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Gene Expression Profiles of the Rat Cochlea, Cochlear Nucleus, and Inferior Colliculus

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ABSTRACT

High-throughput DNA microarray technology allows for the assessment of large numbers of genes and can reveal gene expression in a specific region, differential gene expression between regions, as well as changes in gene expression under changing experimental conditions or with a particular disease. The present study used a gene array to profile normal gene expression in the rat whole cochlea, two subregions of the cochlea (modiolar and sensorineural epithelium), and the cochlear nucleus and inferior colliculus of the auditory brainstem. The hippocampus was also assessed as a well-characterized reference tissue. Approximately 40% of the 588 genes on the array showed expression over background. When the criterion for a signal threshold was set conservatively at twice background, the number of genes above the signal threshold ranged from approximately 20% in the cochlea to 30% in the inferior colliculus. While much of the gene expression pattern was expected based on the literature, gene profiles also revealed expression of genes that had not been reported previously. Many genes were expressed in all regions while others were differentially expressed (defined as greater than a twofold difference in expression between regions). A greater number of differentially expressed genes were found when comparing peripheral (cochlear) and central nervous system regions than when comparing the central auditory regions and the hippocampus. Several families of insulin-like growth factor binding proteins, matrix metalloproteinases, and tissue inhibitor of metalloproteinases were among the genes expressed at much higher levels in the cochlea compared with the central nervous system regions.

Keywords: Gene array, DNA microarray, gene expression, hippocampus, inferior colliculus, cochlear nucleus, cochlea

INTRODUCTION

Expressed genes can, in general, be divided into two classes: (1) housekeeping genes that are ubiquitously expressed and (2) tissue-specific genes. Housekeeping genes are thought to be necessary for the normal maintenance of the structural and functional integrity of most, if not all, cells. Tissue-specific genes, by definition, have a restricted pattern of expression and would be expected to provide important information about the specific structures and functions of a specific tissue. Ideally, one would like to develop complete gene expression profiles of a particular cell or tissue by analyzing all of the 50,000 or so genes predicted to occur in mammalian genomes. That subset of genes expressed in a particular tissue or organism has been termed the "transcriptome," the repertoire of transcribed genes that define that tissue or cell type. However, smaller subsets can still provide considerable information and enhance our understanding of the cells and/or tissue assessed.

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In the auditory system, most functionally important genes and proteins have been identified through mutational studies, through subtractive hybridization, or through differential display. Application of subtractive hybridization to a human fetal cochlear cDNA library identified 1449 previously characterized known genes at http://hearing.bwh.harvard.edu/cochlearcdnalibrary.htm/ as of December 2000. They include several subtypes of collagen genes and peripheral myelin protein (PMP-22), as well as novel cochlear genes (Robertson et al. 1994; Skvorak et al. 1999). A polymerase chain reaction (PCR)-based differential display study of the chick basilar papilla after acoustic trauma revealed that the δ subunit of the neuronal-specific Ca^{2+}/calmodulin-regulated kinase II (CAMK II) and CDC 42, a GTP-binding protein, were up-regulated in the chick basilar papilla exposed to noise (Gong et al. 1996). However, the large number of novel genes identified by this method and the amount of characterization needed to complete these studies in general represents a considerable investment of labor and time.

Gene arrays or DNA microarrays, which contain large numbers of cDNA sequences that represent specific genes, allow the simultaneous assessment of expression of multiple genes in specific tissue types with very little material in a relatively short period of time (for review, see Nature Genetics Supplement 21:1, 1999). Profiling gene expression is a powerful method for characterizing specific tissues, for comparing gene expression between regions, and for determining differential gene expression under varying experimental conditions. For example, a study using DNA microarrays containing probes for almost the complete set of yeast genes showed that genes containing consensus sequences for the transcription factor Ndt80 are important for meiotic prophase in budding yeast during spore development (Chu et al. 1998). DNA microarrays are therefore an excellent tool to provide a global view of biological processes in an efficient way.

This study used a cDNA array to characterize and compare normal gene expression patterns in several regions of the auditory pathway: (a) the cochlear nucleus (CN), (b) the inferior colliculus (IC), (c) the whole cochlea (WC), (d) two cochlear subfractions—the modiolus (MOD) and the sensorineural epithelium (SE). The MOD contains the cell bodies of the auditory nerve and supporting cells, and the SE contains the organ of Corti, supporting cells, and the lateral wall. The hippocampus (HP) was chosen as a well-known and nonauditory tissue to potentially identify auditory region-specific genes. The first level of assessment generated a gene expression profile for each region. A second level of assessment compared expression patterns among regions to determine genes that are differentially expressed. Finally six genes in the cochlea with high expression relative to the other regions were selected and their expression confirmed and compared among regions using semiquatitative reverse transcriptase (RT)-PCR.

METHODS

Gene array

The Atlas™ cDNA expression array (Catalog # 7738-1, Clontech, Palo Alto, CA, USA) is a nylon membrane-type gene array and contains 588 known rat genes plus 9 housekeeping genes. A 200–800 bp long PCR product of each cDNA representing species-specific genes was microspotted in duplicate spots containing 10 ng/dot. The genes are grouped into six blocks. Each block contains functionally related genes (for more detail, see the home page of Clontech at http://www.clontech.com).

RNA extraction

Sprague–Dawley male rats (200–250 g, 8–12 weeks) were deeply anesthesized with 35% (w/v) chloral hydrate (350 mg/kg, IP) and decapitated. Brains were removed and the HP, IC, and CN were dissected and placed in 1 mL of lysis buffer containing 4 M guanidium thiocynate, 25 mM sodium citrate (pH 7.0), 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol per 100 mg wet tissue (Chomczynski and Sacchi 1987). After the brains the temporal bones were removed and dissected in ice-cold RNase-free PBS to collect the cochleae. The otic capsule was removed and the remaining WC with the lateral wall still largely attached was placed in 2 mL of lysis buffer. Alternatively, the WC was further dissected to separate the SE with some lateral wall still attached from the modiolar core containing largely the auditory nerve and spiral ganglion, glial cells, and vascular elements. The cochlear material from six to eight animals was pooled for the WC and the MOD, and from 20 animals for the SE fraction. The tissues were homogenized for 1 min at speed 5 with a Polytron™ homogenizer (Brinkman, Westbury, NY). The homogenized tissues were kept at -70°C until RNA isolation. Total RNA was extracted from the homogenized tissues with SV Total RNA Isolation System (Promega, Madison, WI, USA). The yield of total RNA ranged from 0.5 to 1 μg per WC. Our experience indicated that the proportion of RNA obtained from each subfraction was 1:4 for the SE and the MOD.

Probe synthesis and hybridization

Radiolabeled cDNA was synthesized according to the vendor's protocol with a slight modification (Clontech). Reverse transcriptase reaction was performed

on 2 μg of total RNA in 10 μL of a reaction mixture containing 20 nM each of gene-specific primers; 500 μM each of dCTP, dGTP, and dTTP; 35 μCi of α^{32} P-dATP (3000 Ci/mmol); and 100 U of MMLV reverse transcriptase Superscript II (Life Technologies Inc., Gaithersburg, MD, USA) for 25 min at 50°C. cDNA probe was purified by passage through CHROMA SPIN-200 DEPC–H_2O columns (Clontech), and final probe concentration was adjusted to $1–2 \times 10^6$ cpm/mL in 10 mL of ExpressHyb™ by hybridization solution (Clontech). Hybridization to Atlas Rat cDNA Expression Array was carried out overnight at 68°C. The membranes were washed four times in 2×SSC/1% SDS at 68°C for 30 min each and two more times in 0.1×SSC/0.5% SDS at 68°C for 30 min each and exposed to a Storage Phosphor screen for phosphor imaging overnight. The screen was scanned with phosphor imager 445 SI (Molecular Dynamics, Sunnyvale, CA, USA).

