this pattern suggests that these three mRNAs may be expressed primarily by one of the unique cell types in the mature zone of the olfactory organ: olfactory sensory neurons, their auxiliary cells, or secretory cells of the aesthetasc tegumental gland. Surprisingly, six of the mRNAs (42G09, 18F10, 1C09, 3G08, 21A10, and 44C08) were more abundant in the second antenna and antennular medial flagellum than in the mature zone of the olfactory organ. Their

Table 5. Ratios of mRNA abundance relative to the mature zone of the olfactory organ as measured by quantitative RT-PCR

cDNA Clone	Dactyl	Brain	2A	MF	Per	Eye	PPZ	TZ	Muscle
OET-03 (protease)	0.11	0	0.04	0.07	0	0	0.06	0.12	0
14F03	0.04	0.14	0.50	0.45	0.05	0.05	0.16	0.41	0.07
8G07	0.23	0.07	0.39	0.45	0.08	0.04	0.14	0.05	0.06
Fax	0.14	0.27	0.27	0.28	0.03	0.01	0.20	0.07	0.02
9F01	0.13	0.31	0.31	0.62	0.04	0.03	0.12	0.12	0.03
16E08	0.20	0.91	0.50	1.1	0.24	0.12	0.34	0.12	0.38
44C08	0.24	0.05	10	10	0.07	0.01	1.25	0.22	0.01
21A10	0.38	0.91	5.0	5.0	0.14	0.18	0.50	0.16	0.40
3G08	0.50	0.63	1.7	2.5	0.36	0.12	0.63	0.21	0.17
1C09	1.4	1.0	3.3	3.3	0.24	0.14	0.71	0.22	0.52
18F10*	2.5	10	5.0	3.1	0.28	0.09	2.0	0.59	0.23
42G09*	0.43	0.14	1.2	2.0	0.13	0.06	0.67	0.50	0.14

Dactyl, leg dactyl; 2A, second antenna; MF, medial flagellum of the first antenna; Per, pereopod (walking leg without dactyl); Eye, eye and eyestalk; PPZ, proximal proliferation zone of the olfactory organ; TZ, transition zone of olfactory organ; Muscle, tail muscle. *mRNAs predicted to be lower in the mature zone than leg dactyl; all others were predicted to be higher in the mature zone.

abundance in the leg dactyl also tended to be higher than the other six mRNAs. The common cell types among the second antenna, antennular medial flagellum, and leg dactyl include sensory neurons, glial cells, and epidermis. We hypothesize that these six mRNAs are expressed mostly in one or more of these three cell types. The one mRNA in this group that appeared to best correlate with a specific type of cell was 44C08, whose enrichment in the proximal proliferation zone and scarcity in the brain suggests that its source may be epidermal cells.

Of the cDNAs enriched in either the leg dactyl or mature zone of the olfactory organ, 38 were identified by BLAST as having significant similarity to known mRNAs. This afforded us an opportunity to assess whether functional or cellular relationships might exist between the proteins ostensibly enriched in the mature zone of the olfactory organ. The unique concentration of aesthetasc sensory units in the mature zone and the fact that each sensory unit contains many more neurons than other types of crustacean setae (15, 16) predicts that neuronal mRNAs should be enriched in the mature zone compared with leg dactyl. Indeed, the microarray data predicted that several gene products typically associated with neural tissue, including 14-3-3 ζ , trophinin, Fax, phospholipase C, and an ionotropic glutamate receptor subunit, were enriched in the mature zone. A difference in the relative abundance of neurons between the olfactory organ and leg dactyl would also be consistent with enrichment of mitochondria because neurons typically consume more energy than most cells other than muscle. We detected four mitochondrial mRNAs enriched in the mature zone: two NADH dehydrogenases, cytochrome *b*, and cytochrome *c* oxidase. Cytoskeletal-associated proteins and cell adhesion molecules constituted the largest functional grouping of mRNAs enriched in the mature zone sample. These included 14-3-3 ζ , actins, tubulins, trophinin, Fax, ciboulot, Yel077cp, suppressor of profilin 2, and gelsolin. Finally, the uniqueness of the aesthetasc tegumental gland was further supported by the enrichment of the specific marker of its secretory cells, the serine protease OET-03, in the mature zone sample.

DISCUSSION

Our effort to produce a cDNA library enriched in transcripts involved in olfactory physiology and biochemistry was successful. The subtracted cDNA library we produced was strongly biased toward mRNAs of moderate or low abundance, as evidenced by the frequency distribution of the sequences. The method of backscreening plated colonies of a conventional cDNA library therefore proved to be as efficient as solution hybridization subtraction methods for making subtracted cDNA libraries (e.g., Ref. 78). The only sequence that could be considered poorly subtracted by our approach was one with unusual properties, a 16S rRNA fragment. Being an rRNA, it would have been highly abundant in the starting RNA fraction, it is AT rich (67%), and it has low sequence complexity due to repeated clusters of adenines and/or thymidines. We suspect that these factors contributed to its surviving selection for polyadenylated mRNAs and to its escaping detection during subtraction by backscreening. Only four clones from the other rRNAs were detected among our EST sequences. While subtraction distorted the library's ability to provide information on

the relative abundance of mRNAs by the method of counting sequences, it allowed the library to provide more information about which mRNA species were present. The breadth of sequences detected provides a more balanced view of gene expression in the olfactory organ than has been gathered by other approaches. For example, serine proteases and their inhibitors, which constitute a functionally related group that stood out in previous studies (39, 71) of gene expression in the olfactory organ, were rivaled or surpassed by dehydrogenases, kinases, transporters and channels, and regulators of transcription and translation.

