

Annotated Expressed Sequence Tags and cDNA Microarrays for Studies of Brain and Behavior in the Honey Bee

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To accelerate the molecular analysis of behavior in the honey bee (*Apis mellifera*), we created expressed sequence tag (EST) and cDNA microarray resources for the bee brain. Over 20,000 cDNA clones were partially sequenced from a normalized (and subsequently subtracted) library generated from adult *A. mellifera* brains. These sequences were processed to identify 15,311 high-quality ESTs representing 8912 putative transcripts. Putative transcripts were functionally annotated (using the Gene Ontology classification system) based on matching gene sequences in *Drosophila melanogaster*. The brain ESTs represent a broad range of molecular functions and biological processes, with neurobiological classifications particularly well represented. Roughly half of *Drosophila* genes currently implicated in synaptic transmission and/or behavior are represented in the *Apis* EST set. Of *Apis* sequences with open reading frames of at least 450 bp, 24% are highly diverged with no matches to known protein sequences. Additionally, over 100 *Apis* transcript sequences conserved with other organisms appear to have been lost from the *Drosophila* genome. DNA microarrays were fabricated with over 7000 EST cDNA clones putatively representing different transcripts. Using probe derived from single bee brain mRNA, microarrays detected gene expression for 90% of *Apis* cDNAs two standard deviations greater than exogenous control cDNAs.

[The sequence data described in this paper have been submitted to Genbank data library under accession nos. B1502708–B1517278. The sequences are also available at http://titan.biotech.uiuc.edu/bee/honeybee_project.htm.]

The honey bee (*Apis mellifera*) is an important model for studies of neural and behavioral plasticity, particularly with respect to social behavior, learning, and memory (Fahrbach and Robinson 1995; Robinson 1998; Menzel 2001; Maleszka et al. 2000). The neuroanatomy, neurophysiology, and neurochemistry of the honey bee brain have been studied extensively, and several functions have been mapped to particular brain regions (e.g., Menzel 2001; Fahrbach and Robinson 1995). Honey bees also have been used extensively to study the genetic underpinnings of behavior (Rothenbuhler 1967; Page and Robinson 1991). In the past few years, these lines of inquiry have been extended to the discovery of quantitative trait loci (Hunt et al. 1995, 1998) and analyses of expression levels of genes in the brain (Kucharski et al. 1998, 2000; Fiala et al. 1999; Toma et al. 2000; Shapira et al. 2001; Kucharski and Maleszka 2002).

One strong advantage of working with honey bees is that it is possible to study behavior under both laboratory and natural conditions. The natural social life of honey bees, though arguably as complex as in many vertebrate societies, can be extensively manipulated with precision. Insights gained from both lab and field studies ultimately will enable

information on genes influencing neural and behavioral plasticity to be interpreted from ecological and evolutionary perspectives, contributing to a more comprehensive understanding of genes, brain, and behavior (Robinson 1999).

Molecular analyses in the honey bee have been constrained by the high investment required to identify and clone individual genes and the need to have an *a priori* hypothesis about each gene. The public databases contained only about 101 complete or near-complete *A. mellifera* gene sequences (nonredundant entries in SWISS-PROT and TrEMBL, as of December 2001) and, prior to this study, a total of 800 nucleotide sequences, most of them expressed sequence tags (ESTs) from antennae (H.M.R., unpubl.) or larvae (Evans and Wheeler 2001). The value of studying many genes simultaneously in the honey bee was demonstrated by Evans and Wheeler (2001) who identified gene expression profiles that were characteristic for worker/queen caste differentiation. This study involved the initial identification of 158 candidate clones using subtractive methods, and was thus limited by the small number of genes analyzed. Current DNA microarray technologies allow expression studies of many thousands of genes at the same time (Schena et al. 1995; DeRisi et al. 1997). ESTs provide an economical approach to identifying large numbers of genes that can be used in gene expression and other genomic studies (reviewed by Gerhold and Caskey 1996; see also Dimopoulos et al. 2000 and Porcel et al. 2000).

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Here, we describe a collection of more than 20,000 ESTs generated from the *A. mellifera* brain, putatively representing 8912 different transcripts after sequence assembly. To facilitate gene identification and functional genomic studies in the honey bee, the brain EST set has been annotated using the structured vocabulary provided by the Gene Ontology Consortium (2001), based on molecular studies of gene function in *Drosophila melanogaster*. We describe a DNA microarray resource composed of over 7000 EST cDNA clones putatively representing different transcripts. We demonstrate the utility of this resource by reporting on gene expression measured in single honey bee brains. Additionally, comparative genomics approaches were used to predict or improve predictions for 122 genes in *Drosophila*, as well as to identify 126 genes conserved between *Apis* and other organisms that apparently have been lost from the *Drosophila* genome.

RESULTS AND DISCUSSION

Generation and Assembly of Brain ESTs

A normalized, unidirectional cDNA library was generated from dissected honey bee brains. An initial 7968 clones were sequenced from the 5' end. The library was then subtracted, and 12,288 more clones were sequenced (also from the 5' end). An additional 1152 sequences (3' and duplicate 5' ends) were obtained from previously sequenced clones. Thus, the EST set represents 20,256 cDNA clones and 21,408 total sequences. The 21,408 sequences were trimmed of vector and low-quality sequence and filtered for minimum length (200 bp), identifying 15,311 high-quality ESTs of 494 bp average length (Table 1). The estimated number of ESTs per putative transcript was initially 1.2 when sequencing was initiated and rose to 1.7 at the time sequencing was terminated (based on *phrap* analyses of high-quality ESTs after each batch of sequences; see below).

The 15,311 high-quality ESTs were analyzed with the *CAP3* assembly program to identify those that represent redundant transcripts (Table 2; see Table 8 for all program references). A total of 9481 ESTs were assembled into 3136 contiguous sequences (contigs). The remaining 5830 ESTs did not assemble into contigs (referred to as singletons). Thus, the combined set of contigs and singletons included 8966 sequences (hereafter referred to as "assembled sequences"), putatively representing different transcripts. Only 40 contig sequences contained more than 10 ESTs, and the largest number of ESTs assembled into one contig was 44.

We separately processed the high-quality ESTs using *PHRAP* and *CAP3* using different levels of stringency (Table 2). These different assemblies produced very similar results, and we retained the *CAP3* results for further analyses. Fifty-four assembled sequences were removed from the database (se-

Table 1. Honey Bee Brain EST Summary

Total sequences	21408
cDNA clones sequenced (5' end)	20256
Normalized library	7968
Normalized/subtracted library	12288
Redundant 5' end sequences	960
3' end sequences	192
Total high-quality sequences	15311

EST, expressed sequence tag.