In order to determine the variation among membranes, the cDNA probes of RNA from each of the three brain regions—the HP, IC, and CN—were hybridized to duplicate membranes in the same experiment. Duplicate membranes from each region of the brain were analyzed and compared. The reproducibility of the experimental results was assessed by repeated hybridization of three brain regions (HP, IC and CN) and the WC RNA sample and comparing two experiments in each region.

Data analysis

Image files obtained from phosphor imaging were analyzed with AtlasImage v1.01 software (Clontech). The signal intensity for each gene was the average signal from duplicate spots and corrected for the background signal on each membrane. The signal threshold was set at 100% over background signal ($\{(\text{Signal}_{\text{gene}Z,\text{array1}} - \text{Background}_{\text{array1}})/\text{Background}_{\text{array1}}\} \geq 1$). In the Results section, signals are referred to as either above or below these defined thresholds. For the three brain regions and the WC, two or three membranes from two hybridization experiments were averaged using the AtlasImage software; the averaged values were used for normal gene expression profiles and subsequent comparisons of each region. For the normal gene expression, the expression level of mRNA was displayed as a relative intensity to the background of averaged array. For the comparison among regions, signals from one region were normalized to the other one using at least two out of the nine housekeeping genes in the array to adjust for variation in experimental conditions such as differences in specific activity and the concentration of labeled cDNA, hybridization conditions, and exposure time. The threshold of the ratio of the signal from one array to the other array was set at 2.0 and that of the signal difference was set at the summation of each background signal of two arrays ($\text{Background}_{\text{array1}} + \text{Background}_{\text{array2}}$). For the comparisons of cochlear subfractions, a single array from each region (WC, MOD, and SE) was used.

Sensitivity and threshold considerations

Under our experimental conditions, 40%–50% of the 588 genes on the arrays showed expression over background in the brain tissues. However, when we applied our criterion for a signal threshold of 100% over background (twice background), expression was found in 20%–30% of the genes on the array. In order to evaluate variability among membranes, duplicate membranes were hybridized with the brain tissues. Figure 1A shows membrane variability in the case of the hippocampus. When the corrected signals to the background of one membrane were plotted to *x* axis and those of the other membrane to *y* axis, most of genes with the signal threshold 100% over background fell within the signal ratio 2:0.5 which we set for significant differential expression between two regions. If we lowered the detection limit to 50% over background, then 30%–40% of the 588 genes had positive signals, i.e., had expression above this lower threshold level. However, this significantly decreased the reproducibility of the signals between duplicate membranes. Therefore, we chose to be conservative and defined 100% of the signal threshold (twice background) as the necessary condition for a positive result in this study. It should be noted that many genes whose mRNA is in the low to modest abundance class in the mRNA population being assayed will fall below the detection criterion we have set.

Figure 1B displays the experimental variation of two hybridizations with the hippocampus RNA. The signal differences of the genes with over twice background in both hybridizations, shown in the upper-right corner of the square, were less than twofold. Several genes with the signals below the threshold in either experiment (upper-left and lower-right corner of the square) showed more than a twofold difference between two experiments. For comparison analysis, we therefore applied the very stringent thresholds as the signal difference at twice background as well as the signal ratio at twofold.

Housekeeping genes generally are used for normalizing signals between two arrays being compared. It is possible that the expression levels of the housekeeping genes are themselves different among regions or under experimental conditions. Thus, we compared two different normalization methods. The "global method" calculates a normalization coefficient based on the sum of the values of signal over background for all genes on the arrays. The "housekeeping gene method" calculates the normalization coefficient

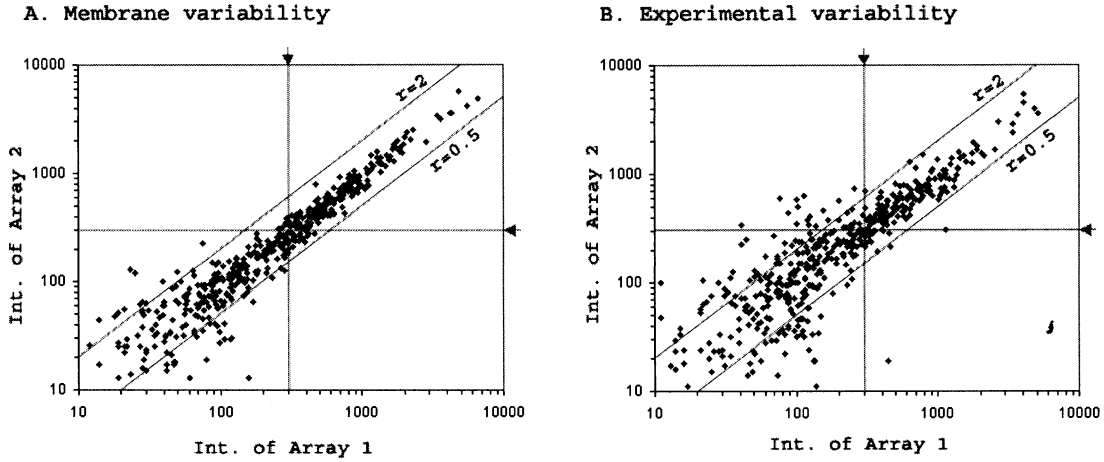

FIG. 1. Experimental variability of gene expression profiles. For each gene, the corrected intensity to the background in one array is given on the *x* axis and the corrected intensity in the other array is plotted on the *y* axis. Twofold changes (r = intensity in array 2/intensity in array 1 > 2 or < 0.5) are indicated. The arrow indicates the signal threshold (twice background) of each membrane. **A** Comparison of duplicate membranes. Hippocampal total RNA (2 μg) was converted into ^{32}P-radiolabeled cDNA and hybridized to duplicate arrays (array 1 and array 2). **B** Comparison of duplicate hybridizations. Hybridization was performed twice with the same hippocampal RNA and after normalization with housekeeping genes. The array 1 of the first experiment was compared with the array 2 of the second experiment.

according to the average of the signal ratios of the housekeeping genes being selected. The two methods resulted in very similar data in the comparisons of two CNS regions or cochlear subfractions but showed a large difference in the comparison of the cochlea to any CNS region. The normalization coefficient of the global method for the comparison of the cochlea to the hippocampus was 50% higher than that of the housekeeping gene method. We believed that this was because, considering that most genes on the array were well characterized in other tissues such as the brain, many more genes on the array could be expressed in CNS regions, biasing results from the global method. The present study therefore chose to make comparisons using the housekeeping gene normalization.

Semiquantitative RT-PCR and data analysis

For RT-PCR, 1 μg total RNA of HP, IC, CN, WC, MOD, and SE was reverse transcribed into single-stranded cDNA with SuperScript II MMLV reverse transcriptase (Life Technologies Inc.) using oligo(dT)$_{12-18}$ as primers in 20 μL of a reaction mixture. The reaction mixture was diluted tenfold with RNase-free water and then stored at -20°C for subsequent PCR analysis.

