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Characterization of Novel and Identified Genes in Guinea Pig Organ of Corti

Takeshi Oshima,*[,1] Takaya Nakajima,† Hiroshi Wada,†
Katsuhisa Ikeda,* and Tomonori Takasaka*

**Department of Otorhinolaryngology, Tohoku University School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai, 980-8574, Japan, and †Department of Mechanical Engineering, Tohoku University, Sendai, Japan*

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A number of proteins are expressed in the organ of Corti and are considered to be responsible for hearing. However, most of them have not been identified. Therefore, to achieve a better understanding of the genetic factors influencing these traits, the first step is to characterize the genes expressed in the organ of Corti. In the present study, a cDNA library was constructed from the guinea pig organ of Corti. After sequencing isolated clones, 196 expressed sequence tags (ESTs) were identified with FASTA analysis: 65 ESTs showed significant sequence homology to previously identified genes in guinea pig, human or other species, and 131 ESTs showed no significant matches to sequences already present in the DNA database DDBJ/GenBank/EMBL. A variety of matching sequences, some of which were known to be cochlea-specific, were found through FASTA analysis of the 65 clones. RT-PCR with a panel of 10 different tissue mRNA revealed the restricted expression of 13 unknown clones. The results of our analysis allowed the establishment of a list of genes expressed in the guinea pig organ of Corti. © 2000 Academic Press

Key Words: **guinea pig; organ of Corti; cochlea; cDNA library; expressed sequence tag (EST); homology search; RT-PCR.**

The cochlea is the principal sensory organ of the auditory system. The organ of Corti, which consists of sensory cells named inner and outer hair cells (IHC and OHC) and supporting cells, resides in the mammalian cochlea and is responsible for the high sensitivity and selectivity to auditory stimulus (1). To unravel the complicated auditory mechanism, the organ of Corti has been investigated by anatomical and cell physiological approaches, however, biochemical or molecular biological studies have not progressed due to limitations in the amounts of samples. Polymerase chain reaction (PCR) is a useful tool to investigate the gene expression, especially in such a small tissue. The cochlear expression of molecules has been identified through reverse transcription (RT)-PCR (2, 3).

There are several reports about the construction of a cDNA library and the following characterization of expressed sequence tags (ESTs) (4–7). Sequencing of ESTs is a rapid and efficient way to establish a detailed profile of genes expressed in a tissue. ESTs can be identified as novel and known genes using the DNA database developed in recent years, and can be used as probes to clone genes from cDNA libraries. This useful approach has been often employed to generate lists of ESTs in different tissues and in several species (8–11). Also several reports have demonstrated a cochlear or OHC profile of gene expression. In rat, 107 ESTs were identified from a whole cochlear cDNA library (12) and 218 ESTs have been characterized from a PCR-amplified OHC cDNA library (13). More than 4000 ESTs also have been developed from a human cochlear cDNA library (14). In the guinea pig, the organ of Corti cDNA libraries have been widely employed to identify novel genes (15–19), however, there has been no available list of ESTs from the guinea pig cochlea. In the guinea pig cochlear tissues, the identification and characterization of ESTs can give us important information on cochlear mechanisms.

In the present study, a cDNA minilibrary was constructed from the guinea pig organ of Corti. The sequences of obtained 196 ESTs were compared with the known genes deposited in the DNA database DDBJ/GenBank/EMBL by FASTA 3.0. Moreover, the tissue distribution of 39 ESTs was investigated in different tissues by RT-PCR. Using these techniques, we have isolated and identified two putative novel and differentially expressed genes, suggesting that they may play important roles in the organ of Corti.

[1] To whom correspondence should be addressed. Fax: +81-22-717-7307. E-mail: oshima@orl.med.tohoku.ac.jp.

