In silico Analysis of 2085 Clones from a Normalized Rat Vestibular Periphery 3' cDNA Library

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Key Words

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Abstract

The inserts from 2400 cDNA clones isolated from a normalized *Rattus norvegicus* vestibular periphery cDNA library were sequenced and characterized. The Wackym-Soares vestibular 3' cDNA library was constructed from the saccular and utricular maculae, the ampullae of all three semicircular canals and Scarpa's ganglia containing the somata of the primary afferent neurons, microdissected from 104 male and female rats. The inserts from 2400 randomly selected clones were sequenced from the 5' end. Each sequence was analyzed using the BLAST algorithm compared to the Genbank nonredundant, rat genome, mouse genome and human genome databases to search for high homology alignments. Of the initial 2400 clones, 315 (13%) were found to be of poor quality and did not yield useful information, and therefore were eliminated from the analysis. Of the remaining 2085 sequences, 918 (44%) were found to represent 758 unique genes having useful annotations that were identified in databases within the public domain or in the published literature; these sequences were desig-

nated as known characterized sequences. 1141 sequences (55%) aligned with 1011 unique sequences had no useful annotations and were designated as known but uncharacterized sequences. Of the remaining 26 sequences (1%), 24 aligned with rat genomic sequences, but none matched previously described rat expressed sequence tags or mRNAs. No significant alignment to the rat or human genomic sequences could be found for the remaining 2 sequences. Of the 2085 sequences analyzed, 86% were singletons. The known, characterized sequences were analyzed with the FatiGO online data-mining tool (<http://fatigo.bioinfo.cnio.es/>) to identify level 5 biological process gene ontology (GO) terms for each alignment and to group alignments with similar or identical GO terms. Numerous genes were identified that have not been previously shown to be expressed in the vestibular system. Further characterization of the novel cDNA sequences may lead to the identification of genes with vestibular-specific functions. Continued analysis of the rat vestibular periphery transcriptome should provide new insights into vestibular function and generate new hypotheses. Physiological studies are necessary to further elucidate the roles of the identified genes and novel sequences in vestibular function.

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Introduction

Considerable effort has been dedicated toward the understanding of the afferent mechanisms of the transduction of forces related to head acceleration and gravity into biological signals. The detection of change in angular and linear acceleration as well as the spatial position of the head in relation to the body is accomplished through specialized mechanoreceptors of the vestibular system. Signals from the type I and type II hair cell mechanoreceptors are transmitted to the central nervous system through bipolar primary afferent neurons whose somata comprise Scarpa's ganglia. Efferent axons, originating in the brainstem, modulate the afferent sensory input to the central nervous system by innervating the afferent dendrites and calyces interfacing with type I hair cells and directly innervating type II hair cells as well as the dendrites associated with type II hair cells. At present, there is a basic understanding of the major neurotransmitters, neuromodulators and their receptors within the vestibular periphery. Likewise much has been learned about the mechanisms of signal transduction, ion channels, transport mechanisms and cellular proliferation within the vestibular epithelia. Despite these advances, significant gaps exist in our knowledge of the wide array of detailed mechanisms subserving vestibular function. These mechanisms require the coordinated expression of a large number of known genes, but also no doubt utilize novel genes which are only expressed in the vestibular periphery. In order to better characterize this transcriptome, various approaches can be used, including reverse transcription-polymerase chain reaction (RT-PCR), cDNA library analysis, microarray expression profiling and serial analysis of gene expression [Halum et al., 2003, 2004; Iijima et al., 2004; Kanzaki et al., 2002; Welling et al., 2002].

Our group has focused on understanding the molecular bases of the efferent-afferent interaction in the vestibular periphery. In addition to immunohistochemical studies localizing specific efferent neuromodulators and receptors receiving efferent input, we have demonstrated the expression of transcripts encoding a wide range of nicotinic acetylcholine receptor subunits [Anderson et al., 1997; Wackym et al., 1995], muscarinic acetylcholine receptor subtypes [Wackym et al., 1996], purinergic receptors [Trojanovskaya and Wackym, 1998] and opioid receptors [Popper et al., 2004]. We have also focused on identifying genes involved in signal transduction and second-messenger systems associated with the efferent-afferent interaction, particularly with G protein α -subunits

[Cioffi et al., 2003; Wackym et al., 2000b; Wackym et al., 2005] and adenylyl cyclase isoforms [Wackym et al., 2000a]. However, physiological data suggest that the precise molecular mechanisms of efferent modulation of afferent vestibular function are not well understood, and a more global assessment of the molecular relationships between these two systems remains to be completed. A comprehensive knowledge of genes that are expressed in the vestibular periphery will allow a more system-based analysis of function and lead to greater insight into normal and abnormal vestibular function.

Characterization of expressed sequence tags (ESTs) derived from cDNA libraries has led to the discovery of several novel inner ear genes [reviewed in Heller, 2002] and greater knowledge of the genes expressed within the cochlea. Transcriptome analysis using these libraries typically involves sequencing randomly picked cDNA clones and comparing these sequences to databases within the public domain. Sequences representing known, characterized genes may reveal genes not previously known to be expressed in the inner ear but important to normal physiology. Sequences not aligning with previously identified sequences may represent novel organ-specific transcripts, possibly conferring specialized function. Mutations in these organ-specific genes resulting in loss of function or impaired function could result in hereditary pathology; consequently these genes represent good candidates for hereditary inner ear dysfunction. Recently, characterization of cDNA libraries from the inner ear of various species, tissues, and cell types using a range of techniques have been reported [Beisel et al., 2004; Coimbra et al., 2002; Harter et al., 1999; Luijendick et al., 2003; Maubaret et al., 2002; Resendes et al., 2001; Robertson et al., 2000].