The sequences of oligonucleotide primers for two housekeeping genes, polyubiquitin and beta actin, and the following six genes were purchased from Clontech: LIM domain kinase-1 (LIMK-1), insulin-like growth factor binding protein-2 (IGFBP-2), IGFBP-6, gelatinase A/matrix metalloproteinase-2 (MMP-2), membrane type MMP-1 (MT MMP-1/MMP-14), and tissue inhibitor of metalloproteinase-3 (TIMP-3). A 5 μL aliquot of the diluted cDNA strand was subjected to PCR amplification using the AmpliTaq Gold™ (Applied Biosystems, Boston, MA, USA) in 25 μL of a reaction mixture. PCR conditions were 94°C for 10 min followed by 30 cycles of 94°C for 1 min and 68°C for 1 min. For the housekeeping genes polyubiquitin and beta actin, the lower number of PCR cycles, 27 and 25 cycles, respectively, were applied. Ten microliters of the PCR reaction aliquot was mixed with 1 μg of ethidium bromide and run on 1.5% of agarose gel. The agarose gel was scanned with a gel documentation system (AlphaEase™ v.3.24; Alpha Innotech Corporation, San Leandro, CA, USA) and the image files from the GelDoc system were quantified with IPLab software (Scanalytics, Fairfax, VA, USA). The variation of the signals from the input of RNA and the efficiency of the RT reaction were normalized with the two housekeeping genes. The values of intensities were averaged from two or three PCR experiments.

RESULTS

In the text, we provide results on genes showing differential expression in auditory regions. Image files for filter hybridization and lists of all genes expressed over background in each region are available on the Kresge Hearing Research Institute webpage at the University of Michigan (www.khri.med.umich.edu/genearray/).

Expression profile of the whole cochlea

In the whole cochlea, approximately 20% (102) of the genes (588) on the array showed expression levels 100% over background. The table containing the list of

genes and the filter hybridization image are available at www.khri.med.umich.edu/genearray/. Twenty-eight of these genes, including TIMP-3, have previously been detected in the Morton human fetal cochlear cDNA library (http://hearing.bwh.harvard.edu/cochlear cdnalibrary.htm). In this study, we demonstrated expression of many additional genes that have not yet been reported in the cochlear cDNA library.

Many genes with significant signals were expected based on previous reports of their expression or the presence of their product in the cochlea, e.g., the trk B receptor (Ylikoski et al. 1993), neuron-specific enolase (Altschuler et al. 1985), and glutathione S-transferase (el Barbary et al. 1993). PMP-22 was reported in the human cochlea cDNA library while we would expect myelin proteolipid protein (PLP) and other proteins to be present based on our knowledge of cochlear histology. These arrays, however, also showed expression of several genes in the whole cochlea that have not been previously reported, including membrane glycoprotein gp130, Crk adaptor protein, PKC inhibitor protein-1, syntaxin binding protein, clusterin, G(i)α2 subunit, IGFBP-6, and TIMP-2.

Genes with particularly high expression in the whole cochlea (at least 5 times over background) included copper–zinc-containing superoxide dismutase 1 (Cu–Zn SOD 1), calmodulin, PMP-22, and the Na^+/K^+–ATPase α1 subunit. Several genes, whose expression would be predicted based on the literature, were expressed over background but fell below the exclusion criterion (twice background). These genes included fibroblast growth factor receptor 1 (http://hearing.bwh.harvard.edu/cochlearcdnalibrary), P2X(2) receptor (Housley et al. 1999; Jarlebark et al.2000), and NMDAR1 (Kuriyama et al. 1993; Niedzielski and Wenthold 1995; Safieddine and Eybalin 1992).

Cochlear subfractions

Figure 2 shows comparison of gene expression profiles among cochlear subfractions. There was little difference between gene expression profiles of the modiolar subfraction and gene expression profiles of the whole cochlea (Fig. 2A), as might be expected, since RNA from the MOD comprises approximately 80% of the whole cochlear RNA. On the other hand, when expression in the SE was compared with that in the WC, six genes showed increased expression in the SE: Ear-3, presomatotropin, synaptobrevin 2 (SYB2), corticosteroid 11-β-dehydrogenase isozyme 1, IGFBP-2, and heparin-binding growth-associated protein (Fig. 2B and Table 1). Four genes had lower expression, suggesting they were much more highly expressed in the modiolus such as (not surprisingly) PLP. A comparison

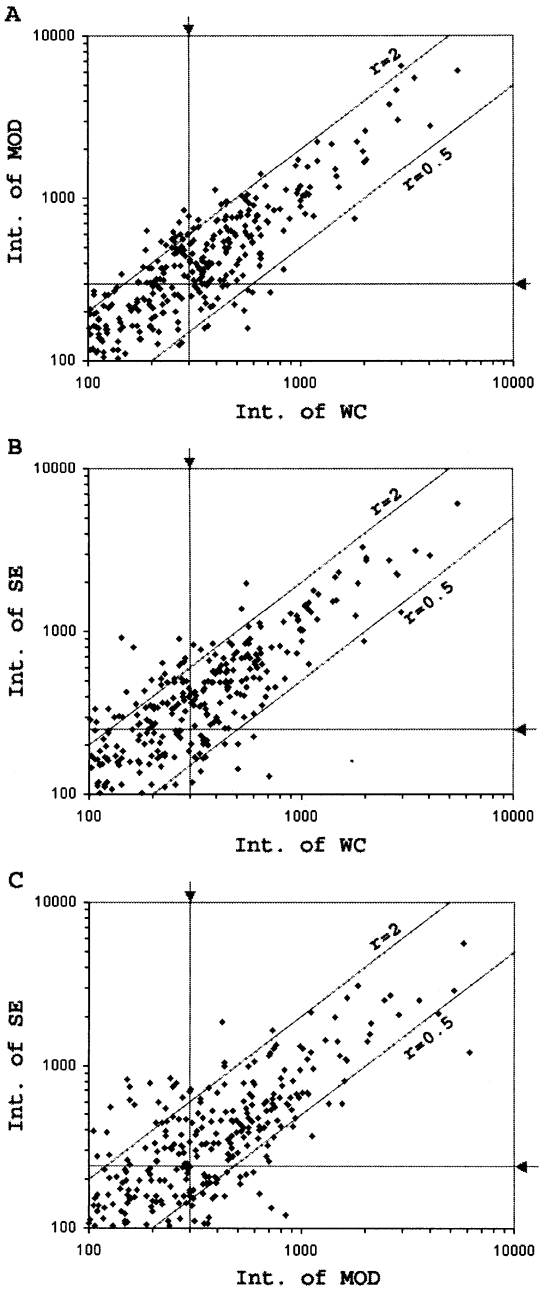

FIG. 2. Comparison of gene expression profiles of the cochlear subfractions. Total RNA (2 μg) from the whole cochlea (WC), the modiolus (MOD), or the sensorineural epithelium (SE) was converted into ^{32}P-radiolabeled cDNA and hybridized to membranes. Twofold changes (r = intensity in array 2/intensity in array 1 $>$ 2 or $<$ 0.5) are indicated. The arrow indicates the signal threshold (twice background) of each membrane. **A** MOD vs. WC. The intensity of the array hybridized with the WC RNA is plotted on the x axis and, after normalization, the intensity of the array hybridized with the modiolar (MOD) RNA is plotted on the y axis. **B** SE vs. WC. The intensity of the array hybridized with the WC RNA is plotted on the x axis and, after normalization, the intensity of the array hybridized with the sensorineural epithelium (SE) RNA is plotted on the y axis. **C** SE vs. MOD. The intensity of the array hybridized with the MOD RNA is plotted on the x axis and, after normalization, the intensity of the array hybridized with the sensorineural epithelium (SE) RNA is plotted on the y axis. Twofold changes (r = intensity in array 2/intensity in array 1 $>$ 2 or $<$ 0.5) are indicated. The arrow indicates the signal threshold (twice background) for each membrane.