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MATERIALS AND METHODS

Construction of cDNA library. Nine young adult Hartley guinea pigs were anesthetized by diethyl ether. The bony bullae were rapidly removed and cochleae were dissected in 0.01 M phosphate buffered saline (PBS) at pH 7.2. The organ of Corti was carefully dissociated from surrounding tissues with a fine needle. Polyadenylated RNA was extracted from the dissected organ of Corti, bypassing the need for intermediate purification of total RNA (QuickPrep *Micro* mRNA Purification Kit; Amersham Pharmacia Biotech, Buckinghamshire, UK). Reverse transcription was performed from the polyadenylated RNA using random hexamers and Moloney Murine Leukemia virus reverse transcriptase, followed by double strand formation and end-blunting (TimeSaver cDNA Synthesis Kit; Amersham Pharmacia Biotech). The cDNA was ligated into a plasmid vector pKF3 (Takara, Otsu, Japan) digested with *Pvu*II. The *E. coli* TH2 competent cells (Takara) were transformed by the plasmid according to the manufacturer's protocol, and then plated at a low density to allow for separation of individual clones.

Determination of cDNA insert size. To evaluate the size of inserts in the cDNA library, the inserted DNA fragments from individual bacterial colonies were directly amplified by PCR using pKF3-specific primers F1′ (5′-TCAGTGAGCGAGGAAGCGGAA-3′) and R3 (5′-CAGGTTGTGTCCTTCACCACCTAT-3′). The PCR amplification was performed in a 25 μl volume reaction with a thermostable DNA polymerase (rTaq DNA polymerase; Toyobo, Osaka, Japan). We performed 30 cycles of amplification (94°C for 30 s, 53°C for 30 s, 72°C for 2 min) using a thermal cycler (GeneAmp PCR System 9600; PE Biosystems, Foster, CA). To estimate the lengths of the inserts, the PCR products were electrophoresed in 2% agarose gels and visualized by ethidium bromide staining and a gel image analyzer (Gel Print 2000 VGA; Genomic Solutions, Ann Arbor, MI).

Sequencing and database analysis. The clones with inserts, the sizes of which were longer than 200 bp, were amplified and purified using GFX *Micro* Plasmid Prep Kit (Amersham Pharmacia Biotech). Sequencing was performed by a fluorescence-coupled autosequencing system (ALFexpress DNA Sequencer; Amersham Pharmacia Biotech) using pKF3-specific primers F1 (5′-GTGAGCGAGGAAGCGGAAGAA-3′) and R4 (5′-TTCGGTTTCTTCGGAGTAGTAGTG-3′) and a sequencing kit (ALFexpress AutoRead Sequencing Kit; Amersham Pharmacia Biotech). The obtained data were analyzed using GENETYX-MAC ver.10.1 software (Software Development, Tokyo, Japan). After the redundant clones were removed, the nucleotide sequences of the clones were compared to human, primate, mammalian, rodent and vertebrate databases in the DDBJ (http://www.ddbj.nig.ac.jp/Welcome-e.html) release 40 using the FASTA 3.0 program. The clones were then classified as known, unknown and contaminated genes according to their match rate to known sequences deposited in the DDBJ/GenBank/EMBL databases. The criteria for scoring a sequence as having a significant match were similarity >70% and E-value < 1e-05 in an overlapping region of at least 70 bp.

Tissue expression analysis using RT-PCR. Thirty-nine clones showing no significant match with known sequences deposited in the DDBJ/GenBank/EMBL databases were selected. Each PCR primer-pair of the selected clones was designed using the utility of GENETYX-MAC ver.10.1 software. The tissue expression of the selected clones was assessed by RT-PCR. The mRNA from 10 different tissues (cochlea, cerebellum, kidney, liver, heart, cerebral cortex, spleen, lung, eye and testis) of the guinea pig was isolated as described above. Four micrograms of mRNA were reverse-transcribed in the presence of random hexamers according to the manufacturer's instructions (First-Strand cDNA Synthesis Kit; Amersham Pharmacia Biotech) and 1/33 of each sample was used as a template for each PCR amplification using the specific primers. Thirty cycles of PCR (94°C for 30 s, 53–58°C for 30 s, 72°C for 1 min) were performed. Simultaneously, glyceraldehyde 3-phosphate dehydrogenase (G3PDH) was amplified as a positive control. The mRNA was used for PCR without reverse transcription as a negative control. The PCR products were electrophoresed on a 2% agarose gel.