While extensive analysis of the genes expressed in many mammalian tissues has been accomplished, analysis of the mammalian vestibular system has been limited to date. Abe et al. [2003] reported the construction and partial characterization of a nonnormalized human vestibular cDNA library by sequencing 506 randomly selected clones and classifying known genes according to their functional profile. In their study, 301 sequences (59.5%) matched known functional genes, 29 clones (5.7%) matched known ESTs, 22 clones (4.3%) were deemed novel, and the remaining clones matched mitochondrial DNA and repetitive sequences [Abe et al., 2003]. Despite the small number of clones sequenced, their study provided insights into the expression profile of the human vestibular system; however, 79 of 301 clones that matched known functional genes were present in

multiple copies [Abe et al., 2003]. Random selection of clones from a nonnormalized cDNA library, such as that used by Abe et al. [2003], favors identification of high abundance transcripts, and consequently there is a high probability of failing to identify low abundance transcripts that could be unique to the tissue of interest. Normalized cDNA libraries help to ameliorate this problem by enriching the library with low copy transcripts and reducing the number of high abundance transcripts. In an effort to more completely describe the transcriptome of the rat vestibular periphery with interest in identifying low copy transcripts, we analyzed a directionally cloned, normalized cDNA library prepared from microdissected vestibular end organs and vestibular nerves including Scarpa's ganglia. To our knowledge, this analysis represents the first gene expression profile study of the rat vestibular periphery.

The gene ontology (GO) project is an effort to provide a structured, precisely defined, consistent and controlled vocabulary that describes gene products in different databases (<http://www.geneontology.org/>) [Ashburner et al., 2000]. The GO collaborators have developed three structured, controlled vocabularies (ontologies) that describe gene products in terms of their associated biological processes, cellular components and molecular functions in a species-independent manner. There are three separate aspects to this effort: first, writing and maintaining the ontologies themselves; second, making associations between the ontologies and the genes and gene products in the collaborating databases, and third, developing tools that facilitate the creation, maintenance and use of ontologies. The use of GO terms by several collaborating databases facilitates uniform queries across them. Any single gene product can be described by numerous terms in each category or by none. GO terms are not names of gene products. The text string for each GO term and the parent-child relationships between terms are defined by the members of the GO Consortium. This combination of a controlled vocabulary with defined relationships between items is referred to as an ontology. For each of the three categories of biological information, a set of terms has been selected and organized as directed acyclic graphs or networks. The difference between a directed acyclic graph and a hierarchy is that in the latter each child can only have one parent; a directed acyclic graph allows a child to have more than one parent. Within an ontology, a child may be a 'part of' or an example ('instance') of its parent [Ashburner et al., 2000]. The greater the level of evidence supporting a specific gene annotation, the higher the assigned level of gene ontology. These values range from

level 2 up to level 5. Software analysis that utilizes GO terms is a convenient and consistent way to organize gene products identified in analysis and has consequently been incorporated into our in silico data analysis scheme.

Materials and Methods

This study was performed in accordance with the United States Public Health Service Policy on Humane Care and Use of Laboratory Animals, the *NIH Guide for the Care and Use of Laboratory Animals* and the Animal Welfare Act (7 U.S.C. et seq.); the animal use protocol was approved by the Institutional Animal Care and Use Committee of the Medical College of Wisconsin.

cDNA Library Construction

Although the construction and normalization of the Wackym-Soares *Rattus norvegicus* vestibular cDNA library has been described in detail before [Cioffi et al., 2003], a brief description follows. 104 adult male and female rats (*Rattus norvegicus*) were anesthetized and decapitated. The 208 temporal bones were quickly dissected on ice and microdissected to retrieve the saccular and utricular maculae, the ampullae of all three semicircular canals, and the superior and inferior vestibular nerves including Scarpa's ganglia (primary afferent ganglia). The tissue was then frozen on dry ice and stored at -80°C until RNA extraction. Total RNA was isolated from the microdissected tissues using standard laboratory methods (TRIzol Reagent; Invitrogen, Carlsbad, Calif., USA). After DNase treatment to eliminate genomic DNA, oligo-(dT) selection (Invitrogen) was used to isolate poly(A)⁺ RNA, prior to first-strand synthesis utilizing a *NotI*-(dT)₁₈ oligonucleotide, followed by second-strand synthesis as described by D'Alessio et al. [1987]. The cDNAs were then blunt-end ligated to *EcoRI* adapters, digested with *NotI* and directionally cloned into a phagemid vector pT7T3-Pac. Bacteria were then transformed with the ligated nucleic acids, and plasmid DNA was prepared using commercially available kits (Qiaprep kit, Qiagen, Valencia, Calif., USA). Nonrecombinant molecules were eliminated from the library by running *NotI* linearized clones on an agarose gel, followed by purification with β -agarase and recircularization. The recircularized DNA was then transformed into bacteria, and plasmid DNA was prepared.

Normalization of the cDNA Library

The directionally cloned library (in single-stranded circular form) was primed with an oligo-(dT)₁₈ primer, and short complementary strands were formed on each circular template using controlled primer-extension reactions. This double-stranded DNA was then HAP (hydroxyapatite) purified to remove any remaining single-stranded circular DNA. The HAP-bound DNA was then melted and reassociated at a low C_0t ($\sim 5\text{--}20$ s mol/l). The remaining single-stranded fraction was purified from the material on the HAP column and converted to double-stranded circular DNA by primer extension. This normalized library was then transformed into bacteria and propagated under antibiotic selection. The resulting normalized vestibular cDNA library had a titer of 8×10^9 CFU/ml and an average insert size of 1–2 kb from the 3' end of the transcript.

Further assessment of quality of the normalized cDNA library was completed by PCR amplification of four rarely expressed transcripts: nicotinic acetylcholine receptor subunit $\alpha 9$ (*Chrna9*) and $\alpha 10$ (*Chrna10*), myosin 6 (*Myo6*) and myosin 7a (*Myo7a*). PCR primers and expected amplicon sizes were: *Chrna9*, TCTCTGGGAGTGACCATCCTAT (forward)/ATATTTTTCGCCAGTGCTTCAT (reverse), 476 bp; *Chrna10*, TCTCTCCCCAAAGTGCTGCTA (forward)/GCTGGAATTACCGTGCTCA (reverse), 203 bp; *Myo6*, ATACGCAGAACTTCGCGATACAA (forward)/TCTCTTCAAAGTGTCTGGGCAAG (reverse), 485 bp; *Myo7a*, CTGGTCACTATGACCCAGACC (forward)/CACCAGGTGTGGAGGGTACTTC (reverse), 624 bp. Negative control PCR experiments were conducted without template for all four amplification targets. The amplicon identity was confirmed by sequence analysis and/or restriction enzyme digestion with *RsaI*, followed by verification of the predicted fragment sizes.

cDNA Library Amplification, Clone Selection and Sequencing

The cDNA library was amplified by methods well described by Soares and Bonaldo [1998]. Briefly, library cDNA was used to transform *E. coli* DH10B electrocompetent cells (Invitrogen) via electroporation, and these cells were incubated using antibiotic selection conditions. The culture was used to prepare frozen 10% glycerol stock solution and stored at -80°C .

