A Subtracted cDNA Library from the Zebrafish (*Danio rerio*) Embryonic Inner Ear

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A database was built that consists of 4694 sequence contigs of ~18,000 reads of cDNAs isolated from the microdissected otocysts of zebrafish embryos at 20–30 hour postfertilization, following subtraction with a pool of liver cDNAs from adult fish. These sequences were compared with those of public databanks. Significant similarity were recorded and organized in a relational database at http://www.genoscope.cns.fr/zie. A first group of 2067 sequences correspond to 1428 known zebrafish genes or ESTs present in the *Danio rerio* section of UniGene. A second group of 302 sequences encode putative proteins that showed significant similarity (50%–100%) with 302 nonzebrafish proteins in the nr databank, a public databank containing an exhaustive nonredundant collection of protein sequences from different species (ftp://ftp.ncbi.nlm.nih.gov/blast/db/nr). The remaining 2325 (49.5%) sequence contigs or singletons showed no significant similarity with sequences available in public databanks. Several genes known to be expressed in the developing inner ear were represented in the present database, in particular genes involved in hair cell differentiation or innervation The occurrence of these genes validates the outcome of this study as the first collection of ESTs preferentially expressed in the zebrafish inner ear during the period of hair cell differentiation and neuroblast delamination from the otic vesicle epithelium. Novel zebrafish genes also involved in these processes are thus likely to be represented among the sequences obtained herein, for which no homology was found in the *D. rerio* section of UniGene.

[The sequence data from this study have been submitted to EMBL under accession nos. AL714032–AL731531].

The establishment of the structure of the inner ear is under the influence of genes controlling complex networks of molecular interactions. Although several genes implicated in inner ear development have been identified in recent years (for review, see Torres and Giráldez 1998), the puzzle is still far from being assembled. Mouse mutants with behavioral abnormalities have been a great aid in the identification and isolation of a large number of genes expressed in the developing ear (Deol 1968, 1970, 1980; Steel 1995). However, in recent years, zebrafish (*Danio rerio*), an aquarium fish originating from the rivers of tropical India, has become a favorite animal model due to a rare combination of attractive features, including large progenies, external fertilization and embryonic development, embryo transparency, can obtain haploid and homozygous diploid individuals by gynogenesis (Streisinger et al. 1981, 1986; Streisinger 1984), and so on.

A zebrafish genome-sequencing project has just begun at the Sanger Centre. Genetic linkage maps now cover the entire zebrafish genome (Knapik et al. 1998; Postlethwait et al. 1998; Gates et al. 1999; Shimoda et al. 1999). In addition, two independent physical maps made from zebrafish–rodent radiation hybrid lines (LN54 and T51) are available, which together cover >90% of the zebrafish genome (Geisler et al. 1999; Hukriede et al. 1999).

Concomitantly, large-scale screenings for embryogenesis defects in zebrafish mutants have been carried out, which have permitted the identification of mutants with defects of the ear development. Twenty of these mutants, defining 13 independent *loci,* have been phenotypically and genetically characterized by Malicki et al. (1996). In an independent study, 95 mutants (39 genes) showing defects in inner ear development have been reported by Whitfield et al. (1996) among zebrafish mutants previously identified through a large-scale mutagenesis screening (Haffter et al. 1996). Another large-scale screening in zebrafish was announced by Amsterdam et al. (1999). The strategy of insertional mutagenesis proposed by this group is expected to accelerate the isolation of the disrupted genes.

Despite some differences in the ear structure of otophysan compared to mammals (otophysan species, including zebrafish, have no middle or outer ear structure and they possess two sound-sensitive maculae in the inner ear instead of the mammalian organ of Corti), the hair cells, the sensory cells that detect sound waves and acceleration are remarkably conserved (Platt 1993; Popper and Fay 1993). The screening of zebrafish mutants for balance phenotypes combined with a candidate gene cloning approach is a potentially powerful strategy for revealing some of the genes that play a crucial role in the developing and functioning of hair cells. However, so

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far, only the myosin VIIA gene, which is defective in *mariner* zebrafish mutants, has been isolated (Ernest et al. 2000) on the basis of the involvement of its orthologs in balance defects and/or deafness in mice (Gibson et al. 1995) and humans (Weil et al. 1995). Improvement of the candidate gene approach requires a more extensive characterization of the genes expressed in the inner ear.