The care and use of the animals in this study were approved by the Institutional Animal Care and Use Committee at Tohoku University School of Medicine.

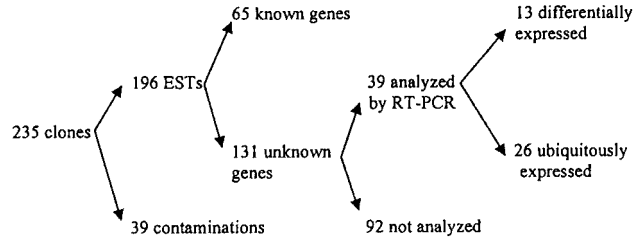

FIG. 1. A comprehensive view of the strategy and the results in this study.

RESULTS

cDNA library and sequencing. The transformed *E. coli* colonies (n = 1948) were picked up from agar plates to check the size of inserts by PCR, which demonstrated that 342 clones carried an insert of >200 bp length. Sequencing analysis of the 342 clones revealed that we could obtain 235 non-redundant clones (Fig. 1), the average size of which was 287 bp. The sequences of 39 out of the 235 clones corresponded to those of ribosomal RNA (n = 31), pKF3 (n = 7) or bacterial genome (n = 1). The other 196 clones were used for the following experiments as ESTs. The 196 nucleotide sequences reported here were deposited in the DDBJ/GenBank/EMBL database (Accession No. AU081352 to AU083347). The FASTA homology search demonstrated that the sequences of 65 out of the 196 clones possessed significant homology to those of previously known genes in guinea pig or other species. The 65 clones were classified into 8 categories shown in Table 1. The full-lengths of 2 clones showed 100% homology to known genes listed in Table 1 under P2X2 receptor splice variant and complete mitochondrial genome. The identity of the other 63 clones to known genes ranged from 70% to 99.5%.

Tissue expression analysis of unknown genes. The sequences of the other 131 clones showed no significant matches to those deposited in the database (Fig. 1). From the 131 clones considered as unknown genes, 39 clones were selected for the tissue expression analysis using RT-PCR. The DNA fragments of the expected size were detected by RT-PCR of all of the 39 clones when using cochlear mRNA as a template. Twenty-six out of the 39 clones exhibited ubiquitous tissue expression, whereas the other 13 clones showed restricted tissue distributions (Table 2). In particular, a clone (AU083235) was exclusively expressed in cochlea, spleen and lung, and another clone (AU083228) was expressed in cochlea, cerebellum and eye (Fig. 2).