Library stock solution was used to inoculate LB_{ampicillin} media and was incubated overnight at 37°C . This solution was serially diluted, plated on LB_{ampicillin} media containing IPTG and X-gal, and incubated at 37°C overnight such that there were approximately 300–500 well isolated colonies per plate. To begin characterizing the normalized transcriptome contained in this rat vestibular cDNA library, 2400 colonies containing inserts were randomly picked and transferred to 96-well plates containing 200 μl of LB broth (100 $\mu\text{g}/\text{ml}$ ampicillin) in each well and incubated at 37°C overnight. 50 μl of a 50% glycerol solution was added to each well (final concentration 10% glycerol), and then 100 μl from each well was transferred to a duplicate 96-well plate. Both sets of plates (stock and duplicate) were sealed and stored at -80°C . The duplicate plates were sent to Seqwright (Houston, Tex., USA) for plasmid isolation and DNA sequencing.

In silico Analysis of the cDNA Library

Raw sequence chromatograms were imported into Chromas 2.23 (Technelysium Pty Ltd., Helensvale, Queensland, Australia) and trimmed according to the following parameters. Vector sequence was removed by identifying the 5' *EcoRI* adapter and 3' oligo-(dT) primer (which contains a tag unique to our library). 5' sequence ends were trimmed until 25 bases contained less than 5 ambiguities. 3' sequence ends were trimmed starting at position 100 and removing the first 25 bases containing greater than 6 ambiguities. In order to assess sequence quality, Phred analysis was performed (Seqwright). On average, 694 bases per sequence had Phred scores >20 , and 624 bases per sequence had Phred scores >30 . These scores correspond to sequencing error rates of <1 and $<0.1\%$, respectively. The insert sequences were then analyzed by comparison to the nonredundant Genbank database (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Nucleotide>) using the BLAST (basic local alignment search tool) algorithm. Sequences that did not align with any previously identified sequence with high homology ($>95\%$ over 100 nucleotides for rat alignments or $>80\%$ for other species over 100 nucleotides) or that aligned with sequenc-

es that have been previously identified but that did not have any informative annotations ascribed to them were further analyzed using four NCBI databases (<http://www.ncbi.nlm.nih.gov/Data-base/index.html>) including Unigene, Entrez Gene, Locuslink and OMIM. In addition, other databases were queried including the UCSC Genome Browser, the Mouse Genome Informatics mouse genome database (<http://www.informatics.jax.org/>) and the Rat Genome Database (<http://rgd.mcw.edu/>). UCSC Rat Genome Assembly v3.0 (June 2003), Unigene Build 138 (rat, November 30, 2004), Unigene Build 143 (mouse, November 28, 2004) and Unigene Build 177 (human, December 5, 2004) were used. Textword-based literature searches using the Pubmed database (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Pubmed>) were performed for clones whose aligned sequence had no functional annotations in any of the databases listed above. Sequences that had no significant alignments or matches in the Genbank nonredundant database were further analyzed with genome-specific BLAST searches within the rat genome data set to identify genomic, EST or trace EST sequences (<http://www.ncbi.nlm.nih.gov/genome/seq/RnBlast.html>) and the mouse genome data set to identify mouse EST sequences (<http://www.ncbi.nlm.nih.gov/genome/seq/MmBlast.html>). The remaining putative novel sequences were then subjected to a BLAT (blast-like alignment tool) alignment to the v3.0 Rat Genome Assembly (June 2003) to identify genome location as well as to determine its gene identity by comparison with non-rat mRNAs (<http://genome.cse.ucsc.edu>).

Classification of Sequences

Sequence alignments were initially analyzed with the FatiGO online data-mining tool (<http://fatigo.bioinfo.cnio.es/>) to identify level 5 biological process GO terms for each alignment in the mouse, rat and human databases and to group alignments with similar or identical GO terms. Alignments that did not have any biological process level 5 GO terms or that had level 4 biological process GO terms or below were manually annotated, and consensus regarding categorization was reached by three of the authors (J.A.C., P.P., P.A.W.) before final assignment was made. The Jackson Laboratory's web-based Visual Annotation Display analysis system (<http://proto.informatics.jax.org/prototypes/vlad/>) was used to allow graphical display of the identified genes based on GO terms. Because of the extremely high annotation density of the Mouse Genome Informatics genome database, this annotation set was utilized in the final analysis.

Results

Figure 1 summarizes the experiments verifying that the Wackym-Soares normalized rat vestibular periphery cDNA library contains known but rare transcripts. The expected amplicon sizes were obtained, and these were: 476 bp (*Chrna9*), 203 bp (*Chrna10*), 485 bp (*Myo6*) and 624 bp (*Myo7a*). The amplicon identities were confirmed by restriction enzyme digestion using *RsaI* and then verifying the predicted fragment sizes. For *Chrna9*, the restriction fragment sizes were 64, 198 and 214 bp. For *Chrna10*, the restriction fragment sizes were 49 and

Fig. 1. Products of PCR amplification with primers for nicotinic acetylcholine receptor $\alpha 9$ (*Chrna9*) and $\alpha 10$ (*Chrna10*) subunits, myosin 6 (*Myo6*) and myosin 7a (*Myo7a*) from the Wackym-Soares normalized rat vestibular periphery cDNA library. Lane 7 shows a 50- to 1000-bp ladder (Roche). Lanes 1 and 2 show the 476-bp $\alpha 9$ and 203-bp $\alpha 10$ nicotinic receptor subunit products of PCR amplification, respectively. Lanes 3 and 4 show the 485-bp myosin 6 and 624-bp myosin 7a products of PCR amplification, respectively. The bands shown in lanes 1–4 were confirmed by sequence analysis and/or restriction enzyme digestion using *RsaI* and subsequent verification of the predicted fragment sizes. Lanes 5 and 6 show negative control PCR experiments with no template which were conducted for $\alpha 9$ and $\alpha 10$, myosin 6 and myosin 7a, respectively.