Table 2. EST Assembly Results

	<i>PHRAP</i> ^a	<i>CAP3</i> ^b	<i>CAP3</i> ^c
Total sequences analyzed	14642	14642	15311
Number of ESTs in contigs	8464	8357	9481
Number of contigs	3119	2910	3138
Number of singletons	6178	6285	5830
Number of putative transcripts (assembled sequences)	9297	9196	8966
Number of contigs containing:			
2–4 ESTs	N/A	2626	2762
5–10 ESTs	N/A	255	334
11–20 ESTs	N/A	28	33
21–40 ESTs	N/A	2	6
>40 ESTs	N/A	1	1

^aDefault settings

^bHigh-quality ESTs assembled using high-quality, vector-trimmed sequence only. Default settings were used except minimum overlap was 40 bp and 95% identity (default is 30 bp, 75% identity).

^cHigh-quality ESTs assembled using high- and low-quality, vector-trimmed sequence, 3' and redundant 5' ESTs were included (these were treated as independent clone sequences to avoid error resulting from manual clone picking). Default settings were used except minimum overlap was 40 bp. These assembly results were used for all analyses in this study, except where noted. EST, expressed sequence tag.

quencing artifacts and/or exogenous contaminants; see Methods), leaving 8912 assembled sequences used in subsequent analyses.

EST Quality Analysis and Sequence Survey

Of the 8912 assembled sequences, 3501 (39%) were similar to known protein sequences in the Non-Redundant Protein (nr) database (*BLASTX*; $E \leq 10^{-5}$). To estimate the proportion of transcript sequences that represent truly novel genes, the assembled sequences were screened to identify only those with clear protein coding capacity. A total of 3449 assembled sequences have an open reading frame (ORF) of at least 450 bp. Of these, 2616 (76%) had matches in the nr database and 833 (24%) had no matches (Fig. 1A). This result indicates that perhaps 24% of the protein-encoding genes expressed in the honey bee brain are highly diverged in primary structure. A total of 5463 assembled sequences did not have an ORF of at least 450 bp; of these, 885 (16%) had matches in the nr database and 4578 (84%) had no matches. Many assembled sequences did not have an ORF of 450 bp because they were too short (916 assembled sequences were <450 bp long). Other assembled sequences may have lacked an ORF for a variety of reasons, including frame shift errors, 5' truncation of cDNA clones (causing ESTs to consist mostly or entirely of 3' untranslated region [UTR]) or ESTs that were not derived from mRNA. Microarray hybridization results indicated that the vast majority of ESTs were derived from legitimate transcripts (see below). To assess 5' truncation of cDNA clones, we examined sequence alignments of 130 ESTs (5') that had matches to *A. mellifera* full-length cDNA sequences in GenBank (matches defined as $\geq 98\%$ identity over at least 200 bp). Nine of these clones were in a backwards orientation (see below). Of the 121 ESTs in a forward orientation, 56 (46%) had 5' sequences that corresponded to the 5' end of the full-length cDNA sequence. The remaining 65 ESTs (54%) were derived from 5' truncated cDNA inserts. This result suggests that a large fraction of noncoding ESTs may have been de-

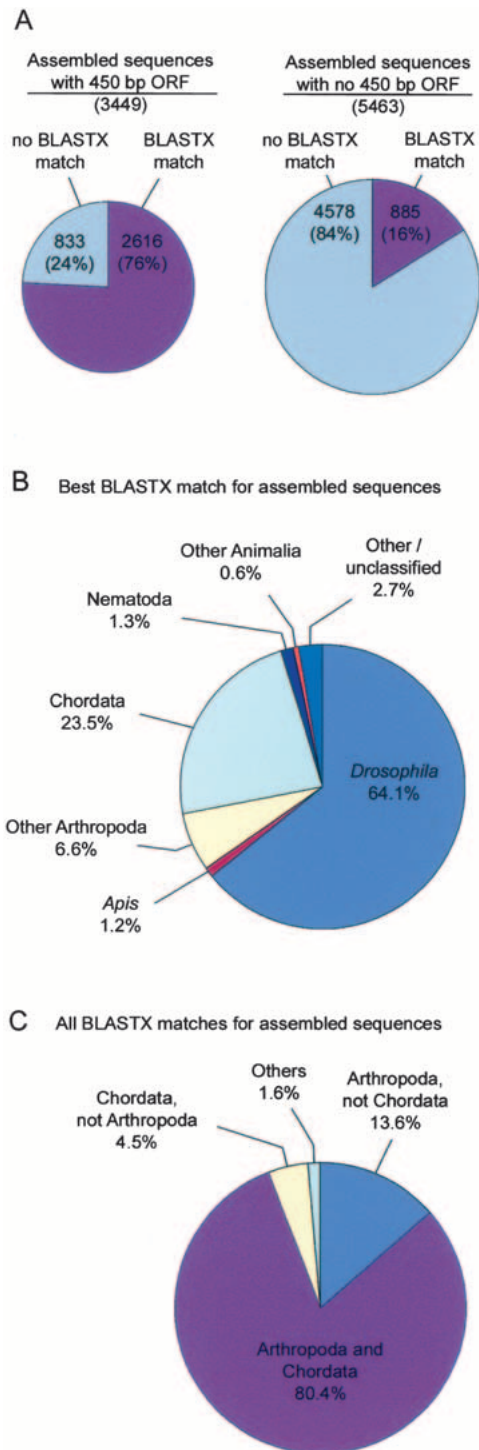


Figure 1 Open reading frame (ORF) and BLASTX results. (A) The proportion of assembled sequences with and without BLASTX matches in the Non-Redundant Protein (nr) database ($E \leq 10^{-5}$) is indicated for assembled sequences with and without an identified 450 bp ORF. Relative area of pie charts indicates number of sequences. (B) *Apis* sequences with matches in the nr database (3501 total) were classified by the organism of the "best hit" protein sequence. (C) *Apis* sequences with matches in the nr database (3501 total) were separately analyzed for matches in Arthropoda and Chordata protein databases (see Table 8 for sub-database creation).

rived from severely truncated cDNAs consisting mostly or entirely of 3' UTR.

ESTs were analyzed to identify a variety of other possible artifacts (see Methods). We estimated that 10% of the clones in the library are at least partially unspliced (often resulting from priming of the oligo(dT) primer within an unspliced AT-rich intron). Approximately 18% of the cDNA clones appear to be inserted in a reverse orientation. Finally, a single chimeric clone was identified that contained linker sequence within an EST flanked by back-to-back poly(A)⁺ sequences. No chimeras were identified by comparing BLASTX matches for 3' and 5' ESTs corresponding to the same cDNA clones (68 clones with 3' and 5' BLASTX matches were tested).