TABLE 1

ESTs Matched to Known Genes in DNA Database

Clones: Accession No.	Matching sequences: Name	Species	Accession No.	E value	Identity (%)	Overlap/total bp
cell signaling/cell communication						
AU083341	BCL2/adenovirus E1B interacting protein 2	Human	U15173	3.2e-29	73.8	352/341
AU083301	beta-cop	Human	AF084457	1.6e-150	93.8	667/667
AU083248	chloride channel protein 3	Human	X78520	3.4e-12	85.7	154/236
AU083319	guanine nucleotide regulatory protein (tim1)	Human	U02082	2.7e-18	70.0	220/222
AU083321	integral membrane protein 1	Mouse	L34260	2.7e-103	87.9	515/511
AU803188	multi PDZ domain protein MUPP1	Human	AF093419	9.8e-21	84.7	144/206
AU083178	olfactory receptor	Human	U78308	1.3e-08	75.7	99/100
AU083316	P2X2 receptor splice variant P2X2-1	Guinea Pig	AF053327	7.3e-80	100.0	312/312
AU083196	prostaglandin D synthase	Human	M61901	6.0e-17	82.3	136/294
AU083219	Rab8-interacting protein	Mouse	U50595	7.6e-42	89.4	219/220
cell structure/motility						
AU08330	alpha II spectrin	Human	U83867	4.0e-52	89.4	257/257
AU083172	brain 4.1	Rat	AB019256	1.1e-09	85.7	77/124
AU083262	collagen Alpha 5(IV) Chain	Human	AL031622	1.8e-24	74.8	254/239
AU083155	dynactin	Human	X98801	1.4e-19	90.5	106/106
AU083317	dynactin subunit (p22)	Human	AF082513	2.4e-73	87.9	408/410
AU083160	ezrin	Mouse	X60671	3.8e-77	89.2	521/521
AU083247	I-plastin	Human	L20826	2.0e-45	91.7	218/218
AU083187	kinesin-2	Human	Y08319	1.7e-45	95.6	185/185
AU083286	matrin 3	Rat	M63485	2.0e-148	96.1	680/691
AU083168	microtubule-associated protein 1a	Human	U38291	1.6e-25	84.0	157/158
AU083250	microbutule-associated protein 1a	Human	U338219	2.0e-32	80.7	228/228
AU083295	microtubule-associated protein 1B	Human	L06237	9.4e-33	93.5	171/168
AU083251	plexin-B1/SEP	Human	X87904	5.7e-19	71.6	233/308
AU083267	spectrin SH3 domain binding protein	Human	U87166	1.2e-65	96.6	295/296
AU083263	tight junction protein (ZO-2)	Dog	L27152	7.6e-29	85.4	179/180
cell/organism defense						
AU083327	GAS-7 protein	Rat	AJ131902	7.3e-47	95.0	222/222
AU083183	heat shock protein 70	Globefish	Y08576	5.7e-42	98.2	175/172
AU083177	heat shock protein 90A	Hamster	L33676	3.9e-30	84.5	168/168
gene/protein expression						
AU083236	calnexin	Human	L18887	4.0e-82	89.2	383/383
AU083340	elongation factor 1 alpha	Rabbit	X62245	2.1e-69	90.9	309/309
AU083159	mitochondrial translational initiation factor	Bovine	L37835	1.1e-26	80.6	248/533
AU083184	ribosomal protein S21	Human	L04483	1.2e-63	88.5	307/311
AU083329	pre-mRNA splicing factor	Human	AF107405	2.4e-52	92.9	240/319
AU083265	RNA binding protein	Mouse	X84692	9.2e-61	94.2	278/317
AU083278	transcription factor	Human	M83233	1.1e-58	91.4	281/281
AU083192	ubiquitin-like/S30 ribosomal fusion protein	Domestic Pig	U72543	2.2e-64	90.0	350/350
mitochondrial						
AU083201	complete mitochondrial DNA sequence	Hedgehod	X88898	5.2e-37	78.0	241/241
AU083190	complete mitochondrial DNA sequence	Rhinoceros	Y07726	3.1e-15	75.0	148/150
AU083342	complete mitochondrial genome	Guniea pig	AJ222767	8.2e-79	100.0	286/286
AU083259	complete mitochondrial genome	Fat Dormouse	AJ001562	7.1e-46	78.1	321/323
hematopoietic						
AU083226	alpha-globin	Rabbit	J00658	2.0e-17	90.5	65/196
AU083270	alpha-globin	Rabbit	J00658	1.1e-15	90.5	95/235
AU083186	alpha-globin	Rabbit	J00658	6.6e-16	91.5	95/247
metabolism						
AU083304	alpha-lactalbumin	Guinea pig	Y00726	4.9e-27	73.6	323/330
AU083222	alpha-lactalbumin	Guinea pig	Y00726	1.2e-51	73.1	611/705
AU083337	co-beta glucosidase	Human	J03077	3.5e-32	84.2	197/304
AU083311	co-beta glucosidase	Human	J03077	4.4e-27	76.0	234/250
AU083288	dihydropyrimidine dehydrogenase	Human	U20938	4.5e-71	91.1	318/318
AU083291	dsRNA-specific adenosine deaminase	Rat	U18942	1.1e-44	83.3	259/259
AU083298	IkappaB kinase complex associated protein	Human	AF044195	4.4e-41	85.6	230/230
AU083200	lipoprotein lipase (gplpl)	Guinea pig	M33379	2.7e-06	73.2	116/657
AU083242	Li-sensitive myo-inositol monophosphatase	Human	AF042729	6.3e-68	90.7	334/335
AU083336	metalloproteinase inhibitor TIMP-2	Guinea Pig	AF127803	4.4e-51	99.5	229/228