154 bp. For *Myo6*, the restriction fragment sizes were 36, 174 and 275 bp. For *Myo7a*, the restriction fragment sizes were 17, 144, 171 and 292 bp. Sequence analyses of the amplicons also confirmed the authenticity of the expected products. The PCR control experiments with no template yielded no amplicons for the *Chrna9* and *Chrna10*, and *Myo6* and *Myo7a* primers.

As summarized in figure 2, of the initial 2400 clones sequenced, the data from 315 of the sequences (13%) were found to be of poor quality and did not yield useful information (e.g. sequencing errors or double priming) and were therefore eliminated from the analysis. Of the remaining 2085 sequences, 918 (44%) were found to represent 758 unique genes (Unigenes) having useful annotations that were identified in databases within the public domain or in the published literature; these sequences were designated as known characterized sequences. 1141 sequences (55%) aligned with 1011 Unigenes that have no useful annotations in the public domain and were des-

Fig. 2. Schematic diagram showing the sequence data distribution of 2400 randomly chosen clones obtained from a normalized rat vestibular cDNA library. cDNA sequences were analyzed using the BLAST against the Genbank nonredundant nucleotide database at NCBI (<http://www.ncbi.nih.gov/Genomes/>). The analysis algorithm schematically designated 'filter' represents the following methods. Sequences that did not align with any previously identified sequence with high homology (>95% over 100 nucleotides for rat alignments or >80% for other species over 100 nucleotides) or that aligned with sequences that have been previously identified but that did not have any informative annotations ascribed to them were further analyzed using four NCBI databases (<http://www.ncbi.nlm.nih.gov/Database/index.html>) including Unigene, Gene, Locuslink and OMIM. In addition, other databases were queried including the Mouse Genome Informatics mouse genome database (<http://www.informatics.jax.org/>) and the Rat Genome Database (<http://rgd.mcw.edu/>). Textword-based literature searches using the Pubmed database (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=Pubmed>) were performed for clones whose aligned sequence had no functional annotations in any of the databases listed above. Sequences that had no significant alignments or matches in the Genbank nonredundant database (potential new ESTs) were further analyzed with genome-specific BLAST searches within the rat genome data set to identify genomic, EST or trace EST sequences (<http://www.ncbi.nlm.nih.gov/genome/seq/RnBlast.html>), and the mouse genome data set to identify mouse EST sequences (<http://www.ncbi.nlm.nih.gov/genome/seq/MmBlast.html>). There was an average redundancy of 14%, and, consequently, 86% of the sequences represented singletons. The entire data set can be found in the Hearing and Balance Gene Expression Database, URL: <http://www.genexpression.info>.

Table 1. New ESTs

	Genbank Accession No.	dbEST	Sequence length bp	Longest open reading frame (A.A.)	Poly(A) signal	Poly(A) tail present	Predicted rat chromosomal location	Human conserved region; potential ortholog
1	CV681225	26255633	154	30	yes ¹	yes	no alignment	not applicable
2	CV681228	26255636	720	44	yes ²	yes	Chr14: 19713939-19714658	Chr4: 73892553-73892695
3	CV681232	26255640	673	57	yes ²	yes	Chr2: 166633531-166634199	Chr3: 169040075-169040659
4	CV681235	26255643	565	64	yes ¹	yes	Chr16: 30817779-30818343	Chr4: 169721500-169721966; 3' region of FLJ20035
5	CV681236	26255644	1174	116	no	yes	Chr12: 3343531-3344687	Chr13: 33054263-33055360
6	CV681238	26255646	572	11	no	yes	Chr2: 139043560-139044019	Chr4: 139364520-139365035
7	CV681240	26255648	467	43	yes ²	no	no alignment	not applicable
8	CV681241	26255649	514	112	yes ³	yes	Chr4: 159534008-159534522	Chr12: 8082080-8082309
9	CV681242	26255650	818	84	yes ³	yes	Chr11: 63829867-63846964	Chr3: 120211963-120227885; β-1,4-galactosyltransferase 4
10	CV681243	26255651	549	142	no	yes	Chr8: 113884663-113885211	Chr3: 48539971-48540642; NCKIPSD
11	CV681244	26255652	647	87	yes ²	yes	Chr8: 73977969-74029670	Chr15: 58098592-58171076
12	CV681246	26255654	1034	135	no	yes	Chr6: 75508516-75509535	Chr14: 33512508-33512902; C14orf24
13	CV681247	26255655	512	99	yes ²	yes	Chr8: 28567268-28567770	Chr11; gap
14	CV681249	26255657	648	42	no	yes	Chr4: 62768610-62769212	Chr7: 134505929-134507295; CNOT4
15	CV681250	26255658	1075	84	yes ¹	yes	Chr15: 66436316-66437188	Chr13; gap
16	CV681251	26255659	727	108	yes ²	yes	Chr20: 30486-31213	not applicable
17	CV681252	26255660	672	55	no	yes	Chr2: 97729065-97729736	Chr8: 78129905-78130250
18	CV681253	26255661	705	65	yes ²	yes	Chr15: 99622307-99623011	Chr13: 90539700-90540161
19	CV681257	26255665	624	65	yes ²	yes	Chr1: 23643610-23644228	Chr6; gap
20	CV681258	26255666	329	62	yes ²	yes	Chr2: 59030408-59030723	Chr5; gap
21	CV681259	26255667	545	45	no	yes	Chr8: 75075651-75076192	Chr15: 56971247-56971785; intron of FLJ13213
22	CV681260	26255668	397	99	yes ¹	yes	Chr6: 129775298-129775692	Chr14: 94737755-94738247
23	CV681262	26255670	437	44	yes ²	yes	Chr5: 137734765-137735167	Chr1; gap
24	CV681264	26255672	888	110	yes ²	yes	Chr2: 172392819-172393570	Chr4: 158468625-158469463
25	CV681267	26255675	1304	120	yes ²	yes	Chr7: 67788587-67789585	Chr12; gap
26	CV681270	26255678	470	63	yes ²	yes	Chr1: 41864236-41864698	Chr6: 160070566-160071064; cDNA FLJ36665

A.A. = Amino acid.