Figure 1B summarizes the top hits (matches with lowest *E* value) for each of the 3501 assembled sequences that had matches in the nr database. As expected, the majority (2245; 64%) were most similar to predicted protein sequence from *Drosophila*. Only 41 (1.2%) were most similar to predicted protein sequence from *Apis* (because of the small number of *Apis* gene sequences in the database). An additional 230 (6.6%) were most similar to sequence from a variety of other Arthropoda, including the insects *Bobyx mori* (28 best matches) and *Manduca sexta* (27 best matches). A surprisingly large number, 823 (24%), were most similar to sequence from Chordata (see Comparative Genomics, below). Others were most similar to proteins from Nematoda (47) or other Animalia (22). Twenty had best matches to various bacterial proteins with amino acid identities ranging from 42% to 92% (specifically, *Mycobacterium* [15], *Caulobacter* [4], and *Agrobacterium* [1]). We suggest that these 20 sequences were derived from unknown bacterial infections or contamination of bee brains or associated tissues. Two sequences appeared to be derived from an uncharacterized virus, having 24% and 39% amino-acid identity to different regions of the 2858 amino-acid poly-protein of the honey bee sacbrood virus.

Separate BLASTX searches of Arthropoda and Chordata protein databases revealed that the majority of assembled sequences with matches (80%) were similar to predicted protein sequences from both Arthropoda and Chordata (Fig. 1C). Others were similar to sequences from Arthropoda but not Chordata (13.6%), from Chordata but not Arthropoda (4.5%), or from non-Arthropoda and non-Chordata organisms only (1.6%). The implications of these findings for *Drosophila* were investigated further (see Comparative Genomics, below).

The assembled EST database was searched for simple sequence repeats using BLASTN and a database of simple sequence repeats of one to four bases (excluding (A)_n repeat). This search identified simple sequence repeats in 767 of the assembled sequences using a highest scoring pair (HSP) cutoff value of 50, and 76 sequences using an HSP cutoff value of 100. These HSP cutoff values roughly correspond to 25 and 50 bp of perfect match, respectively (note that identified repeats are not necessarily contiguous because default BLAST parameters allow gaps in alignment). Repeat sequences are likely to reside primarily in EST noncoding sequence (which constitute a large fraction of the ESTs, see above).

Gene Number

EST assembly is expected to generate an overestimation of the actual number of genes represented, as failure of ESTs to assemble can result from nonoverlapping ESTs, alternate splicing, sequence polymorphism, and sequencing errors. Assuming approximately one-to-one correspondence between genes

in *Apis* and *Drosophila*, the level of redundancy can be estimated based on BLASTX searches of *Drosophila* predicted proteins. A total of 3362 *Apis* assembled sequences had “best hits” to 2672 different *Drosophila* sequences, suggesting 19.6% redundancy in the *Apis* assembled sequence set. Similar levels of redundancy after EST assembly have been estimated in other large EST collections (e.g., roughly 20% in a large mouse cDNA set; see Kawai et al. 2001). Taking 20% as an estimate of redundancy in the 8912 assembled *Apis* sequences, the EST set may represent a total of 7100 genes expressed in the honey bee brain. If *Apis* has about the same number of genes as does *Drosophila*, this would represent roughly 50% of the total number of genes in the *Apis* genome.

A similar estimate of representation was provided by comparison of the 8912 assembled sequences with a set of 101 full- or near-full-length cDNA sequences obtained from an

independent honey bee brain library (sequences kindly provided by R. Maleszka). A total of 55 assembled sequences from the EST set matched 54 different cDNA sequences from the independent brain library (match defined as $\geq 98\%$ nucleotide identity over 200 bp). This result suggested that (based on this small sample set of 101 brain expressed cDNA sequences) the chance of finding a gene in the EST set was about 54%.

Functional Annotation of Bee Brain ESTs

We characterized the *A. mellifera* EST sequences with respect to functionally annotated genes in *Drosophila melanogaster*, taking advantage of the fact that this insect genome has been sequenced and extensively annotated (Adams et al. 2000). Each *Apis* assembled sequence was tentatively assigned Gene Ontology (GO) classification based on annotation of the

Table 3. Molecular Function

Gene ontology term	<i>Drosophila</i> genes ^a	<i>Apis</i> assembled sequences ^b	<i>Drosophila</i> genes represented ^c
All molecular function terms	6260	1958	1509
nucleic acid binding	1052	353	269
DNA binding	696	186	144
transcription factor	495	132	104
RNA binding	265	125	92
translation factor	72	40	32
transcription factor binding	37	18	12
cell-cycle regulator	17	6	6
chaperone	114	50	35
motor	74	31	22
microtubule binding	83	41	28
defense/immunity protein	46	8	6
enzyme	2916	949	752
GTPase	92	51	42
kinase	355	133	108
phosphatase	171	52	41
peptidase	491	110	93
enzyme activator	61	21	20
enzyme inhibitor	74	7	7
apoptosis activator	3	1	1
apoptosis inhibitor	10	3	3
signal transducer	677	193	141
receptor	443	107	70
ligand-dependent nuclear receptor	22	9	7
transmembrane receptor	400	79	55
G-protein coupled receptor	207	21	17
olfactory receptor	58	1	1
receptor signaling protein	161	76	61
ligand	71	9	9
cell adhesion	53	27	17
storage protein	7	0	0
structural protein	354	106	92
transporter	792	296	195
ion channel	141	72	46
voltage-gated ion channel	43	29	16
neurotransmitter transporter	13	14	6
ligand binding or carrier	1095	451	335
protein tagging	6	5	3
antioxidant	8	7	5

Classification is hierarchical: indented terms are children of parent terms listed above.

Genes may be assigned to more than one term. Also note that child terms may have more than one parent term (e.g., “ligand-dependent nuclear receptor” is a child of both “receptor” and “transcription factor”) (see The Gene Ontology Consortium 2001).

^aTotal number of *Drosophila* genes assigned to each Gene Ontology term (from databases listed in Table 8).

^bNumber of *Apis* assembled sequences that match *Drosophila* genes assigned to each term in (a). Match means that the *Drosophila* gene was “best hit” for the *Apis* sequence (and $e\text{-value} \leq 10^{-5}$).

^cNumber of different *Drosophila* genes matched by *Apis* sequences.

single “best hit” match in BLASTX searches of *Drosophila* predicted proteins ($E \leq 10^{-5}$). Functional assignments of *Apis* ESTs described here are at the “inferred from electronic annotation” (IEA) level of evidence (see The Gene Ontology Consortium 2001). We take a conservative approach and avoid using *Drosophila* annotations that are, themselves, assigned at the IEA level of evidence. We do not exclude *Drosophila* annotations that are assigned at the “inferred from sequence similarity” (ISS) level of evidence (which requires human judgment and is therefore a higher level of evidence than IEA).