TABLE 1—*Continued*

Clones: Accession No.	Matching sequences					
	Name	Species	Accession No.	E value	Identity (%)	Overlap/total bp
AU083285	PAF acetylhydrolase 45 kDa subunit	Bovine	D30615	1.9e-99	92.3	469/461
AU083202	seminal vesicle secretory protein	Guinea pig	U59722	4.6e-07	70.4	122/230
AU083214	sphingolipid activator protein	Human	M81355	7.9e-40	88.2	229/229
AU083169	ubiquitin-orotein ligase E3-alpha	Human	AF067384	1.3e-10	83.5	146/279
unclassified						
AU083246	atrophin-1	Human	U23851	4.4e-40	88.6	230/224
AU083303	chromosome 16	Human	AC004493	1.1e-30	93.1	262/258
AU083238	chromosome 5	Human	AC005753	2.1e-06	74.2	101/244
AU083223	coch-5B2	Human	AF006740	1.0e-43	88.2	222/222
AU083314	KIAA0836 protein	Human	AB020643	1.8e-64	86.6	336/337
AU083232	OS-9	Human	U41635	6.3e-53	92.4	251/251
AU083217	retinoblastoma-related protein Rb2/p130	Mouse	U36799	7.7e-55	82.8	304/304
AU083157	trg mRNA	Rat	X68101	2.9e-49	75.2	376/371

Note. The criteria for scoring a sequence as having a significant match were similarity >70% and E value <1e-05 in an overlapping region of at least 70 bp.

G3PDH could be amplified by RT-PCR with the G3PDH-specific primers and mRNA purified from the each tissue. No band could be detected by RT-PCR without a RT reaction.

DISCUSSION

To identify and characterize genes expressed in the organ of Corti, we constructed the guinea pig organ of Corti cDNA library. In total, 196 ESTs were randomly isolated and classified according to their match rate to known sequences deposited in the DDBJ/GenBank/EMBL databases. In the present study, 67% of ESTs (131/196) were considered to be unknown genes. Lower percentages of unknown genes have been demonstrated in other cochlea-derived libraries: rat cochlea, 37% (12); rat OHC, 22% (13); human cochlea, 13% (14). Among the 65 characterized clones in our library, only 7 (10.7%) corresponded to previously identified guinea pig genes and 58 were homologous to genes from other species, including rat, mouse and human. As mentioned above, in the cochlear libraries higher percentages of clones homologous to identified genes of the same species as sacrificed have been observed; 36% in rat cochlea library (12), 72% in rat OHC library (13) and 33% in a human cochlear library (14). The DNA entries of human and rat sequences deposited in the DDBJ/GenBank/EMBL database are much larger than those of the guinea pig (Human, 2720458 entries; Rat, 82789 entries; Guinea pig, 396 entries. DDBJ release 40. Jun. 2000). The high percentage of unknown genes in our library is possibly due to the small number of entries for the guinea pig. In this study, we used guinea pig as the source of the cDNA library in spite of

TABLE 2

Tissue Distribution of Differentially Expressed 13 Clones Detected by RT-PCR

Accession No.	Co	Ce	Ki	Li	He	Cc	Sp	Lu	Ey	Te
AU083188	+	+	+	+	+	+	−	+	+	+
AU083209	+	+	+	+	+	+	+	+	−	+
AU083224	+	+	−	+	+	+	−	+	+	+
AU083228	+	+	−	−	−	−	−	−	+	−
AU083231	+	+	+	+	+	+	+	+	−	+
AU083235	+	−	−	−	−	−	+	+	−	−
AU083256	+	+	+	+	+	+	+	+	−	−
AU083269	+	+	+	+	−	+	+	+	+	+
AU083274	+	+	−	+	+	+	+	+	+	+
AU083277	+	+	+	−	−	+	+	−	+	+
AU083279	+	+	−	+	+	+	+	+	−	+
AU083282	+	+	−	+	−	+	+	+	+	+
AU083287	+	+	−	+	+	+	+	+	+	+