Several sequences corresponded with functional properties of the olfactory organ and are therefore candidates to execute those functions. In many cases, these functions are associated with specific cell types. The ecdysteroid receptor presumably mediates the preparations for molting induced by ecdysone, most critically in the epidermis of the olfactory organ. Phosphodiesterase-1 β is an ectoenzyme in mammals, so the lobster homolog we detected might provide the ectonucleotidase activity that helps to clear nucleotide odorants from the lymph of the aesthetasc setae (32, 74, 75). A sequence similar to *Drosophila* glutamate synthase, but only weakly similar to bacterial glutamate synthetases, corresponds with the hypothesis that lobster olfactory sensory neurons are glutamatergic. The ionotropic GABA and glutamate receptors correspond to evidence of modulation of olfactory receptor neuron sensitivity, especially at their axon terminals (39, 40, 76, 83, 84). The G γ ₁ heterotrimeric G protein subunit corresponds to the missing partner of G α - and G β -subunits previously identified as mediators of olfactory transduction (80, 82). Chloride channels are important to the physiology of lobster olfactory sensory neurons, regulating the membrane potential and accounting for at least part of the hyperpolarizing receptor potential (22, 51). The chloride channel ESTs we detected were most similar to bestrophins or Clic5. Bestrophins are calcium-activated chloride channels highly sensitive to cell volume changes (27), raising the hypothesis that osmosensitive cells exist in the olfactory organ. In fact, osmosensitive cells have been described in spiny lobster sensory organs (29). Clic5 channels are more enigmatic, acting as chloride channels in vitro but in vivo localizing intracellularly with the cytoskeleton, Golgi apparatus, or microvilli in cell type-dependent patterns (8). We also detected a homolog of Rga, a mammalian protein that appears to act as a chaperone for Trp channels (5). As-yet-unidentified Trp channels are thought to be critical for lobster olfactory transduction (10).

Some of the ESTs suggest new avenues of investigation. Orcokinin were originally identified as myotropic hormones but are broadly expressed in the crustacean central nervous system, where they have neuromodulatory actions (64). The identification of an orcokinin precursor mRNA in the olfactory organ suggests that the olfactory sensory neurons might express this hormone and may be sensitive to it. Lobster olfactory sensory neurons have relatively long axons to generate and maintain, something that may have been reflected in the detection of several mRNAs encoding axonal proteins, cytoskeletal-associated proteins and cell adhesion molecules such as Fax, ciboulot, Cdc42, dyneins, kinesins, dynactin 3, fasciclins, basigin, Dscam, and neuroligin. Fax is critical for the formation of axon connections in *Drosophila*, and disruption of it or of a basigin homolog alters olfactory-guided behavior (3, 38).

Dscam is critical for the proper targeting of *Drosophila* olfactory axons to glomeruli (41). That it would perform a similar function in lobsters seems a reasonable hypothesis.

Several additional ESTs were also homologous to gene products implicated in the olfactory systems in other species. Quick-to-court is expressed in *Drosophila* olfactory organs, and a quick-to-court mutant has an elevated frequency of sexual behavior (28). Several lines of evidence link olfactory cilia formation and function with that of other ciliated or flagellated cells, but especially with spermatogenesis (43, 62). Calmegin is critical for the function of sperm in mice, so the presence of a calmegin ortholog among the ESTs suggests that although outer dendrites do not have the microtubule arrangements of cilia, they might depend on similar mechanisms for their formation (56). Mammalian olfactory sensory neurons are also enriched in several gene silencing and chromatin remodeling mRNAs (62). Among the lobster ESTs were YY1, bicoid interacting protein 1a, and a homolog of SWI/SNF complex protein 170. These three genes encode proteins that often help to repress gene expression by chromatin remodeling via roles in polycomb complexes, SWI/SNF complexes, or histone deacetylation (63, 67). Cells involved in immune and defense responses are also present in the mammalian olfactory periphery and can be detected there by microarray analysis (31, 62, 72). The hemocytes of crustaceans are the major source of innate immune responses (44, 65) and participate in the damage-induced replacement of olfactory sensory neurons of lobsters (61). Hemocyte markers among the ESTs included antipolysaccharide factor, crustin, prophenoloxidase, and peroxynectin (7, 48, 66, 77). The detection of hemocyte markers among the ESTs is consistent with our method of freezing of the olfactory organs immediately after their excision, thereby limiting the drainage of hemolymph.

In summary, we generated a subtracted cDNA library that is representative of the cellular diversity of the mature zone of the olfactory organ. It includes cDNAs for all the known markers for cell types in the tissue. Sequence analysis shows enrichment of neuronal mRNAs in the library, consistent with the preponderance of neurons in the mature zone. Several of the newly identified sequences are candidate sources of physiological functions previously measured. Others suggest new functions not previously recognized in the olfactory organ. We have grown and stored 5,586 cDNA clones in individual wells to facilitate their recovery for future uses. These uses include a cDNA microarray, which was employed successfully to identify mRNAs differentially expressed between the mature zone of the olfactory organ and leg dactyl.

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