Tables 3 and 4 summarize assignments of *Apis* sequences to major molecular functions and biological processes, respectively. A broad range of functions and processes are represented in the brain ESTs. Table 5 lists *Apis* sequences that match *Drosophila* genes implicated in synaptic transmission (GO:0007268). Fifty-four (out of 116) *Drosophila* genes implicated in synaptic transmission were “best hit” for at least one *Apis*-assembled sequence. Table 6 lists *Apis* sequences that match *Drosophila* genes implicated in behavior. Note that current GO annotation for *Drosophila* includes only 42 genes implicated in behavior (as of December 2001). To provide information for comparative analysis, we generated a list of 106 genes directly implicated in behavior based on mutant analysis and/or transgenic experiments in *Drosophila* (compiled from FlyBase and J. Hall, pers. comm.). Genes were listed if at least one mutant allele or transgene affected a specific

aspect of behavior, such as rhythmicity, mating, feeding, or learning and memory. (Global locomotor effects such as paralysis, uncoordinated movement, or shaking were not considered in this analysis, although many of the genes listed do exhibit global locomotor or lethal phenotypes when mutated to the null state.) Using this criteria, 47 (out of 106) *Drosophila* behavior genes were “best hit” for at least one *Apis*-assembled sequence. Annotation of *Apis* EST sequences with respect to all GO terms for molecular function, biological process, and cellular component are regularly updated and can be accessed at http://titan.biotec.uiuc.edu/bee/honeybee_project.htm.

We expect that ongoing improvements in GO annotation for *Drosophila*, human, mouse, and *Caenorhabditis elegans* will lead to significant improvements in *Apis* gene annotation in the near future. The current annotation of *Apis* sequences, based solely on matches to *Drosophila* proteins, allowed useful comparative analyses but had several drawbacks. We often found *Apis* sequences that clearly encoded members of important gene families of known function, but nevertheless were not annotated. In every case examined, this occurred because the “best hit” gene in *Drosophila* was not yet assigned GO annotation. Conversely, *Apis* sequences sometimes were assigned function based on fairly weak matches (i.e., close to the E -value cutoff of 10^{-5}), resulting from the short length of the *Apis* EST. Annotation also was limited by a high proportion of ESTs in this project that contain transcript noncoding sequence (e.g., 3' UTR). Additional ESTs, especially from full-

Table 4. Biological Process

Gene Ontology term	<i>Drosophila</i> genes	<i>Apis</i> assembled sequences	<i>Drosophila</i> genes represented
All biological process terms	2746	906	696
cell growth and maintenance	2102	766	597
metabolism	1493	531	424
protein metabolism and modification	887	300	239
ion homeostasis	7	9	6
intracellular protein traffic	158	88	67
vesicle transport	150	83	62
synaptic vesicle transport	108	67	48
stress response	120	22	16
response to external stimulus	390	73	51
cell organization and biogenesis	253	108	79
cell cycle	187	42	34
apoptosis	29	2	2
cell communication	772	257	178
cell adhesion	46	25	15
cell recognition	43	33	20
neuronal cell recognition	14	3	3
synaptic target recognition	5	2	2
signal transduction	274	84	67
cell-cell signaling	117	79	54
synaptic transmission	116	79	54
neurotransmitter release	111	71	50
developmental processes	406	153	106
embryogenesis and morphogenesis	227	99	67
neurogenesis	95	59	36
imaginal discs development	63	35	27
sex determination	8	4	3
dosage compensation	8	6	4
metamorphosis	6	3	2
physiological processes	30	6	3
perception external stimulus	196	31	23
behavior	42	18	15

See notes for Table 3.

Table 5. *Apis* Matches to *Drosophila* Synaptic Transmission Genes

<i>Apis</i> sequence	<i>Drosophila</i> gene	Alignment length (aa)	HSP	e-value	Identities
Contig276	<i>amphiphysin</i>	295	390	1E-108	65%
BB160003A10D03	<i>AP-47</i>	251	411	1E-115	82%
BB170001B10H02	<i>AP-50</i>	234	407	1E-115	88%
BB160013B20B02	<i>Arf51F</i>	172	349	1E-96	97%
Contig 1946	<i>Arf72A</i>	179	340	8E-94	93%
BB160006B10F12	<i>Arf79F</i>	171	344	2E-95	98%
BB170032A10C06	<i>BcDNA:LD23336</i>	200	95	3E-20	30%
BB170005A10D08	<i>CaMKII</i>	59	123	1E-28	96%
BB160014B20D10	<i>Caps</i>	225	372	1E-103	77%
Contig2785	<i>Cdk5</i>	264	443	1E-125	80%
BB160022A10H03	CG10617	93	61	6E-10	40%
BB16000BA20A11	CG1107	121	137	6E-33	55%
Contig 1704	CG14296	178	335	2E-92	91%
BB170005B20G04	CG15694	149	212	2E-55	63%
Contig1152	CG17762	186	92	3E-19	33%
Contig 1768	CG2381	201	399	1E-111	91%
Contig2868	CG2903	51	62	3E-10	47%
BB170016A20B10	CG3020	38	53	2E-09	57%
Contig2061	CG3029	210	340	1E-93	81%
Contig190	CG5014	89	101	2E-38	58%
BB160022A20H05	CG5627	220	263	7E-71	60%
BB170011A20D07	CG5678	164	287	3E-78	85%
BB160024A20D05	CG7034	199	202	2E-52	51%
BB160003B20B01	CG7127	235	130	8E-31	37%
Contig2640	CG7321	213	192	2E-49	49%
BB160020B10H05	CG7736	208	115	1E-42	37%
BB160009A10E12	CG8608	131	198	4E-51	70%
Contig1193	<i>Chc</i>	295	446	1E-126	74%
Contig924	<i>Csp</i>	237	218	4E-57	51%
BB160022A10D06	<i>Dap160</i>	202	155	3E-38	46%
BB170019A20D08	<i>dlg1</i>	266	377	1E-105	72%
Contig1272	<i>gammaSnap</i>	247	283	8E-77	55%
BB160010B20F08	<i>Gdi</i>	109	181	3E-46	75%
BB160015B20E03	<i>I(2)gl</i>	193	96	1E-20	33%
Contig1207	<i>lqf</i>	102	66	3E-11	45%
BB170026B10H11	<i>Nrx</i>	142	215	9E-57	67%
Contig1277	<i>Nsf2</i>	108	148	9E-37	66%
BB170025B20H08	<i>n-syb</i>	73	135	2E-32	91%
Contig1852	<i>Rab3</i>	198	367	1E-102	89%
Contig2442	<i>Rop</i>	147	222	8E-59	70%
Contig1960	<i>Sed5</i>	150	141	4E-34	50%
Contig734	<i>Snap</i>	279	427	1E-120	72%
BB170015A10F09	<i>Snap24</i>	32	51	4E-07	78%
BB160017B20C04	<i>Stam</i>	219	253	5E-68	57%
Contig2134	<i>syt</i>	330	559	1E-160	85%
Contig80	<i>SytIV</i>	190	274	6E-74	70%

Synaptic transmission (GO:0007268)

length, enriched, normalized, and subtracted libraries (e.g., Carninci et al. 2000), would enhance *Apis* gene annotation by allowing more ESTs to be assembled into larger contig sequences.

