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Molecular markers for cell types of the inner ear and candidate genes for hearing disorders

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ABSTRACT **To identify genes expressed in the vertebrate inner ear, we have established an assay that allows rapid analysis of the differential expression pattern of mRNAs derived from an auditory epithelium-specific cDNA library. We performed subtractive hybridization to create an enriched probe, which then was used to screen the cDNA library. After digoxigenin-labeled antisense cRNAs had been transcribed from hybridization-positive clones, we conducted *in situ* hybridization on slides bearing cryosections of late embryonic chicken heads, bodies, and cochleae. One hundred and twenty of the 196 clones analyzed encode 12 proteins whose mRNAs are specifically or highly expressed in the chicken's inner ear; the remainder encode proteins that occur more widely. We identified proteins that have been described previously as expressed in the inner ear, such as β-tectorin, calbindin, and type II collagen. A second group of proteins abundant in the inner ear includes five additional types of collagens. A third group, including Coch-5B2 and an ear-specific connexin, comprises proteins whose human equivalents are candidates to account for hearing disorders. This group also includes proteins expressed in two unique cell types of the inner ear, homogene cells and cells of the tegmentum vasculosum.**

Developmental analysis of the inner ear is hindered by the paucity of molecular markers for specific cell types. At present, most cells in the cochlea and vestibular organs cannot be unambiguously identified before they display their characteristic mature forms. The cloning of cDNAs that encode proteins specific to hair cells, supporting cells, and other cells of the inner ear is, in turn, retarded by the limited numbers of these cells in an animal. This disadvantage potentially can be circumvented through enrichment of specific cDNAs by subtractive hybridization (1–3) and library normalization (4). That only a few inner ear-specific cDNAs have been obtained from libraries constructed by this approach (2, 3), however, suggests that the efficient cloning of cDNAs specific to sensory-epithelial cell types requires libraries constructed from more abundant sources than whole cochleae. To address this problem, we constructed a cDNA library from sensory epithelia of the chicken's cochlea and developed an enriched probe with which to identify cDNAs encoding proteins expressed by specific cell types of the inner ear.

The identification of proteins specific to the internal ear is also potentially useful in the study of heritable human deafness, which afflicts about one individual in a thousand. Several proteins that have been identified as markers for specific aural cell types recently have been advanced as candidate genes for human hearing disorders (for reviews see refs. 5–8). Some of the genes identified by the strategy described in this paper are likely to have human equivalents that cause hearing disorders when mutated.

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MATERIALS AND METHODS

Library Construction and Screening. Basilar papillae from 250 late-embryonic (E, embryonic day) (8% E14, 14% E15, 16% E16, 26% E17, 20% E18, and 16% E19) chickens (*Gallus gallus*) of the white Leghorn strain (SPAFAS, Norwich, CT) were removed and transferred into PBS solution at pH 7.2. Sensory epithelia were carefully dissected from surrounding tissues and frozen in liquid nitrogen. Total RNA was isolated by isopycnic centrifugation through a cesium trifluoroacetate gradient (9). After enrichment for poly(A)+ RNA (PolyATtract, Promega), 5 μg of poly(A)+ RNA was used as starting material for the construction of a unidirectional cDNA library (HybriZAP Two-Hybrid cDNA Cloning Kit, Stratagene).

An independent subtractive procedure was used to generate a probe for library screening. The tester was constructed by reverse transcription of 500 ng poly(A)+ RNA from E17 auditory epithelium; 50 μg poly(A)+ RNA from a mixture of E17 retina, brain, and liver served as a source for the driver (PCR-Select cDNA Subtraction Kit, CLONTECH). After the probe had been normalized, it was enriched by a single subtraction. High-stringency screening was performed with randomly primed (10), ^{32}P-labeled DNA probes at 65°C (11). *In vivo* excision of the recombinant pAD-GAL4 phagemid vectors from the HybriZAP vectors (12) was conducted with the helper bacteriophage ExAssist and XL1-Blue MRF′ (Stratagene) as the host strain of *Escherichia coli*.