¹ ATTAAA (noncanonical).² AATAAA (canonical).³ AGTAAA (noncanonical).

ignated as known but uncharacterized sequences. The remaining 26 sequences (1%) did not correctly correspond to any previously identified rat EST (table 1). Predicted rat chromosomal location and corresponding human ortholog location were identified in 24 of the 26 new ESTs. Of the 2 ESTs which we were unable to place in the context of the rat genome, one (Genbank accession CV681240) lacked a poly(A) tail and therefore may represent a cloning artifact, while the other (Genbank accession CV681225) had a noncanonical poly(A) signal and a poly(A) tail [Beaudoing and Gautheret, 2001]. Both of these ESTs had very short predicted open reading frames, 43 and 30 respectively. None of the new ESTs corresponded to human chromosomal locations currently known to be associated with deafness loci. There was an average redundancy of 14%, and, consequently, 86% of

the sequences represented singletons. The known, characterized sequences were analyzed with the FatiGO online data-mining tool (<http://fatigo.bioinfo.cnio.es/>) to identify level 5 biological process GO terms for each alignment and to group alignments with similar or identical GO terms into groups. Several of these groups were combined to form six categories, all summarized in sections within table 2 (see online supplemental material): (1) synaptic mechanisms; (2) cell surface linked signal transduction; (3) intracellular signaling cascade; (4) transport; (5) RNA transcription and metabolism, and (6) cell proliferation, neurogenesis, cell growth and programmed cell death. Genes with biological process GO terms at level 4 and lower, and genes without biological process GO terms, were annotated and assigned by the authors into these six categories.

Table 1 summarizes the dbEST (database for expressed sequence tags) number, Genbank accession number, sequence length and presence of a poly(A) tail, longest predicted open reading frame, predicted rat chromosomal location, human conserved region and potential ortholog for each of these 26 new ESTs. The Genbank accession numbers, associated dbEST numbers and other details regarding the entire data set can be found in the rat vestibular periphery cDNA library section of the Hearing and Balance Gene Expression Database [Wackym, 2005].

Table 3 (see online supplemental material) summarizes the description and number of unique clones belonging to the same Unigene cluster. For the known annotated function group, there were 918 sequences that corresponded to 758 Unigene clusters. Individual Genbank accession numbers assigned to each clone can be found within the rat vestibular cDNA library section in the Hearing and Balance Gene Expression Database [Wackym, 2005]. There were no duplicate clones; however, 288 sequences matched 128 Unigene clusters: 108 had only 2 matches per cluster; 14 had 3 matches per cluster; 3 had 5 matches per Unigene cluster; 2 had 4 matches per Unigene cluster, and 1 had 7 matches per Unigene cluster. For the unknown function group, there were 1141 sequences that corresponded to 1011 Unigene clusters. Individual Genbank accession numbers assigned to each clone can be found within the rat vestibular periphery cDNA library section within the Hearing and Balance Gene Expression Database [Wackym, 2005]. There were no duplicate clones; however, 228 sequences matched 100 Unigene clusters: 85 had only 2 matches per cluster; 12 had 3 matches per cluster; 1 had 4 matches per Unigene cluster; 1 had 5 matches per Unigene cluster, and 1 had 13 matches per Unigene cluster. By definition, the 26 new EST sequences did not match Unigene clusters with the currently available Unigene builds.

The Visual Annotation Display analysis provided a graphical summary of the identified genes based on GO terms. The analysis and graphical display of molecular function and cellular component was also completed (data not shown). The genes corresponding to each GO term used in this analysis can be found in the Hearing and Balance Gene Expression Database within the rat vestibular periphery cDNA library section [Wackym, 2005].

Discussion

The experiments designed to determine if known but rare transcripts were represented in the library, together with a relatively large insert size and the robust library titer all demonstrate that the Wackym-Soares normalized rat vestibular periphery cDNA library is of high quality. Moreover, the data presented in table 3 and the detailed data set found in the Hearing and Balance Gene Expression Database [Wackym, 2005] show that multiple Genbank accession numbers are associated with each Unigene cluster. The 86% rate of singletons further reflects the quality of the normalization process.

While challenging to present the large volume of data resulting from this study, we have reported the 26 new ESTs (table 1), the gene names and descriptions within six main categories which are relevant to the function and regeneration of the vestibular periphery (table 2), and all of the associated data have been placed in the Hearing and Balance Gene Expression Database [Wackym, 2005]. In addition, figure 2 shows the relative distribution of genes identified by sequence analysis and associated Unigene representation. It is also important to recognize that transcriptome analysis of a normalized cDNA library will not determine the relative abundance of expressed genes, and that we have sampled only 2085 clones. Consequently, many other genes relevant to the unique function of the vestibular periphery have not yet been identified in this study, but may be present in our library. We have sequenced an additional 20000 clones and are now beginning the Genbank submission process. We then plan to undertake the sequence analysis and annotation. It is also fair to state that the rat genome sequence is far from being completely sequenced or annotated. Therefore, we must rely on currently available information as we await verified functional information on the 35000 or so genes in the rat genome. Certainly, additional information will evolve as the genome is annotated but does not negate the initial annotation. Indeed, GO trees are in constant flux and change each day. GO based upon domain homology or cross-species homology is a reasonable first step; however, even a gene defined in another species may well have a modified function in another. We can only know this when each gene's function is experimentally characterized. With this study we have taken a snapshot of the information at this time, and it must be acknowledged that this data set will evolve. Despite this limitation, the data reported herein are relevant and potentially important to our research community.