TABLE 1

List of genes differentially expressed in the cochlear subfractions[a]

Location	*Protein/gene*	*Ratio*[b]	*Difference*[c]
	Higher expression in SE compared with WC		
A4k[e]	Insulin-like growth factor binding protein 2 (IGF-binding protein 2; IGFBP2; IBP2)	3.6	1446
F2h	Heparin-binding growth associated protein	2.6	855
D2h	Corticosteroid 11-beta-dehydrogenase isozyme 1 (11-DH)	—[d]	772
A2n	Ear-3; V-erbA related protein; COUP-TFI transcription factor	—	639
F3h	Presomatotropin	—	634
C7f	Synaptobrevin 2 (SYB2); vesicle-associated membrane protein 2 (VAMP2)	—	567
	Lower expression in SE compared with WC		
C3f	Myelin proteolipid protein (PLP); DM-20; lipophilin	0.4	−1651
A7d	Copper-zinc-containing superoxide dismutase 1 (Cu-Zn SOD1)	0.4	−1108
B6f	Sec1; syntaxin binding protein 1; UNC-18A; UNC-18-1; N-SEC1; RBSEC1	0.4	−813
C6c	Neuronatin	—	−574
	Higher expression in SE compared with MOD		
A4k[e]	Insulin-like growth factor binding protein 2 (IGF-binding protein 2; IGFBP2; IBP2)	4.4	1423
D5b	40S ribosomal protein S17 (RPS17)	2.3	921
D2h	Corticosteroid 11-beta-dehydrogenase isozyme 1 (11-DH)	—	809
C7n	P2X purinoceptor 2; ATP receptor P2X2; purinergic receptor	—	669
A3l	Sky proto-oncogene; Tyro3; Rse; Dtk	2.4	614
A2n	Ear-3; V-erbA related protein; COUP-TF1 transcription factor	—	594
A6k	DNA topoisomerase II alpha (TOP2A)	—	588
F3h	Presomatotropin	—	582
	Lower expression in SE compared with MOD		
C3f	Myelin proteolipid protein (PLP); DM-20; lipophilin	0.2	−4969
C3h	SR13 myelin protein; peripheral myelin protein 22 (PMP-22); CD25 protein	0.5	−2302
B6f	Sec1; syntaxin binding protein 1; UNC-18A; UNC-18-1; N-SEC1; RBSEC1	0.4	−974
C2c	Rac-alpha serine/threonine kinase (RAC-PK-alpha); protein kinase B (PKB); AKT1	—	−929
B3k	LIM domain serine/threonine kinase 1 (LIMK1)	—	−759
C5e	Neuroendocrine protein 7B2 precursor; secretogranin V; SGNE1	0.4	−756
C6c	Neuronatin	—	−729
D4j	60S ribosomal protein L44; L36A	—	−594

[a]Single array for each region was used for comparison.
[b]Ratio = adjusted intensity of SE array/adjusted intensity of WC array (or MOD).
[c]Difference = adjusted intensity of SE array - adjusted intensity of WC array (or MOD).
[d]— = the signal ratio was not calculated when the adjusted intensity is background level in either array.
[e]Gene chosen for RT-PCR assay.

between the SE and MOD subfractions also identified genes with differential expression (Fig. 2C and Table 1). For instance, the P2X(2) receptor was more enriched in the SE subfraction than in the MOD. This result is consistent with the report of its localization (Housley et al. 1999; Jarlebark et al. 2000).

Expression in the cochlear nucleus

In the CN, approximately 25% of the genes on the array showed expression levels 100% over background (www.khri.med.umich.edu/genearray/). Many of these levels were consistent with levels published in previous literature which looked at the specific gene or its product. They include trkB receptor (Hafidi et al. 1996), neurotransmitter receptor-related genes such as somatostatin (Wynne and Robertson, 1997), glycine receptor (GlyR) α1 (Friauf et al. 1997; Sato et al. 1995), NR-1 (Sato et al. 1998), GluR-4 (Hunter et al. 1993), and GABA-BR (Juiz et al. 1994). Other genes whose expression might be expected include channel-related genes such as Na^+/K^+–ATPase α1 and β3 subunits, chloride channel 1 (RCL1), delayed-rectifier K^+ channel, and Na^+ channel 1, as well as synapsins, glutamate transporter (GluT), and the glycine transporter.

Several genes, whose expression had been reported in the cochlear nucleus or was expected based on what is known about CN function, had expression levels above background but fell below our criterion for 100% over background: the neuronal acetylcholine receptor (AchR) β subunit, GluR2, GluR1, GluR3, mGluR1, GABA-ARγ, and 2GABA-ARδ.

Expression in the inferior colliculus

In the inferior collicus (IC) approximately 30% of the genes on the microarray showed expression levels 100% over background (www.khri.med.umich.edu/

TABLE 2

List of genes differentially expressed in the central auditory brainstem[a]

Location	*Protein/gene*	*Ratio*[b]	*Difference*[c]
	Higher expression in the CN compared with the IC		
D7i	Signal transducer CD24 precursor, heat stable antigen (HAS); nectadrin	6.6	1693
C7c	Glycine receptor (GlyR) alpha-1 chain precursor (48 kDa); strychnine binding subunit	2.7	890
E6f	Cardiac delayed-rectifier potassium channel protein	2.8	734
B5j	Transducin beta-1 subunit; GTP-binding protein G(i)/G(s)/G(t) beta subunit 1	2.3	683
F2g	Insulin-like growth factor II (IGF-II)	8.0	600
A4k	Insulin-like growth factor binding protein 2 (IGF-binding protein 2; IGFBP2; IBP2)	—[d]	780
C7e	Synaptobrevin 1 (SYB1); vesicle-associated membrane protein 1 (VAMP1)	—	779
	Lower expression in the CN compared with the IC		
C4h	Neuromodulin; axonal membrane protein GAP43; PP46; B-50	0.1	−1805
C7k	GABA-B receptor 1a + GABA-B receptor 1b	0.4	−1154
E7l	GABA-A receptor beta-2 subunit precursor	—	−913
B1e	BDNF/NT-3 growth factor receptor precursor; trkB tyrosine kinase	0.4	−694
C6c	Neuronatin	0.4	−616

[a]Averaged values of three arrays for each region were used for comparison.
[b]Ratio = adjusted intensity of CN array/adjusted intensity of IC array.
[c]Difference = adjusted intensity of CN array - adjusted intensity of IC array.
[d]— = the signal ratio was not calculated when the adjusted intensity is background level in either array.

genearray/). Many of these were consistent with previous literature including neurotransmitter-related genes such as substance P (Wynne and Robertson 1997). Other genes showing expression, which was expected, include neurotransmitter receptor genes, channel-related genes, synapse-related genes such as the synapsins SYN1 and 2A, and neurotrophic factor receptors such as RET2 (GDNF receptor subunit) and trk B.