This study is the first large-scale sequencing project aimed at gaining information on gene expression in the inner ear of developing zebrafish. The inner ear of zebrafish and other vertebrates differentiates from the otic placode, a dorsolateral thickening of the surface ectoderm, adjacent to the rhombencephalon (Waterman and Bell 1984; Platt 1993; Haddon and Lewis 1996). In the zebrafish, the otic placode can be identified at 16 hr postfertilization (hpf). The placode gives rise to the otic vesicle (at 18 hpf), from which neuroblasts, the precursors of sensory neurons, will later delaminate to form the vestibuloacoustic ganglion. The peak of neuroblast delamination is between 22 and 30 hpf. At ~24 hpf, the first hair cells differentiate within two sensory maculae. The three semicircular canals form between 43 and 72 hpf from the walls of the otocyst, and three additional clusters of hair cells soon become visible in the sensory cristae of these canals. Thus, by the end of the first week, all key structures of the ear are present, but before the ear is completely mature, thousands more hair cells and neurons will be produced by subsequent division of precursors (Waterman and Bell 1984; Haddon and Lewis 1996). Specifically, we focused this study on the 20–30 hpf stage, corresponding to the period of differentiation of the first hair cells and to the peak of neuroblast delamination (Waterman and Bell 1984; Haddon and Lewis 1996). The strategy of cDNA library subtraction was chosen because it has already been successful in identifying genes preferentially or specifically expressed in the human, mouse, and chicken auditory sensory organ (Robertson et al. 1994, 1998; Cohen-Salmon et al. 1997; Heller et al. 1998; Yasunaga et al. 1999; Simmler et al. 2000; Verpy et al. 2000, 2001). A pool of cDNAs from the liver was used as driver to minimize representation of housekeeping genes in the subtracted library.

RESULTS AND DISCUSSION

After trimming and assembling (see Methods) the 17,468 individual reads of >100-bp long, the final database contained 4694 contigs and singletons averaging 530 bp. Forty-four percent (2067/4694) of these sequences matched at least one *D. rerio* sequence cluster in the UniGene databank (Table 1).

Table 1. Analysis of Clone Sequences

	***n* (%)**
Total reads generated from independent clones	21848
Reads selected for further study	17468
Total of contigs and singletons after clustering	4694 (100%)
Sequences matching previously described *Dario rerio* sequences	2067 (44%)
Sequences matching proteins in nonredundant	302 (6.5%)
Sequences with no match	2325 (49.5%)