Honey Bee Brain Microarray

To allow functional genomic studies of brain and behavior in the honey bee, we generated cDNA microarrays from the annotated EST set described above. A total of 7329 cDNAs (putatively representing different transcripts) were successfully amplified as “single-band” PCR product and spotted on the microarray. Pilot studies indicated that fluorescent probe derived from single-brain mRNA (amplified by *in vitro* transcription; see Methods) could be used to label the vast majority of *Apis* cDNA spots on the microarray. Data obtained from one microarray experiment are presented in Table 7 and Figure 2.

In this experiment, two dissected adult bee brains were combined and mixed during homogenization, then split into two equal samples. Each of the two samples was used to generate an independent probe (one Cy5-labeled probe [635 nm] and one Cy3-labeled probe [532 nm]). The two probes were combined and hybridized to a single microarray. A total of 7300 and 7305 cDNAs produced hybridization signal at least two standard deviations (SD) greater than background at 635 and 532 nm, respectively. To determine whether this hybridization signal was specific, we compared signal produced by *Apis* cDNA spots with exogenous negative control cDNA spots on the microarray (derived from vertebrate and plant genes). A total of 6647 (91%) and 6631 (90%) of the *Apis* cDNAs produced signal at least two standard deviations greater than exogenous control cDNAs at 635 and 532 nm, respectively. Signal intensities between 635 and 532 nm were highly corre-

Table 6. *Apis* Matches to *Drosophila* Behavior Genes

<i>Apis</i> sequence	<i>Drosophila</i> gene	Alignment length (aa)	HSP	e-value	Identities
Contig3015	<i>14-3-3zeta</i>	246	452	1E-127	91%
BB170004B20H03	<i>acj6</i>	45	92	2E-19	97%
BB160024A10A12	<i>Adar</i>	169	185	3E-47	54%
Contig 1753	<i>ap</i>	83	143	2E-34	81%
Contig467	<i>ari-1</i>	55	103	1E-22	81%
BB170007B20D03	<i>Atpalpha</i>	97	177	3E-45	89%
BB160016A10E02	<i>CadN</i>	126	117	4E-27	47%
Contig2335	<i>Cam</i>	145	228	5E-60	74%
BB170005A10D08	<i>CaMKII</i>	59	123	1E-28	96%
BB170029A10F02	<i>Cha</i>	87	55	4E-08	36%
BB170016A10D05	<i>chp</i>	32	47	5E-06	62%
BB160005A10C01	<i>CoVa</i>	144	151	3E-37	53%
Contig924	<i>Csp</i>	237	218	4E-57	51%
BB170022A10F08	<i>dare</i>	155	101	1E-32	38%
Contig377	<i>Dat</i>	167	113	9E-26	39%
BB170029B10C01	<i>dnc</i>	66	120	8E-29	84%
BB160007B10G06	<i>dsx</i>	47	50	2E-06	46%
Contig1083	<i>e</i>	147	86	2E-17	36%
BB160017B10B10	<i>Fas2</i>	199	189	1E-48	47%
BB160010A10H12	<i>for</i>	193	291	2E-79	75%
BB170016A20G06	<i>fru</i>	103	168	3E-42	76%
BB160008B10H11	<i>G-salpa60A</i>	234	357	4E-99	73%
BB170024A20D07	<i>Hk</i>	28	52	4E-07	78%
Contig12	<i>lark</i>	367	380	1E-106	58%
BB170007B10A02	<i>mas</i>	167	340	4E-94	94%
Contig923	<i>mle</i>	375	355	3E-98	50%
Contig362	<i>nbA</i>	159	70	2E-12	33%
BB160015B10F09	<i>Nf1</i>	210	226	1E-59	57%
BB170031B10G07	<i>ninaA</i>	172	212	2E-55	55%
BB170001A10G09	<i>ninaE</i>	163	239	1E-63	69%
BB170022A10G08	<i>nompC</i>	161	101	4E-22	40%
BB160020B20C08	<i>para</i>	34	76	1E-14	97%
Contig730	<i>Pka-C1</i>	197	402	1E-112	95%
BB160004A20E12	<i>plx</i>	53	82	3E-16	71%
Contig397	<i>Pp1-87B</i>	141	282	1E-76	94%
BB170012A20E10	<i>rdgB</i>	160	58	4E-09	28%
Contig2777	<i>Reg-5</i>	90	69	3E-12	40%
BB160003A20A12	<i>Rya-r44F</i>	197	316	2E-89	73%
BB160013A20B02	<i>sbb</i>	53	52	2E-06	54%
BB160004A10F11	<i>sd</i>	260	360	1E-100	68%
Contig830	<i>sgg</i>	285	522	1E-148	87%
Contig3064	<i>Shab</i>	88	98	3E-21	59%
Contig 1958	<i>Shal</i>	72	95	3E-20	58%
Contig2624	<i>slo</i>	90	184	5E-47	95%
Contig2399	<i>tipE</i>	64	107	7E-24	78%
Contig1139	<i>vri</i>	247	99	2E-21	30%
Contig2819	<i>w</i>	68	108	2E-56	73%

Behavior genes defined in text. *Drosophila* genes tested but not found: *Ace*, *Acp70A*, *Adf1*, *amn*, *bi*, *Btk29A*, *Caki*, *Ca-alpha1D*, *clk*, *CrebB-17A*, *Crg-1*, *crl*, *cry*, *cyc*, *Cyp4e2*, *dco*, *Ddc*, *disco*, *Dr*, *dsf*, *dy*, *eag*, *gk*, *G-alpha65A*, *Hdc*, *inaC*, *lat*, *lio*, *lush*, *lz*, *mnB*, *mud*, *mys*, *ninaC*, *nompA*, *nompB*, *nonA*, *norPA*, *ogre*, *otu*, *Pdf*, *per*, *Pka-R1*, *ppl*, *qtc*, *rb*, *rut*, *scb*, *Sh*, *Shaw*, *shi*, *sol*, *spin*, *sws*, *tim*, *to*, *tutl*, *Ubc47D*, *W*.

lated in this experiment ($r = 0.9926$) indicating that technical variation (from RNA isolation, mRNA amplification by in vitro transcription, and fluorescent labeling of probe) is very low. Results from additional microarrays were qualitatively similar using different bee brains as source material (data not shown). These results indicate that genomic scale gene expression profiling is feasible in single honey bee brains using the microarrays and protocols described here.

Microarray hybridization data have been used for the validation of gene sequences (e.g., Andrews et al. 2000; Shoemaker et al. 2001). The results presented above indicate that

the vast majority of bee ESTs were derived from legitimate brain-expressed gene transcripts.