Genes with particularly high expression (at least 5 times over background) included Cu–Zn SOD, calmodulin, protein kinase C inhibitor protein 1, clusterin, neuron-specific enolase, secretogranin II, secretogranin V, signal transducer CD 24, carboxypeptidase E, GABA-BR, β-alanine-sensitive GABA transporter, Na^+/K^+–ATPase α1, somatostatin, and GAP-43. Several genes, whose expression was expected based on the literature or what is known about IC function, had expression levels above background but fell below our criterion for 100% over background. They include receptor subunits such as GluR3, D(2) dopamine receptor, AchR M2, RET1, and insulin-like growth factor 1 receptor α and channel related genes such as voltage-gated Ca^{2+} channel α1, voltage-gated K^+ channel RK5, Na^+ channel 6, and G-protein-activated K^+ inward rectifier.

Comparison of IC and CN

When expression in the CN was compared with expression in the IC, there were several genes that had a significant difference in expression between the two regions. The CN showed higher expression in GlyR α1 than the IC and the IC expressed a higher level of GABA-AR β1 and GABA-AR 1a/1b than the CN, consistent with the literature (Sato et al. 2000a, 2000b). Na^+/K^+–ATPase α1 and β3 subunits were expressed more highly in the IC while β2 was expressed more highly in the CN. Other genes differentially expressed between the two regions include signal transducer CD24, IGFBP-2, SYB 1, cardiac delayed-rectifier K^+ channel protein, and GAP43 (Table 2).

Comparison of auditory regions and the hippocampus

Each region of the auditory pathway was compared separately to the HP. A greater number of differentially expressed genes were identified when comparing the peripheral (cochlear) regions with the central regions (CN, IC, HP) than when comparing the three central regions. For instance, there were many more genes with significant differences in expression when the WC was compared with the HP (Fig. 3B and Table 4) than when the IC was compared to the HP (Fig. 3A and Table 3). Two central auditory regions were compared; although there were a few more genes differentially expressed between the IC and the CN (Fig. 3C and Table 2) than between the IC and the HP, the comparison patterns of IC vs. HP and CN vs. IC were very similar. The comparison patterns of WC vs. IC (Fig. 3D) and WC vs. HP (Fig. 3B) were very similar as well.

In general, the expression patterns of proto-oncogenes, signaling molecules, and metabolic enzymes were similar in the CNS and cochlear regions, although a few genes showed distinct expression among regions, such as $p27^{Kip1}$ (A5n) in the cochlear regions and cell adhesion kinase β (B41) in the HP.

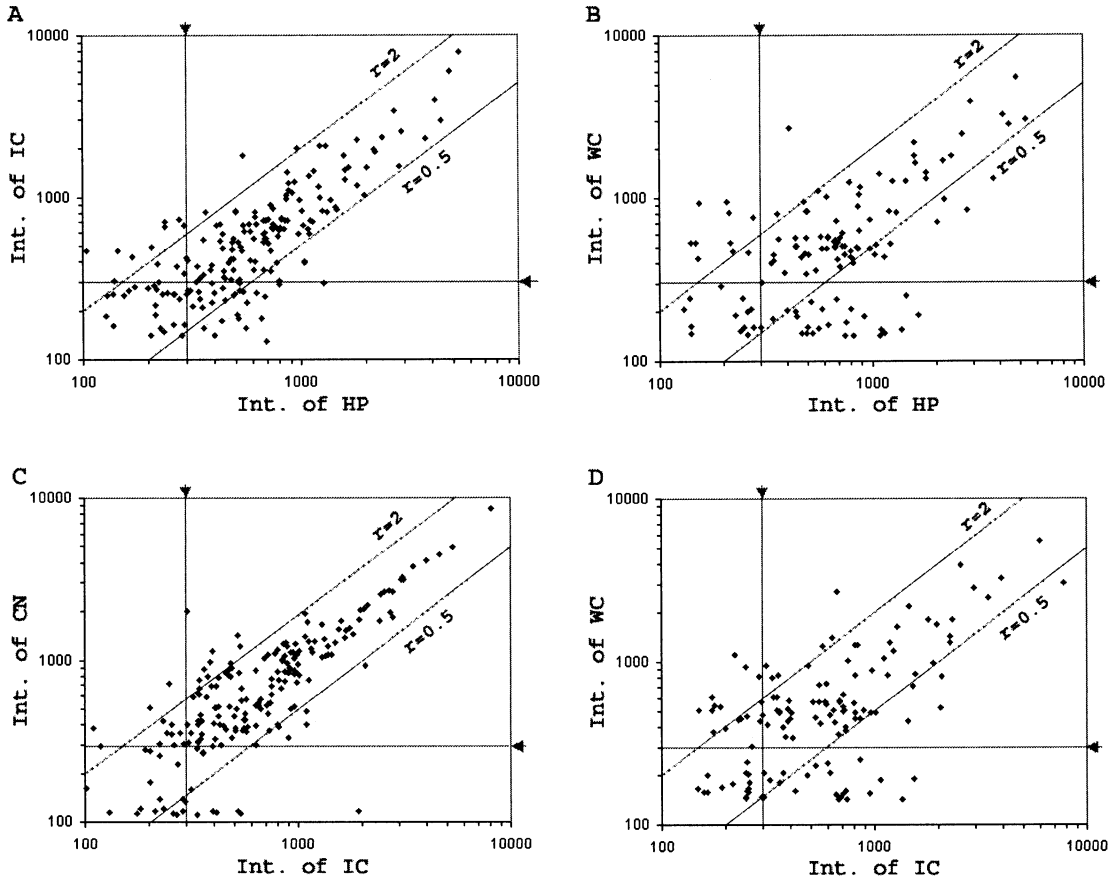

FIG. 3. Comparison of gene expression profiles. Two central auditory regions and the whole cochlea were compared with either the hippocampus (HP) (**A, B**) or to the inferior colliculus (**C, D**). Twofold changes (r = intensity in array 2/intensity in array 1 > 2 or < 0.5) are indicated. The arrow indicates the signal threshold (twice background) of each membrane.

TABLE 3

List of genes differentially expressed between the IC and the HP[a]

Location	*Protein/gene*	*Ratio*[b]	*Difference*[c]
	Higher expression in the IC compared with the HP		
E7j	Beta-alanine-sensitive neuronal GABA transporter	3.3	1256
C7d	Secretogranin II precursor (SGII; SCG2); chromogranin C (CHGC)	2.1	1040
	Lower expression in the IC compared with the HP		
B4l	Cell adhesion kinase beta (CAK beta); calcium-dependent; FAK family	0.1	−1145
C7l	Glutamate receptor 1 precursor (GluR-1); GluR-A; GluR-K1	0.2	−987
B5j	Transducin beta-1 subunit; GTP-binding protein G(i)/G(s)/G(t) beta subunit 1	0.4	−651
B6a	Ras-related protein Rab2	0.4	−632
E7g	Ehk 3; ephrin type-A receptor 7; tyrosine kinase (Eph-related); EphA7	—[d]	−752

[a]Averaged values of three arrays for the IC and four arrays for the HP were used for comparison.
[b]Ratio = adjusted intensity of IC array/adjusted intensity of HP array.
[c]Difference = adjusted intensity of IC array - adjusted intensity of HP array.
[d]— = the signal ratio was not calculated when the adjusted intensity is background level in either array.