A total of 1428 *D. rerio* UniGene clusters were significantly similar to 2067 sequences in our database. Sixty-one percent (880/1428) corresponded to *D. rerio* known genes or to *D. rerio* ESTs previously classified as highly, moderately, or weakly similar to known (or predicted) genes in other organisms. A number of these genes are known to be expressed in the developing inner ear of zebrafish or other vertebrates during the early stage of hair cell differentiation (Holme et al. 2002). These include the genes encoding the transcription factors msxc, pax2, eya1, dlx2, and genes encoding signaling molecules that drive hair cell fate determination and proliferation (deltaA, deltaB, and deltaD, Lunatic fringe, and retinoic acid receptor gamma). The gene encoding the transcription factor pax2 is expressed early in the otic placode, and its expression is maintained in a restricted region of the otic vesicle. Whereas *eya1*, encoding a transcription coactivator underlying the branchio-oto-renal syndrome (Abdelhak et al. 1997; Kalatzis et al. 1998), and *pax2* are both expressed in the ventro-medial wall of the otic vesicle (i.e., the presumptive area of the sensory epithelia; Rinkwitz-Brandt et al. 1996; Sahly et al. 1999), the homeobox-containing gene *msxc* is expressed in the dorso-lateral aspect of the vesicle. *Msxc* continues to be expressed during the differentiation period, and eya1 persists even after differentiation has taken place (Ekker et al. 1992; Sahly et al. 1999). Finally, *dlx2* is expressed in the sensory patches during the period of hair cell differentiation (Robinson and Mahon 1994). Several different families of signaling molecules have been shown to be involved in the generation and maintenance of the complex cell architecture patterns of hair cells and supporting cells in the sensory epithelia. The notch-signaling pathway regulates sensory cell commitment in the vertebrate inner ear. In zebrafish, the *delta* homologs *deltaA*, *deltaB*, and *deltaD*, coding for ligands of the notch receptor, are expressed in nascent neurons and probably also in nascent hair cells (Dornseifer et al. 1997; Haddon et al. 1998). According to the lateral inhibition hypothesis (Corwin et al. 1991; Lewis 1991), the nascent hair cells, by expressing the delta protein, would inhibit their neighbors from becoming hair cells, "forcing" them to be supporting cells instead (Haddon et al. 1998). Lunatic fringe (encoded by *Lnfg*) is another component of the notch pathway, which seems to play a role in the commitment of the inner hair cell precursors. Unlike *delta*, *Lfng* is expressed in nonsensory supporting cells of the mouse cochlea (Zhang et al. 2000). Retinoic acid and retinoid receptors also seem to play a role in early developmental events including hair cell proliferation and commitment (Represa et al. 1990; Raz and Kelley 1997; Pasqualetti et al. 2001). Previously known zebrafish genes playing a role in inner ear innervation were also found among the sequences of our database, including *BETA2/NeuroD1*, which encodes *NeuroD1*, a transcription factor expressed in both sensory ganglia and sensory epithelia of the developing inner ear. It is implicated in neuroblast delamination from the otic vesicle epithelium. *BETA2/NeuroD1* was the first gene shown to regulate neuronal and sensory cell differentiation in both the auditory and vestibular systems (Liu et al. 2000). The aforementioned genes validate the present database as a valuable tool to decipher the molecular basis of inner ear development. The remaining 548 *D. rerio* UniGene clusters represented in this database were made of ESTs not related to any known gene or predicted protein. Putative human orthologs were identified for 919 of the 1428 *D. rerio* genes or ESTs. The positions of 820 (89%) of these human orthologs on the GoldenPath draft of the human genome were determined; 177 genes or ESTs colocalized with published *loci* of nonsyn-

dromic deafness or Usher syndrome (i.e., a syndrome including sensory hearing loss) and are therefore candidate genes for these defects.

Eleven percent (302/2627) of the sequences that showed no similarity to sequences in the *D. rerio* section of UniGene could be tentatively assigned to 302 proteins in nr databank, a public databank containing an exhaustive nonredundant collection of protein sequences from different species (ftp://ftp.ncbi.nlm.nih.gov/blast/db/nr). Considering a sliding window of 100 amino acids, the numbers of sequences in each range of percentage of similarity (positivity) to proteins in nr were as follows: 34 sequences between 50% and 60%, 47 between 61% and 70%, 47 sequences between 71% and 80%, 71 between 81% and 90%, and 103 sequences >90%.

The remaining 2325 contigs and singletons showed no similarity with sequences in public databanks. Some of these sequences may be internal fragments of genes or ESTs already represented in public databases by their 5′ and 3′ ends. However, novel genes involved in inner ear development, including hair cell differentiation and innervation, are likely to be represented in this group.

The complete results of this study are presented in a relational database on the Web server of the Génoscope (Centre National de Séquençage). A user-friendly Web interface (http://www.genoscope.cns.fr/zie) allows easy query of the data resumed herein.

METHODS

Embryo Microdissection

Both otocysts were excised from 1000 zebrafish embryos at 20 to 30 hpf. The microdissection technique consisted of two slanting cuts as close as possible to the anterior and posterior otocyst edges, allowing the extrusion of the entire otocyst. Some surrounding tissue was inevitably carried with the otocyst.