Note. RT-PCR was performed with 10 different tissues (Co, cochlea; Ce, cerebellum; Ki, kidney; Li, liver; He, Heart; Cc, cerebral cortex; Sp, spleen; Lu, lung; Ey, eye and Te, testis). +, a positive result; −, a negative result of RT-PCR in a given tissue.

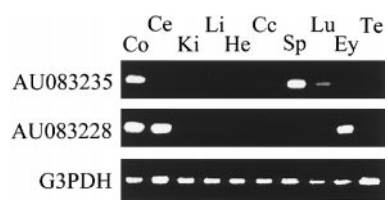

FIG. 2. RT-PCR of cochlea-preferentially expressed clones. Products obtained from mRNA extracted from different tissues were analyzed on agarose gel stained with ethidium bromide. The upper panel revealed the restricted expression (cochlea, spleen and lung) of the clone (AU083228). Evident bands were observed only on the lanes Co, Ce and Ey of the middle panel (AU083235), indicating the cochlea-preferential expression. The bottom panel indicated the G3PDH amplification as a positive control. The abbreviations of tissues are the same as those in Table 2.

the paucity of information on its ESTs. The guinea pig has been the most versatile animal for the cochlear anatomy and physiology. Therefore, cDNA libraries and ESTs of the guinea pig organ of Corti will be helpful to clarify the pathophysiology of the auditory system.

Among the ESTs obtained in this study, some possible contaminations were observed. For example, the hematopoietic tissue transcript such as alpha globin is more likely to originate from contaminating blood rather than from the organ of Corti. We obtained two clones homologous to microtubule-associated protein 1a. This neuron-specific molecule is reported not to be expressed in the organ of Corti (20). It is considered to be almost impossible to entirely remove other tissues binding the organ of Corti. Another putative contaminant was mitochondrial genome. Four clones homologous to the mitochondrial genome were obtained. These clones might be due to contamination of mitochondrial DNA or nuclear transcripts derived from mitochondrial DNA-like nuclear genomic sequences. The possible contamination of the mitochondrial genome in our library may demonstrate that mitochondria are abundant in the organ of Corti. In fact, mitochondrial DNA are closely associated with auditory pathology (21, 22).

Our library seems to be highly representative of the gene expression in the organ of Corti. For example, Coch-5B2, to which the clone (AU083223) was homologous, is reported to be expressed at very high levels in the cochlea and vestibule (23). Autosomal dominant deafness DFNA9 is caused by a mutation of its human counterpart (24). We obtained the clone with a significant match to P2X2 purinergic receptor which is reported to be localized in the guinea pig organ of Corti (19). Alpha II spectrin was also detected as a homologous gene. Spectrin molecules are abundant in OHCs and contribute to the cortical lattices of the OHC lateral wall (25, 26).

The tissue expression of 39 unknown clones was examined by RT-PCR. Two clones were expressed exclusively in only 3 tissues including the cochlea out of 10 different tissues. The sequences of the 2 clones possessed no open reading frames, suggesting that they were non-coding regions of novel transcripts. We failed to obtain their full-length sequences by a conventional cDNA library screening method with the clones as probes, suggesting that the amounts of their transcript were very small or there were other difficulties in the screening. It cannot be excluded that their encoded proteins play unique roles in hearing even though their transcripts were expressed faintly.

The cDNA library in this study will be a valuable tool to access novel genes that are preferentially expressed in the organ of Corti. Further investigations should be performed to identify the hundreds of genes obtained in this study. Such studies will give us important information on auditory physiology and pathology.

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