Comparative Genomics in *Apis* and *Drosophila*

A total of 823 of the assembled sequences (24% of those with matches) were most similar to protein sequence from Chordata (Fig. 1B). The high level of *Apis* "best hits" to Chordata could arise from a high rate of sequence divergence or gene loss in *Drosophila* and/or be related to deficiencies in *Drosophila* gene prediction. To distinguish between these possibilities, *Drosophila* genome sequence and EST databases were

Table 7. Signal analysis of an example microarray

	Number of spots	635 nm				532 nm			
		Feature		Background		Feature		Background	
		Avg.	SD	Avg.	SD	Avg.	SD	Avg.	SD
<i>Apis</i> cDNAs	7329	3.42	0.47	2.23	0.05	3.43	0.48	2.15	0.04
Exogenous control 1	16	2.40	0.07	2.24	0.05	2.35	0.07	2.15	0.04
Exogenous control 2	16	2.43	0.07	2.23	0.04	2.41	0.06	2.15	0.04
Exogenous control 3	16	2.32	0.08	2.23	0.05	2.26	0.08	2.15	0.04
Exogenous control 4	16	2.38	0.06	2.24	0.04	2.32	0.05	2.15	0.04
Exogenous controls 5–48	43	2.49	0.20	2.23	0.05	2.47	0.22	2.15	0.05
All controls spots	107	2.43	0.15	2.23	0.05	2.39	0.17	2.15	0.04

	635 nm	532 nm
# <i>Apis</i> cDNAs > background + 1 SD	7308	7311
# <i>Apis</i> cDNAs > background + 2 SD	7300	7305
# <i>Apis</i> cDNAs > control spots + 1 SD	7029 (96%)	7035 (96%)
# <i>Apis</i> cDNAs > control spots + 2 SD	6647 (91%)	6631 (90%)

All intensity values were log₁₀ transformed. Feature and background readings are indicated for each cDNA spot (based on median pixel intensity) for 635 and 532 nm wavelengths. Average (Avg.) and standard deviation (SD) are indicated. Exogenous controls are described in Methods.

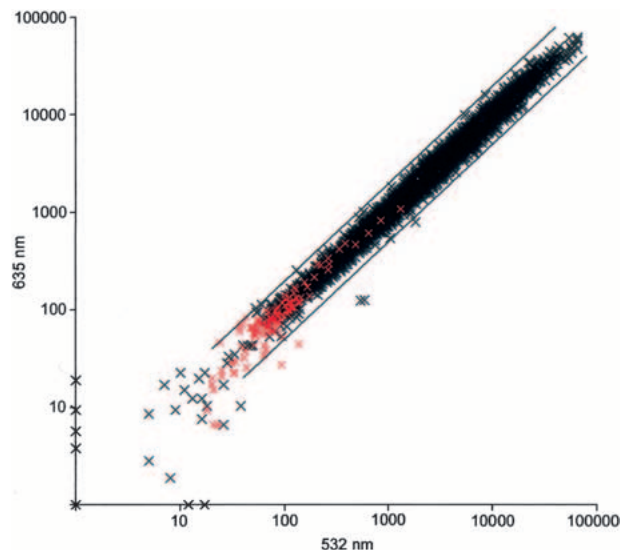


Figure 2 Signal intensities from an example microarray. Values plotted are feature minus background intensity at 635 and 532 nm wavelengths for each cDNA spot (see Methods). Values were normalized such that the median ratio (635:532 nm) equals 1.0. *Apis* cDNAs are shown as black x's, exogenous negative control cDNAs are shown as red x's. Cy3-labeled probe (532 nm) and Cy5-labeled probe (635 nm) were independently derived from the same starting sample (using in vitro transcription to amplify starting mRNA; see Methods). The starting sample consisted of a mixture of two dissected adult bee brains (one bee observed foraging and one bee observed caring for brood). The coefficient of correlation (r) between 635 and 532 nm values was 0.9926 (based on log-transformed values). Divergence of values from the diagonal (ratio = 1) reflects technical variation introduced during RNA isolation, mRNA amplification by in vitro transcription, and fluorescent labeling of probe. The two diagonal bars indicate ratios (635:532 nm) equal to 0.5 and 2.0.

searched for matches to *Apis*-assembled sequences using TBLASTX. Matches were screened individually to identify true gene alignments based on plausible exon structure and amino-acid composition. In 99 cases, predicted proteins in *Drosophila* were missing one or more exons (predicted by alignment between *Apis* ESTs and *Drosophila* genome sequence). This caused a weak or no match to the *Drosophila*-predicted protein sequence and a misleading “best hit” to Chordata. In 23 cases, genes were identified in *Drosophila* genomic sequence (based on alignment with *Apis* sequence) that were not represented in *Drosophila* predicted protein or EST databases. Suggestions for annotations of these 122 *Drosophila* genes have been communicated to FlyBase.

Of the 701 remaining cases where the best match for the *Apis* sequence was to Chordata, 574 (16% of *Apis*-assembled sequences with matches) had likely orthologs in *Drosophila*, but these *Drosophila* genes were so diverged that better matches for the *Apis* sequences were identified in human, mouse and/or other non-Arthropoda. In 126 cases (3.6% of *Apis* assembled sequences with matches), the *Apis* sequence had significant and clear matches to proteins from human, mouse and/or other organisms, but no plausible ortholog was identified in searches of *Drosophila*-predicted protein, genome, or EST databases. These *Apis* sequences appear to define genes that have been lost from the *Drosophila* genome. Detailed analysis of these highly diverged genes and gene loss events in *Drosophila* will be presented in a subsequent manuscript.

Future Prospects

The relationship between genes and behavior is complex and is only beginning to be understood. Honey bees exhibit a wide variety of behavioral phenomena that are not observed in *Drosophila*, such as kin recognition, complex communication via the dance language, socially regulated division of labor, and a larger variety of forms of learning. The honey bee also is haplodiploid and has the highest known recombination rate of any animal (Hunt and Page 1995), traits that can

facilitate genetic analyses of behavior. A wide range of naturally variable behavior traits has been described in honey bees, including defensive behavior (Hunt et al. 1998), foraging preferences (Hunt et al. 1995), and differences in socially regulated division of labor (Robinson 1992; see also Brillet et al. 2001). A comprehensive, web-based atlas of the bee brain currently in development (see <http://www.neurobiologie.fu-berlin.de/Menzel.html>) also will be helpful in providing a stronger neurobiological foundation for the study of genes and behavior in the honey bee. Early efforts to develop transgenic bees (Omholt et al. 1995; Ronglin et al. 1997; K. Robinson et al. 2000) suggest that there are no barriers to harnessing this technology. The work described here provides additional resources that should contribute to molecular analyses of honey bee behavior, using candidate gene studies, positional cloning, and functional genomic approaches.

METHODS

Bees

Approximately 600 adult workers were collected from a typical field colony at the University of Illinois Bee Research Facility. The colony had about 40,000 adult bees and was derived from a naturally mated queen. The bees in this area are a mixture of various races of European honey bees, predominantly *Apis mellifera ligustica* (Pellett 1938). Bees were collected when they were 1, 5, 10, 15, 20, 25, and 30 days old, which spans the typical lifespan during the active season (Winston 1987). This collection scheme ensured a broad representation of behavioral states, because bees specialize on different tasks at different ages (Robinson 1992). To obtain bees of known age, frames of pupae were removed from the colony and placed in an incubator (33°C). About 3500 one-day-old bees were marked with a spot of paint (Testor's Pla) on the thorax and then returned to their natal colony. We supplemented these age-based collections with samples of bees taking preforaging orientation flights (Capaldi et al. 2000) and foragers returning with either pollen or nectar loads. Collections were made both in the early morning and late in the afternoon. Bees were collected directly into liquid nitrogen (Toma et al. 2000) to minimize the possible effects of collection on gene expression. Brains were dissected on dry ice.