Subtracted cDNA Libraries

Poly(A)$^+$ RNA was extracted from the microdissected otocysts and from the liver of adult zebrafish. Ear and liver cDNA were produced with the CLONTECH SMART PCR cDNA synthesis kit (CLONTECH). Liver cDNA was subtracted from the ear cDNA using the CLONTECH PCR-Select cDNA subtraction kit. Manufacturer's instructions were followed, except that two rounds of subtraction (instead of one) were performed against liver cDNA. The efficiency of the subtraction was estimated by hybridizing samples of the cDNA pools obtained before and after subtraction with a ^{32}P-labeled zebrafish-actin probe. Signals analyzed visually showed ~90% removal of housekeeping cDNAs. After subtraction, cDNA was amplified and the resulting PCR products were run on agarose gel. Three batches, containing fragments with sizes ranging from 300 to 600 bp, 600 to 1 Kbp, and >1 kbp, respectively, were eluted and subcloned in the pMOS vector (Amersham Pharmacia Biotech Europe GmbH). Ten thousand independent clones were grown and frozen, and plasmid DNA was extracted for sequencing.

High-Throughput Sequencing

The reactions were performed with a Big Dye terminator cycle sequencing kit and analyzed by an ABI-377 XL automated sequencer (Applied Biosystems). Sequences were base-called using the program PHRED.

Assembling Sequences

The sequences of the cloning vectors and adaptors were removed from the selected high-quality parts of 21,848 reads in the cDNA library from the *D. rerio* inner ear region. Sequences <100 bases were discarded. The resulting 17,468 trimmed sequences were masked for repeated sequences using RepeatMasker (A.F. Smit and P. Green, unpubl. data. http://repeatmasker. genome.washington.edu/cgi-bin/RM2_req.pl) and the *D. rerio* repeats library. Unmasked versions of these 17,468 nonassembled sequences were submitted to EMBL (accession nos. AL714032 to AL731531). Mate pairs (i.e., paired clone ends) were fused when a significant overlap was detected, generating what we call meta-sequences. For sequence assembling, meta-sequences or remaining single reads sharing at least 95% identity over 100 bases (as estimated with BLASTN) were clustered with the Single Linkage clustering algorithm, implemented in the software LASSAP (Gene-it), and each cluster was analyzed with PHRAP version 11.0 for constructing contig sequences. The final database contained 4694 contigs and singletons averaging 530 bases.

Sequence Comparison Against Public Databanks

The 4694 contigs and singletons were used as queries for BLASTN (default parameters) searches against the section *D. rerio* of UniGene (ftp://ftp.ncbi.nlm.nih.gov/repository/UniGene/Dr.seq.all). Sequences were considered identical if they shared at least 95 % identity in a window of at least 100 bases.

The translations in the six phases of the 2627 sequences in this database without significant similarity with sequences in *D. rerio* UniGene clusters were compared to nr (ftp://ftp.ncbi.nlm.nih.gov/blast/db/nr). In this case, any match with at least 50% positivity in a window of at least 100 amino acids was considered significant.

Further Sequence Annotation

A Perl script was written that retrieves the most probable putative human ortholog for each *D. rerio* gene or EST present in our database from the HomoloGene table (ftp://ftp.ncbi.nlm.nih.gov/pub/HomoloGene/) and then parses UniGene annotations for zebrafish and human clusters (gene name, title, linkage group/chromosome, tissue expression, and reference sequence). In the next step, the script retrieves the location of these human orthologs on the GoldenPath (UCSC Human Genome Project Working Draft, December 22, 2001, assembly hg9; tables refGene.txt and all_est.txt available at http://genome.cse.ucsc.edu/) and compares them to the nucleotide positions of the published *loci* for nonsyndromic deafness and Usher syndrome (nucleotide positions of delimiting markers being previously extracted from table stsMap.txt of GoldenPath). Co-located genes are labeled. Finally, the script screens our database for genes known to be expressed in the inner ear during the developmental stage this study focused on Holme et al.'s "table of gene expression in the developing ear" (http://www.ihr.mrc.ac.uk/hereditary/genetable/index.shtml/).

The results of sequence comparison and further annotation were organized in a relational database using MySQL. A user-friendly Web-based interface was programmed, allowing easy query of all the data of this study (http://www.genoscope.cns.fr/zie)

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