There were several genes identified that potentially have high clinical relevance. These genes and the associated or possibly associated disease included: natriuretic peptide receptor 3 (*Npr3*; Ménière's disease), *Ush3a* also known as clarin 1 (Usher syndrome type III), potassium voltage-gated channel, Isk-related subfamily, member 1 [*Kcne1*; Jervell and Lange-Nielson syndrome (JLNS2)], thyroid-hormone-receptor-associated protein 2 (*THRAP2*; Noonan syndrome) and estrogen-related receptor γ (*Esrrg*; age-related hearing loss in women and acoustic neuroma biology). Because mutations in protocadherin 15 (*PCDH15*; DFNB23), 6 different myosins (*MYO1A*, *MYO3A*, *MYO6*, *MYO7A*, *MYO15*, *MYH9*; DFNA48, DFNB30, DFNB37, DFNA22, DFNB2, DFNA11, DFNB3, DFNA17), 2 different transcription factors (*POU4F3*, *POU3F4*; DFNA15, DFN3) and a solute carrier family 26, member 4 gene (*SLC26A4*; Pendred syndrome) cause deafness [Van Camp and Smith, 2004], and very similar genes were found in our vestibular cDNA library (*Pcdh13*, *Myo5a*, *Myr8*, *Pou4f2*, *Slc3a1*, *Slc4a4*, *Slc7a1*, *Slc9a6*, *Slc12a12*, *Slc12a5*, *Slc12a7*, *Slc13a3*, *Slc16a1*, *Slc22a6*), it is intriguing to speculate whether mutations in any of these genes would produce vestibular dysfunction.

The expression of *Npr3* in the vestibular periphery suggests the possibility that natriuretic peptides play a role in regional fluid homeostasis within the inner ear. High-affinity atrial-natriuretic-peptide-binding sites have been identified in the guinea pig inner ear [Meyer zum Gottesberge and Lamprecht, 1989], and upregulation of all 3 *Npr* genes has been demonstrated in the rat inner ear after round window injection of atrial natriuretic peptide [Dornhoffer et al., 2002]. Natriuretic peptides represent an important candidate group for the development of therapeutic agents in the management of hypertension [Vanderheyden et al., 2004], which suggests that a similar role may be developed in the pharmacological management of endolymphatic hydrops. The mechanisms of regulation of endolymphatic fluid volume are emerging [Salt, 2001]; however, the fundamental pathology responsible for Ménière's disease and the histological correlate of endolymphatic hydrops remains unknown [Wackym and Sando, 1997].

The autosomal recessive disease Usher syndrome type III is characterized by sensorineural deafness and progressive retinitis pigmentosa, with vestibular dysfunction [Ahmed et al., 2003]. Thus, the presence of *Ush3a*, also known as clarin 1, in our vestibular cDNA library is of clinical interest. It is known that *Ush3a* is expressed in the inner and outer hair cells of the organ of Corti and the

spiral ganglion cells of the cochlea [Adato et al., 2002]; however, the pattern of expression of this gene in the vestibular periphery has not been explored to date. Although the function of clarin 1 remains unknown, Adato et al. [2002] have speculated that clarin 1 (*Ush3a*) plays a role in hair cell synapses due to the sequence homology to the AMPA-receptor-interacting protein stargazin [Cuadra et al., 2004]. Ultrastructural localization of epitopes of this protein would serve to clarify this issue.

Jervell and Lange-Nielson syndrome is characterized by the phenotype of profound congenital sensorineural hearing loss associated with syncopal episodes which are caused by ventricular arrhythmias [Tyson et al., 1997]. Although the phenotypes are indistinguishable, there are two genetic defects responsible for this syndromic disorder. One form (JLNS2) has a mutation in a delayed rectifier potassium channel that corresponds to the potassium voltage-gated channel, Isk-related subfamily, member 1 (*Kcne1*) gene which we identified in our cDNA library. Although vestibular function has not been studied in these patients and that vestibular dysfunction has not been described, based on the presence of *Kcne1* in the vestibular periphery, it is likely that patients with JLNS2 also have vestibular dysfunction. If the gene responsible for JLNS1 (*Kvlqt1*) is not expressed in the vestibular periphery, it may be possible to distinguish JLNS1 from JLNS2 by vestibular function testing. Additional basic and clinical studies are needed to test this hypothesis, including vestibular testing to determine if vestibular dysfunction also defines the phenotype of this disorder. Although less well studied in patients with long QT syndrome (JLNS) another gene implicated in this disorder is ankyrin 2 (*Ank2*) [Haack et al., 2004], which was also identified in our library.

There are a wide range of conditions that result in hypothyroidism and consequently cause hearing loss and vestibular dysfunction. There were 2 thyroid-related genes identified in this initial library analysis. The first is thyroid-hormone-receptor-associated protein 2 (*Thrap2*) which has been implicated in Noonan syndrome [Musante et al., 2004]. Noonan syndrome is characterized by sensorineural and conductive hearing loss as well as vestibular dysfunction. The second is thyroid hormone receptor interactor 12 (*TRIP12*). While no pathologies have been associated with mutations of this gene, the presence of this gene implies functional significance in the vestibular periphery, and this gene may play a role in hypothyroidism-mediated vestibular dysfunction. Another disorder that results in hypothyroidism, hearing loss and vestibular dysfunction is Pendred syndrome,

which is an autosomal recessive genetic disorder characterized by severe to profound congenital hearing loss, abnormalities of the bony labyrinth, particularly a wide vestibular aqueduct, goiter and impaired vestibular function as a result of compensated hypothyroidism [Campbell et al., 2001]. Pendred syndrome is caused by a mutation in a gene encoding a solute carrier family 26, member 4 protein (*SLC26A4*); however, there are 2 thyroid-related genes identified in our transcriptome analysis that could potentially play a role in inner ear dysfunction. In addition, while *Slc26a4* was not found in the transcriptome sample that we report herein, it should be noted that 9 solute carrier family genes were identified (table 2).

While sexual dimorphism has not been demonstrated in the auditory or vestibular systems, estrogen-deficient individuals with Turner syndrome experience hearing loss [Stenberg et al., 2003]. In addition, Monsell and Wiet [1990] reported that 19% of acoustic neuromas (vestibular schwannomas) express estrogen receptors and that there was no difference in estrogen receptor expression between men and women. Our cDNA library contains estrogen-related receptor γ (*Esrrg*) which is an orphan nuclear receptor that shares significant amino acid identity with the estrogen receptors and plays a role in constitutive transcriptional activity [Huppunen et al., 2004].