However, the expression patterns of apoptosis-related genes and neuronal genes, growth factor receptors and channels, and proteases and inhibitors were very different between CNS and the cochlear regions. The cochlear regions expressed two types of myelin proteins: PLP (C3f) and PMP-22 (C3h), while CNS expressed largely PLP. The cochlear regions showed a high level of expression in TGFβ II receptor (E1n) and sensory neuron-specific proton gated cation channel (E6n), while the CNS expressed high levels of glutamate transporter (E6i) and GABA transporter (E7j). The CNS expressed a high level of somatostatin

TABLE 4

List of genes differentially expressed between the WC and the HP[a]

Location	*Protein/gene*	*Ratio*[b]	*Difference*[c]
	Higher expression in the WC compared with the HP		
C3h	SR13 myelin protein; peripheral myelin protein 22 (PMP-22); CD25 protein	6.6	2295
A5n	$p27^{Kip1}$	6.0	776
F6b[e]	Metaloproteinase inhibitor 3 precursor; tissue inhibitor of metalloproteinase 3 (TIMP3)	4.6	750
D2a	Fatty acid-binding protein (heart; H-FABP)	3.8	599
F2b[e]	Insulin-like growth factor-binding protein (rIGFBP6)	—[d]	970
E1n	Transforming growth factor-beta II receptor precursor (TGF-beta II receptor; TGFBR2)	—	825
E6n	Proton gated cation channel drasic; "AQ6" sensory neuron specific	—	757
F4d[e]	Matrix metalloproteinase 14 (MMP14); membrane-type MMP 1 (MT-MMP1)	—	698
D3f	Lecithin:cholesterol acyltransferase (EC 2.3.1.43; LCAT)	—	606
E2k	Insulin receptor precursor (INSR; IR)	—	605
F6i[e]	Gelatinase A	11	447
	Lower expression in the WC compared with the HP		
B6i	14-3-3 protein zeta/delta; PKC inhibitor protein-1	0.4	−2432
F5i	Carboxypeptidase E; carboxipeptidase H	0.3	−1992
C7k	GABA-B receptor 1a + GABA-B receptor 1b	0.1	−1469
C5e	Neuroendocrine protein 7B2 precursor; secretogranin V; SGNE1	0.4	−1316
C5d	Neuron-specific enolase (NSE); gamma enolase (EC 4.2.1.11)	0.5	−1224
C4l	N-methyl-D-aspartate receptor (NMDAR1); glutamate receptor subunit zeta 1 precursor	0.1	−1219
C5j	Synapsins IA and IB (SYN1)	0.2	−1200
B3i	Extracellular signal-regulated kinase 2 (ERK2); mitogen-activated protein kinase 2	0.1	−995
B4d	Protein kinase C beta-I type (PKC-beta I) + protein kinase C beta-II type (PKC-beta II)	0.1	−954
F5a	Dipeptidyl aminopeptidase-related protein (DPP6)	0.1	−944
E3c	Calcium-independent alpha-latrotoxin receptor	0.2	−733
B5i	Guanine nucleotide-binding protein alpha 12 subunit (G alpha 12; GNA12)	0.4	−720
C6g	PMCA; ATP2B2; calcium-transporting ATPase plasma membrane (brain isoform 2)	0.4	−705
C3k	G protein beta-adrenergic receptor kinase 1 (beta-ARK1; EC 2.7.1.126)	0.2	−671
D5i	SHPS-1 receptor-like protein with SH2 binding site	0.2	−608
C5k	Synapsin 2A	—	−1967
E6i	GluT and GluT-R glutamate transporter	—	−1327
C7l	Glutamate receptor 1 precursor (GluR-1); GluR-A; GluR-K1	—	−1279
B4l	Cell adhesion kinase beta (CAK beta); calcium-dependent; FAK family	—	−1227
C7f	Synaptobrevin 2 (SYB2); vesicle-associated membrane protein 2 (VAMP2)	—	−1218
B4g	Protein kinase C epsilon type (PKC-epsilon)	—	−1095
D6b	Glutamate receptor 2 precursor (GLUR-2; GLUR-B; GLUR-K2)	—	−1029
C6i	Glia maturation factor beta (GMF-beta; GMFB)	—	−1017
C7d	Secretogranin II precursor (SGII; SCG2); chromogranin C (CHGC)	—	−957
F2l	Somatostatin	—	−944
C4h	Neuromodulin; axonal membrane protein GAP43	—	−871
D5c	Elongation factor SIII P15 subunit	—	−835
A3i	c-Kit proto-oncogene	—	−773
B5m	Ras-related protein m-ras	—	−765
B7j	PKI-alpha; cAMP-dependent protein kinase inhibitor (muscle/brain form)	—	−734
E6e	Proton-coupled dipeptide cotransporter	—	−729
F5g	Proteasome subunit RC10-II	—	−688
F5b	Proteasome delta subunit precursor; macropain delta	—	−679
A4d	c-H-ras proto-oncogene; transforming G-protein p21	—	−678
A4f	c-K-ras 2b proto-oncogene; transforming G-protein p21	—	−611
D1c	Epidermal fatty acid-binding protein (E-FABP); cutaneous fatty acid-binding protein	—	−607

[a]Averaged values of two arrays for the WC and four arrays for the HP were used for comparison.
[b]Ratio = adjusted intensity of WC array/adjusted intensity of HP array.
[c]Difference = adjusted intensity of WC array - adjusted intensity of HP array.
[d]— = the signal ratio was not calculated when the adjusted intensity is the background level in either array.
[e]Genes chosen for RT-PCR assay.

(F21), dipeptidyl aminopeptidase-related protein (F5a), and carboxypeptidase E/H (F5i). However, the cochlear regions expressed a high level of IGFBP-6 (F2b), MT-MMP 1 (F4d), and TIMP-3 (F6b). The filter hybridization images of six regions are available on our webpage (www.khri.med.umich.edu/genearray/).

RT-PCR analysis

RT-PCR was used to verify expression of six genes that showed higher expression in the cochlear regions than in the CNS regions and that had not been previously reported to be expressed in the cochlea: LIMK-1, IGFBP-2, IGFBP-6, gelatinase A/MMP-2, MT MMP-1/MMP-14, and TIMP-3. The differences in the expression levels of IGFBP-6, MT MMP-1, and TIMP-3 were large enough to meet our criteria for a significant difference; twofold ratio and twofold background in signal difference (Table 4). RT-PCR confirmed that these three genes were highly expressed in the cochlear regions (Fig. 4). IGFBP-2 was differentially expressed between the MOD and the SE in the gene array study (Table 1) and RT-PCR confirmed the highest expression level of IGFBP-2 in the SE (Fig. 4). Gelatinase A was also chosen for RT-PCR verification, although the expression difference between the WC and the HP did not meet the criteria. RT-PCR revealed a high expression of gelatinase A in the cochlear regions and the CN. The expression level of gelatinase A was in fact not detectable in the IC and HP but detectable with a significant level of intensity in the cochlear regions and the CN. This example shows that while our stringent threshold for a significant difference provides for high confidence in the results, it can allow genes with a small difference between two regions to be missed. LIMK-1 was expressed at high level in the WC and the modiolar subfraction. Our studies that used membranes with a larger number of genes (unpublished data) showed that the WC expressed a high level of cofilin that is phosphorylated only by LIMK-1 (Yang et al. 1998). LIMK-1 and cofilin are known to be involved in the actin dynamics (Arber et al. 1998). RT-PCR confirmed the higher expression of LIMK-1 in the cochlear regions than in the CNS (Fig. 4).

DISCUSSION

These gene expression profiling studies detected expression of genes in the cochlea, CN, and IC that had not previously been reported. For example, Crk adapter protein, syntaxin binding protein, Sec1, clusterin, TIMP-2, and others were expressed at high levels in all auditory regions examined in this study. Our gene expression data are largely consistent with previous studies. We could detect genes, such as $p27^{Kip1}$ and P2X(2) receptor, whose expression was previously reported in the whole cochlea. TIMP-3, PMP-22, TGFBR2 and 3, and others were also reported in the human fetal cochlear cDNA library. This gene profiling study also revealed differences in gene expression among the six regions tested. We found, not unexpectedly, greater differences between the CNS regions and the cochlea than between the auditory and nonauditory brain regions. These facts suggest that even small subsets of genes can provide fruitful information of gene expression profiles.