Brain cDNA Libraries

Total RNA was isolated from 400 bee brains (ca. 500 µg) with Rneasy total RNA isolation kit (Qiagen) followed by treatment with Dnase (1 unit RQ1 Dnase; Promega). Poly(A)⁺ RNA was purified and cDNA was synthesized and directionally cloned into *NotI* and *EcoRI* digested pT7T3-Pac phagemid vector as in Bonaldo et al. (1996). cDNA inserts are flanked by linker sequences 5'-*NotI*-GTTGC-3' (library specific, 3' linker) and 5'-*EcoRI*-GGCACGAGG-3' (5' linker). The library was normalized and (subsequently) subtracted as in Bonaldo et al. (1996).

Sequencing and Sequence Analysis

Plasmid DNA was extracted and sequenced using ABI 377 and 3700 sequencers. The sequencing primer used was 5'-AGCGGATAACAATTTACACACAGGA-3'. Base-calling was performed with *phred* (see Table 8 for all programs and databases used). Vector sequences were trimmed using *Cross-match*. Low-quality bases (quality score <20) were trimmed from both ends of sequences using *Qualtrim* and *Simpletrim*. Those ESTs having a length of more than 200 bp after both vector and quality trimming were considered "high-quality" ESTs. The repeat sequences in these ESTs then were masked by *RepeatMasker* program using *Drosophila* re-

Table 8. Databases and software used

	Version and/or date downloaded	Source
Sequence analysis and assembly		
<i>phred</i>	0.000925.c	1
<i>Cross match</i>	0.990319	1
<i>Qualtrim</i>	September, 2000	2
<i>Simpletrim</i>	July, 2000	2
<i>RepeatMasker</i>	July, 2000	1
<i>phrap</i>	0.990319	1
<i>CAP3</i>	July, 2000	3
<i>Flip</i>	2.0	4
Sequence similarity searches		
<i>Stand-alone BLAST</i>	Oct., 01 and later	5
<i>nr</i>	Aug., 2001	5
<i>nt</i>	May, 2001	5
<i>EST_Human</i>	May, 2001	5
<i>EST_Mouse</i>	May, 2001	5
<i>EST_Other</i>	May, 2001	5
<i>aa_gadfly.dros.RELEASE2</i>	RELEASE2	6
<i>na_arms.dros.RELEASE2</i>	RELEASE2	6
<i>na_EST.dros</i>	May, 2001	6
<i>nr_Arthropoda</i>	Aug., 2001	7
<i>nr_Chordata</i>	Aug., 2001	7
Functional annotation		
<i>function.ontology</i>	2.99; Sep., 2001	8
<i>process.ontology</i>	2.88; Sep., 2001	8
<i>gene_association.fb</i>	1.29; Sep., 2001	8

¹University of Washington Genome Center; <http://www.genome.washington.edu/UWGC>

²Keck Center for Comparative and Functional Genomics, University of Illinois at Urbana-Champaign; <http://www.biotech.uiuc.edu/keck.htm>

³Huang and Madan (1999)

⁴Organelle Genome Megasequencing Project, University of Montreal; <http://megasun.bch.umontreal.ca/ogmproj.html>

⁵National Center for Biotechnology Information (NCBI); <http://www.ncbi.nlm.nih.gov>

⁶Berkeley *Drosophila* Genome Project (BDGP); <http://www.fruitfly.org>

⁷Sub-databases were extracted from *nr* using NCBI gene identification (*gi*) numbers for each taxonomic group.

⁸The Gene Ontology Consortium (2001); <http://www.geneontology.org>

peat sequences as reference. The masked sequences were further screened for bacterial chromosomal DNA, RNA, insect viral DNA, rRNA, and mitochondrial DNA using *BLASTN*. Further screens for possible contaminants were conducted by *BLASTN* searches of the Non-Redundant Nucleotide Sequences (*nt*), *EST_human*, *EST_mouse*, and *EST_others* databases. Eighty-one ESTs were removed that corresponded to clear contaminants likely derived from other library and/or sequencing projects (from mouse or rat [49], cattle [9], human [6], pig [2], undetermined vertebrate [2], and various non-*Escherichia coli* bacteria [9]). No other ESTs were found to be ≥90% identical (over any 100 bp span) to nucleotide sequence from any non-*Apis* species, suggesting that the EST set did not include contamination from *Drosophila* or other sources not identified here. An additional 101 ESTs were removed as informatic artifacts (e.g., sequencing lanes that should not have produced sequence). Some EST screening was conducted after assembly, resulting in 54 contig sequences that were composed of contaminant or artifact ESTs. These 54 sequences were removed from the "assembled sequence" database and did not affect analyses presented here.

ESTs were analyzed to identify chimeric, backward, or unspliced inserts. Chimeric clones could be indicated by back-

to-back poly(A)⁺ tails or vector linker sequences within ESTs. BLASTN searches for these instances identified only one chimera (out of all 21,408 ESTs). In this instance the 3' linker sequence was found in the middle of an EST, flanked by back-to-back poly(A)⁺ tails from two different transcripts. Furthermore, in all cases where 3' ESTs had BLASTX matches ($E \leq 10^{-20}$) to a *Drosophila* predicted protein (68 cases), 5' ESTs from the same cDNA matched the same *Drosophila* protein. To estimate the total number of backward cDNA inserts, singlet ESTs with BLASTX matches to *Drosophila*-predicted proteins were analyzed. Out of 1919 singlet EST matches, 364 (19%) had a negative reading frame, indicating a backward cDNA insert. Of 720 individually analyzed ESTs with BLASTX matches to proteins from other organisms, 72 (10%) had clear instances of unspliced intron sequence (based on alignment with putative orthologs, ORF analysis, and identification of putative splice junctions); many of these clones appear to have resulted from priming of the oligo(dT) primer within an unspliced AT-rich intron.

ESTs were assembled using CAP3 and phrap (see Table 2 for settings).

ORFs were identified using FLIP with the minimum length set to 150 amino acids (450 bp). All BLAST searches were conducted on a desktop PC or local server using stand-alone BLAST software and sequence databases indicated in Table 8. All *E*-value cutoffs were 10^{-5} , except where indicated otherwise. GO databases were installed on a local server. A GO browser was designed and implemented at the W.M. Keck Center for Comparative and Functional Genomics (University of Illinois at Urbana-Champaign) and used for functional annotation of the assembled EST sequences.