There were a number of interesting genes associated with synaptic mechanisms and transport (table 2). Since glutamate is the hair cell neurotransmitter, it was not unexpected that a number of glutamate receptors were identified. There were however a number of interesting genes integral to synaptic function. These included: agrin (*Agrn*) which is associated with the aggregation of nicotinic acetylcholine receptor subunits, synaptosomal-associated protein (*SNAP25*), one of the SNARE proteins concentrated at both poles of auditory hair cells [Safieddine et al., 2002], collagen-like tail subunit of asymmetric acetylcholinesterase (*Colq*) which is important to efferent cholinergic synapses, clarin 1 (*Ush3a*) whose function has been discussed above, early endosome antigen (*Eea1*) which is associated with internalization of G-protein-coupled receptors, vesicle-associated membrane protein 4 (*Vamp4*) and the unconventional *Myo5a*. Immunohistochemistry has been used to localize myosin V epitopes in the afferent calyces surrounding vestibular type I hair cells of the guinea pig, and *Myo5a* has been implicated in the trafficking of intracellular vesicles in neurons and other secretory cells [Coling et al., 1997].

Other interesting genes associated with transport include: the large number of potassium channels (*Kcna6*,

Kcnc2, *Kcnh1*, *Kcne1*, *Kcnj13*, *Kctd15*), genes associated with lipid rafts (*Cav2*, *Edg1*) [Marchand et al., 2002], sorting nexins (*Snx1*, *Snx4*, *Snx9*, *Snx10*, *Snx15*), calcium channels (*Cacna1a*, *Cacna1d*, *Ecac1*), chloride channels (*Clcn2*, *Clns1a*) and solute carrier transcripts (*Slc3a1*, *Slc4a4*, *Slc7a1*, *Slc9a6*, *Slc12a12*, *Slc12a5*, *Slc12a7*, *Slc13a3*, *Slc16a1*, *Slc22a6*). All 5 of the potassium channels identified are voltage-gated channels and therefore are likely to be associated with membrane potential function; however, because *Kcne1* can play a role in renal electrolyte exchange, it is possible that this gene plays a role with dark cell K^+ recirculation [Peters et al., 2004]. Because of the biological function of each of these genes in other systems, further investigation of their role in the vestibular periphery is warranted.

Within this sample of the rat vestibular periphery transcriptome there were relatively large numbers of genes associated with G proteins and Ca^{2+} metabolism (table 2). Because of our work in signal transduction, the G-protein-related genes were of particular interest. In earlier studies we have identified a number of the G α -subunits in the vestibular periphery [Cioffi et al., 2003; Cioffi et al., 2005; Wackym et al., 2005] including some novel alternatively spliced transcripts. The ascertainment of β - and γ -subunits (*Gnb1*, *Gng12*), α -subunits (*Gnai1*, *Gna13*), regulatory proteins (*Rgs7*) and other G-protein-coupled receptors (*Gpr81*, *Gpr88*, *Gpr126*) leads to the question of functional significance which can only be answered after anatomical localization, pharmacological and physiological studies have been completed. There were also a large number of genes associated with Ca^{2+} metabolism. One important gene identified is calbindin 2 (*Calb2*), also known as calretinin. Calretinin immunohistochemistry has demonstrated that this protein is found predominantly in the caliceal vestibular primary afferent dendrites within the center of the crista ampullaris, although in the rat calretinin also labels approximately 5% of type I hair cells and 20% of type II hair cells [Desai et al., 2005]. Within the primary afferent neurons, the exclusive expression of calretinin within the caliceal units suggests that it plays an important role in their function. Other transcripts associated with Ca^{2+} regulation included: calcium-binding protein 39 (*Cap39*), calcium-binding protein p22 (*Chp*), calcium-modulating ligand (*Camlg*), calcium/calmodulin-dependent protein kinase II γ (*Camk2g*), centrin 2 (*Cetn2*), annexin A7 (*Anxa7*), striatin and striatin 3 (*Strn*, *Strn3*) and SPARC-related modular calcium-binding 1 (*Smoc1*).

Another important group of identified genes are those associated with transcription, cell proliferation, neuro-

genesis, cell growth and programmed cell death (table 2). Although the majority of effort of other groups interested in these processes has been focused on auditory hair cell regeneration, vestibular hair cell recovery and regeneration remain an active area of investigation. In addition, there is evidence that vestibular hair cell regeneration is clinically relevant [Lambert, 1994; De Waele et al., 2002]. The majority of experimental paradigms have utilized an injury-regeneration model [Kevetter et al., 2000; Matsui et al., 2000; Boyle et al., 2002; Gale et al., 2002], and evidence of functional recovery has been presented [Boyle et al., 2002]. For damaged, but surviving hair cells, stereocilia repair has been shown, revealing an example of intracellular self-repair in the absence of mitosis [Gale et al., 2002]. The basic mechanism of stereocilia functional architecture and self-renewal has been described recently [Rzadzinska et al., 2004].

There were several genes identified in our study that have been implicated in vestibular hair cell regeneration. Kim et al. [2004] have recently demonstrated that β -catenin may play a role in the induction of cell proliferation in the vestibular epithelium of the rat. β -Catenin is known to play an important role in both cell-cell adhesion and signal transduction associated with cell proliferation. E-cadherin has been reported to be expressed in supporting cells and downregulated in differentiating hair cells, and that constitutive expression in a conditionally immortal utricular macula cell line inhibits the progression of hair cell differentiation [Hackett et al., 2002]. Protocadherin $\alpha 13$ (*Pcdha13*) is an associated gene that was identified in our library. These genes are expressed at synaptic junctions and may be involved in the determination of synaptic density [Noonan et al., 2003]. Fibroblast growth factor 2 (*Fgf2*) has been shown to be expressed in vestibular hair cells and upregulated in dissociated supporting cells suggesting that *Fgf2* may play a role in regulating the proliferation of supporting cells [Cristobal et al., 2002]. While *Fgf2* was not found in the sampling of the rat vestibular periphery transcriptome reported herein, we did find the expression of *Fgf10* and *Fgf12*.