Neurotransmitter receptors

Our gene expression profiles showed differences among the HP, IC, and CN in the expression of many neurotransmitter-related genes, and these results were consistent with previous findings. The neurotransmitter receptor genes reported as positive are indeed those which have been reported to have high expression, such as NMDAR1 (Barnes–Davies and Forsythe 1995; Bilak et al. 1996; Kuriyama et al. 1993; Niedzielski et al. 1997; Safieddine and Eybalin 1992; Sato et al. 1998). The CN has low expression of GluR1 compared with other regions (Hunter et al. 1993). The HP shows a much higher expression of GluR1 than the IC (Wenthold et al. 1996). There is high glycinergic input to the CN compared with the HP with the IC intermediate and no known glycinergic input to the cochlea (Altschuler et al. 1986a, 1986b; Sato et al. 1995, 2000a). The GlyR α1 subunit is expressed more highly in the CN than in the IC and below the threshold in the HP and cochlear regions. GABA is well represented in the IC and HP, with inputs also present in the CN and cochlea (Altschuler et al. 1986b; Sato el al. 2000b). The HP and the IC had the greatest expression of GABA-AR subunits, with the β2 subunit highest in the IC. While no GABA-AR subunit was detected in cochlear fractions, the GABA-B 1a/1b receptor was expressed in the MOD and WC, consistent with the literature (Siddique et al. 2000). The GABA-B 1a/1b receptor was also very highly expressed in the HP, IC, and CN.

The SE subfraction expressed the P2X(2) receptor but the MOD did not, suggesting a role in the organ of Corti or stria vascularis, consistent with previous reports (Housley et al. 1999; Jarlebark et al. 2000). It was not expressed above our threshold for any of the brain regions assessed. The positive expression of somatostatin in the IC and CN and of substance P in the IC but not in the CN is consistent with previous reports of their expression in these areas (Wynne et al. 1995; Wynne and Robertson 1997).

Cyclins, CDKs, and CKIs; $p27^{Kip1}$

The Atlas Rat cDNA Expression Array contains genes for seven cyclins (A4n–A5f), five CDKs (A5g–A5k), and four CKIs (A5l–A6a). Most of these genes were expressed at a low level in all six regions, whereas $p27^{Kip1}$, one of the CKIs, was detected at a higher level in the cochlea and its subregions than any other CNS regions examined in this study.

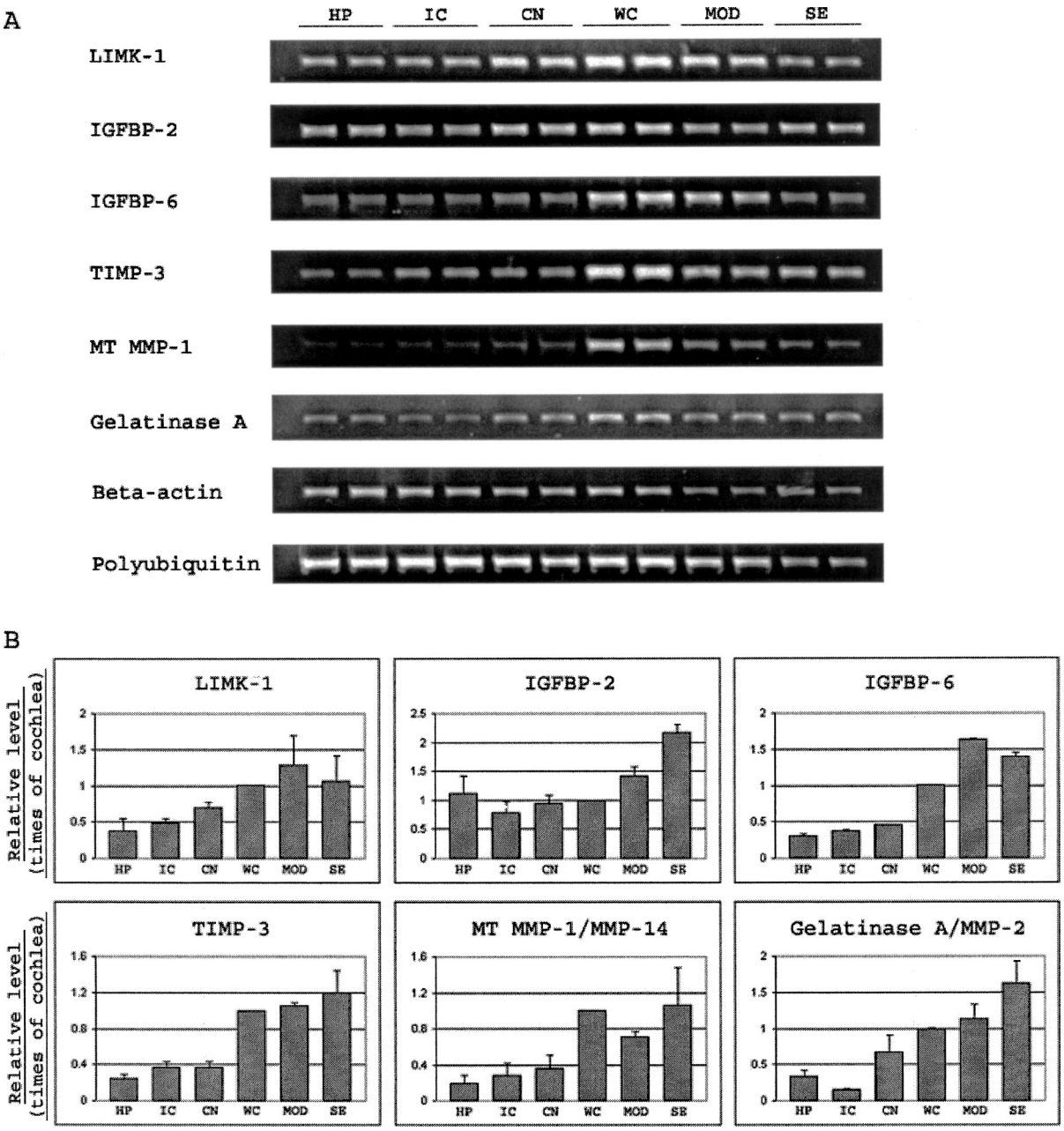

FIG. 4. RT-PCR analysis of mRNA expression in the auditory regions and the hippocampus. RT-PCR assay was performed on the total RNA from the hippocampus (HP), the inferior colliculus (IC), the cochlear nucleus (CN), the whole cochlea (WC), the modiolus (MOD), or the sensorineural epithelium (SE) for mRNA expression of the indicated genes. **A** Agarose gene electrophoresis of PCR products. Aliquots (10 μL) of the PCR reactions were loaded in duplicate onto agarose gels. The PCR reactions were sampled at 30 cycles for the first six genes and at 25 and 27 cycles for β-actin and polyubiquitin, respectively. **B** Quantitative analysis of the PCR products. The value of the mean + standard deviation from two or three PCR experiments is plotted. The raw intensity volumes of the PCR bands obtained from IPLab software were converted into a relative value to the whole cochlea (the intensity of the whole cochlea is given as 1) and then normalized to the housekeeping genes β-actin and polyubiquitin.