Microarray Fabrication

A single EST cDNA clone was selected to represent each assembled sequence (putatively unique transcript). For contigs with multiple ESTs, the rule followed was to select the 3'-most EST that had at least 300 bp of high-quality sequence. This procedure biases the cDNAs on the microarray toward the 3' end but ensures that at least 300 bp of cDNA is spotted on the array. A total of 8872 cDNA clones were selected. These clones were picked from the library stock plates (384-well bacteria clones) and rearranged to a new set of 384-well plates. These clones were grown overnight followed by sequence verification (see Clone Tracking, below).

Creation of the microarrays was essentially as described by Brown and Botstein (1999). Bacteria clones were inoculated to 96-well plates with LB and Amp and grown overnight. Plasmid inserts were amplified by PCR using 1 μ L of the overnight bacteria inoculant and modified M13 (5'-CCAGTCACGACGTTGTAAAACGAC-3') and M13 reverse (5'-GTGTGGAATTGTGAGCGGATAACAA-3') primers in 50 μ L volume reactions. Amplifications were performed in a MJ PTC-200 thermocycler (MJ Research). PCR reaction mixes contained 5 μ L 10x reaction buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 2.0 mM MgCl₂, 100 μ M dNTPs, 0.2 μ M each primer, and 1U Amplitaq Gold (Perkin Elmer). An initial 9-min denaturation was followed by 35 cycles of 40 sec denaturation at 94°C, 40 sec annealing at 65°C, and 3.5 min elongation at 72°C. The reaction ended with an additional incubation of 5 min at 72°C. Products were cleaned using Sephadex G-50 columns. Five microliters of each clean PCR product was analyzed on a 1% agarose gel. cDNA amplification products were visually examined and subjectively classified as follows: "strong single band" (86%), "weak or absent band" (13%), or "multiple bands" (1%). Only cDNAs that were amplified as "single strong band" and successfully spotted on the array (see below) were used in subsequent data analysis (7329 total).

PCR products were dried and resuspended in 8 μ L 3x SSC, 1.5 M betaine. Betaine was used as in Diehl et al. (2001) to

improve spot homogeneity and to increase hybridization signal on the microarray. All cDNAs were printed as single spots on Telechem Superamine slides (Arrayit) using a Cartesian Technologies spotter. Exogenous control cDNAs derived from cattle (phosphoglycerate kinase 1 and β -2-microglobulin) and soy (rubisco small chain 1 and chlorophyll ab binding protein) were spotted on the array 16 times each, such that they were represented on each of the 16 subgrids on the microarray ("exogenous controls 1–4", respectively, in Table 7). An additional 43 vertebrate-derived cDNAs (singly spotted at random positions throughout the microarray) were used as control spots ("exogenous controls 5–48" in Table 7).

Spot and printing quality were assessed visually after printing. cDNA spots do not fully evaporate after arraying (as a result of 1.5 M betaine) allowing inspection of spot morphology under a dissecting scope. A few slides (about one in every five) exhibited minor defects (e.g., a single spot missing or several spots damaged by dust or lint particles). The majority of slides exhibited no defects (no spots missing, no spots joined, and all spots uniform in size).

DNA was crosslinked to slides by baking at 80°C for 1 h. Slides were blocked in 0.2% SDS for 4 min, followed by two washes in water. Slides were denatured in boiling water for 2 min, spun dry, and stored.

Microarray Hybridization, Scanning, and Data Analyses

Frozen brains were dissected from bees of known age and behavioral state as above. mRNA was amplified exactly as in Baugh et al. (2001), using only one round of in vitro transcription. Amplified RNA (aRNA) was analyzed by spectrophotometer and gel electrophoresis. Negative control reactions (no template and genomic DNA only) conducted in parallel produced no aRNA. aRNA was labeled by reverse transcription as follows: 5 μ g of aRNA was mixed with 5 μ g of random primer (Roche) (10 μ L volume), denatured at 70°C for 4 min, and placed on ice. Labeling reaction (6 μ L of 5x 1st Strand Buffer [Gibco]; 3 μ L of 100 mM DTT; 6 μ L of low T dNTPs [2.5 mM each dATP, dCTP, dGTP and 1.0 mM dTTP] (Sigma), 3 μ L of 1 mM Cy3- or Cy5-dUTP [Amersham Pharmacia] and 2 μ L of 200 U/ μ L SuperScript II [Gibco]) was prepared on ice, mixed with aRNA and primer, then incubated at 42°C for 1 h. One microliter of SuperScript II was added and the reaction was incubated at 42°C for an additional hour. RNA was removed by adding 1 μ L of 0.25 mg/mL RNase A (NEB) and 0.5 μ L of 2 U/ μ L RNase H (Stratagene) and incubating at 37°C for 30 min. Labeled cDNA was purified using the Qiagen PCR Purification Kit.

Thirty microliters of purified, labeled cDNA was mixed with blocking oligos dT-T7 (20 μ g; see Baugh et al. (2001)) and dT₃₀ (40 μ g), boiled for 3 min, allowed to anneal at 60°C for 10 min and then room temperature for 10 min, mixed with an equal volume of 2x hybridization buffer (50% formamide, 10x SSC, and 0.2% SDS), and then hybridized to microarray at 42°C overnight. Excess probe was removed by a series of 4 min washes in 1x SSC, 0.2% SDS at 42°C; 0.1x SSC, 0.2% SDS at room temperature; and 0.1x SSC at room temperature. Slides were scanned using an Axon 4000B scanner, and images were analyzed with GenePix software.

All data analyses were conducted using log-transformed values (median pixel intensities) generated by the GenePix software.

Clone Tracking

To identify and correct possible errors in clone tracking, 420 cDNA clones (of the initial set of 20,256) were resequenced from the stock bacterial 384-well plates. Two clones were selected from different positions from each 96-well quadrant (there are four quadrants per 384-well plate). These sequences

were tested against existing EST sequences in the database. A PERL script was used to identify expected matches, possible lane-tracking errors, quadrant or plate swaps, or errors in quadrant or plate orientation. In the majority of cases, one or two sequences were obtained from each quadrant and matched expected database sequences, thus confirming tracking accuracy. In cases where a sequence was not obtained or did not match the expected sequence, two additional clones were grown and sequenced. Tracking errors affecting whole quadrants were indicated for 16 (of 212 total) quadrants, including quadrant swaps, duplicate sequencing of quadrants, and quadrants in which database sequences were in an upside-down orientation with respect to the actual clones. The exact nature of each quadrant error was determined (in all cases, the initial determination was confirmed by additional sequencing) and corresponding sequence entries in the database were corrected to reflect their true plate positions. Lane-tracking errors (i.e., ABI 377 generated sequences that drift from one lane into a neighboring lane) were not observed.

After rearraying the 8872 clones to be used for the microarray, an additional 192 cDNA clones were regrown and sequenced to verify tracking integrity (two clones were picked from each 96-well quadrant, as above). From these, 136 high-quality sequences were obtained and tested for identity with the expected EST. Only one sequence of the 136 tested did not match the expected EST, suggesting that clone tracking was close to 99% accurate at this stage.

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