When considering other identified genes belonging to the cell proliferation category, there are several that may prove to play an important role in vestibular epithelium proliferation.

The final group of genes with known identity and function will be discussed in the context of vestibular schwannoma (acoustic neuroma). Acoustic neuromas arise from the vestibular nerve, affect approximately 1.5% of the population and arise as a result of a spontaneous or inherited

mutation of the gene encoding schwannomin found on chromosome 22. Despite this relatively high prevalence, little is known about the basic mechanisms of tumor biology that define the wide array of phenotypes; however, recent applications of cDNA library analysis, microarray expression profiling and serial analysis of gene expression have been completed [Halum et al., 2003, 2004; Welling et al., 2002]. In the present study there were 8 genes or groups of related genes that were of particular interest (table 2). These include: v-*maf* musculoaponeurotic fibrosarcoma oncogene family protein B (*Mafb*), members of the Ras oncogene family (*Rab1*, *Rab7*, *Rab12*, *Rab21*, *Rab33b*, *Rasa1*, *Ralgds*, *Rheb*, *Rab2c*, *Ran*, *Rhot1*, *Rangap1*, *Rab2b*, *Rap1ga1*), ski-like which is a unique proto-oncoprotein (*Skil*), CGI-121 protein which is a novel p53-related protein-kinase-binding protein (*Cgi-121*), forkhead box 5 which is a candidate tumor suppressor gene (*Foxn5*), Ahi-1 which is closely linked to the *c-myc* proto-oncogene and may be involved in tumor development (*Ahi1*), nibrin which is associated with chromosomal instability and radiation sensitivity plus tumor development (*Nbn*) and vascular endothelial growth factor A (*Vegfa*). *Mafb* is associated with the basic leucine zipper transcription factor *c-maf*, and we have found *c-maf* in a cDNA library that we constructed from an acoustic neuroma resected from a patient with neurofibromatosis type 2 (unpubl. data, Genbank AF540388). This particular gene has also been shown to be expressed in lens and spinal cord, both areas that develop characteristic diseases in neurofibromatosis type 2 – cataracts and spinal cord tumors [Sakai et al., 1997]. The final particularly intriguing gene is nibrin. The human gene *NBS1* encodes the protein nibrin which when defective is associated with chromosomal instability, radiation sensitivity and tumor development [Demuth et al., 2004]. The expression of this gene in the vestibular periphery has clinical relevance because of the concern regarding radiation-induced malignancy in patients with acoustic neuromas who elect to undergo radiation as their treatment option [Wackym et al., 2004].

Of the remaining 26 sequences (1%), 24 aligned with rat genomic sequences but none matched previously described rat ESTs or mRNAs. No significant alignment to the rat or human genomic sequences could be found for the remaining 2 sequences. Of these 26 ESTs, 13 contained the canonical poly(A) signal while 6 contained noncanonical poly(A) signals [Beaudoing and Gautheret, 2001]. We were unable to identify any type of poly(A) signal in 7 of these new ESTs. The possibility exists that these may represent cloning artifacts although 3 sequenc-

es (CV681243, CV681249, CV681259) map to locations of potential human orthologs. Since some of these new ESTs have the potential to represent novel vestibular-specific genes or at least vestibular-relevant genes, it is important to pursue this direction of investigation. Therefore, we are in the process of testing and screening these new ESTs using RT-PCR to determine expression in the rat within major organs and tissues including the brain, liver, lung, kidney, heart and skeletal muscle. It is also necessary to determine the cellular location of at least a subset of these 26 unknown genes within the vestibular periphery in order to ascertain which genes are expressed by vestibular hair cells, ganglion cells and supporting cells. One approach would be to use *in situ* hybridization to anatomically define expression patterns; however, we have found this approach to be less sensitive in the vestibular end organs than other molecular approaches. Consequently, we are now approaching this problem with a combination of laser capture microdissection, RT-PCR and microarray expression profiling [Cristobal et al., 2005] using a custom array containing these 26 unknown as well as control sequences. In addition, we are further characterizing these potentially novel transcripts in order to obtain full-length cDNAs.

Future studies will include determination of rat chromosomal locations and predicted human ortholog loci for useful sequences resulting from the analysis of the 22400 clones that we have sequenced to date, as well as detailed annotation. Comparison to known deafness loci will then be completed which may yield potential candidate genes. However, it should be noted that very little information and objective vestibular testing is available for patients with syndromic or nonsyndromic hearing loss. Because very few deaf patients undergo vestibular testing there are gaps in our knowledge regarding this. Consequently, it would be useful to better define the phenotypes of these deaf individuals via collection of vestibular histories, vestibular system physical examinations and objective vestibular testing.

Finally, it is acknowledged that our normalized vestibular periphery cDNA library was constructed from vestibular end organs and ganglia, therefore cell types other than hair cells and primary afferent ganglion cells were also included. Dark cells, supporting cells, endothelial cells, Schwann cells and subepithelial fibroblasts are all represented in this library. Consequently, cellular localization of specific genes is necessary to better understand the context of this transcriptome.

Conclusions

Initial *in silico* analysis of the rat vestibular periphery transcriptome revealed numerous genes that have not been previously shown to be expressed in the vestibular system. Further characterization of the new ESTs identified may lead to the identification of genes with vestibular relevant functions. Continued analysis of the rat vestibular periphery transcriptome could provide new insights into vestibular function and generate new hypotheses. Physiological studies are necessary to further elucidate the roles of the identified genes and novel sequences in vestibular function. The comprehensive analysis of the vestibular periphery transcriptome should provide further insight into normal vestibular function and the mechanisms responsible for specific vestibular diseases.

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Supplemental Table Files

Table 2. Genes identified in the Wackym-Soares normalized rat vestibular periphery cDNA library with Gene Ontology terms and organized by function. Genes with Gene Ontology terms which can be categorized into the subheadings included are found in this paper. The entire expression database for the Wackym-Soares normalized rat vestibular periphery cDNA library can be found in the Hearing and Balance Research Database (URL: <http://www.genexpression.info>).

Table 3. Description and number of unique clones belonging to the same Unigene cluster.

For further information please refer to <http://www.karger.com/doi/10.1159/000087348>.

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