Many roles have been assigned to $p27^{Kip1}$ such as tumor suppressor and cell differentiation factor. Decreased expression level of $p27^{Kip1}$ is associated with several human cancers (Lloyd et al. 1999). $p27^{Kip1}$-deficient mice in adulthood are larger than normal mice without gross morphological deformation and they develop pituitary tumors at a later stage in life (Fero et al. 1996; Kiyokawa et al. 1996; Nakayama et al. 1996). Recent studies on the knockout mice reported that $p27^{Kip-/-}$ mice have abnormal morphology in the organ of Corti (supernumerary hair cells and supporting cells) and impaired hearing with 30–

50 dB shifts throughout broad ranges of hearing frequency (Chen and Segil 1999; Lowenheim et al. 1999). The expression of $p27^{Kip1}$ in the primordial organ of Corti is detected between E12 and E14 when the developing hair cells and supporting cells undergo their terminal division; $p27^{Kip1}$ continues to be expressed in the supporting cells of adult animals (Chen and Segil 1999). These studies suggest an important role of $p27^{Kip1}$ in inner-ear development. Our gene profile results strengthen the conclusion that $p27^{Kip1}$ is also important in the mature ear. Since the expression of $p27^{Kip1}$ is limited to supporting cells in the organ of Corti, determination of the $p27^{Kip1}$ cellular localization in the modiolus will be valuable.

MMPs and TIMPs

Matrix metalloproteinases (MMPs) are proteolytic enzymes that degrade extracellular matrix (ECM) proteins such as collagens and proteoglycans, and TIMPs regulate the activity of MMPs. MMPs and TIMPs are also regulated at the level of transcription, which means an environmental stimulus can modulate the expression level. These complex aspects subject MMPs to a very precise temporal and spatial control (Crawford and Matrisian 1996).

The Atlas Rat cDNA Expression Array includes six MMPs: MMP-2 (F6i), MMP-7 (F4b), MMP-10 (A4m), MMP-11 (C2n), MMP-14 (F4d), and MMP-16 (F7b) and three TIMPs: TIMP-1 (F4g), TIMP-2 (F4h), and TIMP-3 (F6b). In our gene profiling and RT-PCR assays, MMPs and TIMPs showed distinct expression patterns between three CNS regions and the cochlear tissues; the cochlea expresses exclusively a high level of MMP-2/gelatinase A, MMP-14/MT MMP-1, and TIMP-3 compared with the CNS regions.

Putative physiological functions of the MMP and TIMP system are listed as tissue remodeling, angiogenesis, and wound healing (Yong et al. 1998). MMPs and TIMPs are also highly implicated in human disease such as cancer (Crawford and Matrisian 1996; Okada et al. 1995; Sternlicht et al. 1999) and multiple sclerosis (Yong et al. 1998). $MMP\text{-}9^{-/-}$ mice exhibit abnormal angiogenesis and ossification of the skeletal growth plate in young animals (Sternlicht et al. 1999). $MMP\text{-}14^{-/-}$ mice have defects in the formation of skeletal connective tissue and die between days 50 and 90 (Holmbeck et al. 1999). Patients with Sorsby's fundus dystrophy (SFD) have a mutation on TIMP-3 (Weber et al. 1994). SFD is a hereditary, autosomal dominant, macular degenerative disease resulting in irreversible visual loss, although it is not clear whether patients with SFD have abnormal hearing or not. The observation that the cochlea expresses a higher level of MMP-2, MMP-14, and TIMP-3 mRNAs than the CNS region is intriguing, although the precise function of these proteins in the cochlea is not known yet.

IGFs and IGFBPs

IGFs are growth factors and the accessibility of IGFs to the tissues is controlled by IGFBPs (Ferry et al. 1999; Rosenfeld et al. 1999; Wetterau et al. 1999). The Atlas Array contains two IGFs: IGF-I (F2j) and IGF-II (F2g), two IGFRs: IGFI-Rα (E2b) and IGF-2R (A4l), and four IGFBPs: IGFBP-1 (F1a), -2 (A4k), -3 (F1b), and -6 (F2b). Our profiles showed that the expression pattern of IGFBPs was different between the CNS regions and the cochlea. IGFBP-2 (A4k) was expressed in all six regions but the expression level was highest in the SE subfraction. IGFBP-6 was detected in the cochlea and its subfractions at a very high level but not in the CNS regions. Morton's human fetal cochlear cDNA library reported the expression of IGF-I and IGFBP-1, -3, and -5 and our gene expression profile showed IGF-2 and IGFBP-2 and -6. The expression of IGFBPs could be regulated at developmental stages (Cerro et al. 1993; Green et al. 1994).

The expression patterns of IGFBPs are also tissue-specific. IGFBP-1 is the major IGFBP in the amniotic fluid, IGFBP-3 in the serum, and IGFBP-2 and -6 in the cerebrospinal fluid (Ferry et al. 1999). The biological functions of the IGFBP complex—IGFBP-2, -4, and -6—are inhibitory for IGF-I whereas IGFBP-5 potentiates the IGF-I action (Murphy 1998). Some of the IGFBPs are known to have IGF-independent effects as well; IGFBP-1 has an effect on migration of vascular smooth muscle cells *in vitro* regardless of the presence of IGF-I (Ferry et al. 1999; Murphy 1998). IGFBP-2 was elevated in CSF from children with malignant CNS tumors (Muller et al. 1994). The phenotype of the IGFBP-2 knockout mouse was very subtle (Wood 1995; Wood et al. 1993). Therefore, the evaluation of the physiological roles of IGFBP-2 and -6 in the cochlea will remain for future studies.

Methodological considerations

One question we had was whether the WC preparation would be biased toward genes expressed in the modiolus, which represents the greatest fraction (80%) of the tissue and thus cause us to miss expression of genes specific to the organ of Corti and/or stria vascularis. We found, for the most part, that genes selectively expressed in the SE vs. the MOD could also be detected in the WC. The P2X(2) receptor, the Ear-3 transcription factor, bFGF-R, and IGF II were all detected in both the WC and the SE subfractions but not in the MOD. Thus, we can conclude that most genes expressed in the organ of Corti will be detected in a WC preparation. This may be related to a technical

advantage of this Clontech gene array. The use of gene-specific primers in order to make the radiolabeled cDNA probe could increase the sensitivity. On the other hand, growth hormone was expressed in the SE but was not detected in the MOD or WC. The enrichment provided by subdividing may be necessary to see less abundantly expressed genes. This suggests that a further subdivision of the SE into enriched organ of Corti and lateral wall subfractions might be of benefit in future studies.

Clearly, our results identified many genes with low expression that fall below the threshold levels that we set. Several neurotransmitter receptors were reported as expressed in auditory brainstem regions were detected but were below the threshold in this study. For example, expression of GluR 2/3 has been shown in spiral ganglion cells of the cochlea (Niedzielski et al. 1995); however, expression in WC or MOD preparations was below our threshold in the current study. Therefore, it is important that a negative result in the current study not be interpreted to mean that there is no expression of that gene. All that can be concluded is that the expression is relatively low compared with genes that could be detected by this method.

CONCLUSION

Normal gene expression profiling using gene arrays provides a global picture of the biology of a specific region, and the differences in gene expression among regions reflect the morphological and functional differences among tissues. We believe that gene expression profiling will help us to understand the biological events occurring in the auditory system under normal conditions and to follow abnormal stimuli resulting from environmental stresses or from disease states.

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