***In Situ* Hybridization.** Digoxigenin-labeled antisense probes were synthesized (DIG RNA Labeling Kit, Boehringer Mannheim) from 500 ng of linearized plasmid DNA and resuspended in 50 μl water. *In situ* hybridization on cryosections was conducted by a modification of a published protocol (13). In brief, 14-μm frozen sections were cut with a cryomicrotome (CM3000, Leica), collected on silylated slides (PGC Scientific, Gaithersburg, MD), dried at 37°C for 45 min, and stored frozen at −70°C. The standard slides included sections of cochleae, heads, and bodies from E16 or E17 embryos.

For hybridization, the sections were brought to room temperature, rehydrated in 100 μl diluted probe (1:100) in 50% (vol/vol) formamide/10% (wt/vol) dextran sulfate/1 mg·ml^{-1} yeast RNA/1× Denhardt's solution/185 mM NaCl/5.6 mM NaH_2PO_4/5 mM Na_2HPO_4/5 mM EDTA/15 mM Tris at pH 7.5. After coverslipping and overnight incubation at 65°C in a chamber humidified with 50% (vol/vol) formamide in 150 mM NaCl/15 mM trisodium citrate, pH 7 (1× SSC), the coverslips were removed in 5× SSC and the slides were washed twice for 30 min each in 50% (vol/vol) formamide/0.1% (vol/vol) polyoxyethylene sorbitan monolaurate (Tween-20) in 1× SSC

Abbreviations: E, embryonic day; Cx, connexin.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. AF012252 (chicken Coch-5B2), AF072699 (connexin 31), AF042795 (homogenin), and AF072698 (otokeratin)].

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at 65°C. Thereafter, the slides were washed for 15 min in 0.2× SSC and for 15 min in PBS at room temperature.

For antibody detection, the sections were blocked for 1 hr in 0.5% (wt/vol) blocking powder (Boehringer Mannheim)/10% (vol/vol) heat-inactivated goat serum/0.1% (vol/vol) Tween-20 in PBS. The slides were then incubated for 2 hr at room temperature in blocking solution preincubated for 1 hr with alkaline phosphatase-conjugated anti-digoxigenin Fab fragments (1:1,500; Boehringer Mannheim). Unbound Fab fragments were removed by washing twice for 30 min each in 0.1% (vol/vol) Tween-20 in PBS and twice for 20 min each in PBS. For detection, the sections were covered with 100 μl of nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate substrate (1-STEP NBT/BCIP, Pierce), coverslipped, and incubated overnight at room temperature in a humidified chamber. Photography was conducted with slide film (Ektachrome 160T, Eastman Kodak) in a MC80 camera on an Axiovert 135 microscope (Zeiss).

Northern Blotting. Twenty-five micrograms of denatured total RNA was separated on 1% formaldehyde-agarose gels. The RNA was transferred (11) to Hybond-N (Amersham) and incubated at 70°C with randomly primed (10), ^{32}P-labeled probes in hybridization solution (ExpressHyb, CLONTECH). After having been washed with 0.1% SDS in 0.2× SSC at 68°C, the filters were exposed to film (XAR-5, Eastman Kodak).

Sequence Analysis. cDNAs were sequenced in the Protein/DNA Technology Center of The Rockefeller University. Sequence analysis was performed on Macintosh computers (Apple) running the LASERGENE software package (DNAstar, Madison, WI). Amino acid sequences were analyzed (14, 15) with LASERGENE software and PSORT (http://psort.nibb.ac.jp).

RESULTS

To identify cDNAs encoding proteins expressed in specific cell types of the inner ear, we constructed a library from mRNA derived from 500 auditory epithelia of chicken embryos (E14–E19). Because it was not always possible to entirely remove the auditory ganglion, the source material included hair cells, supporting cells, ganglion cells, and other cells bounding the sensory epithelium. Analysis of 20 randomly selected clones revealed an average insert size of 1,500 bp, with sizes ranging from 550 bp to 3,200 bp.

Screening for Cochlea-Specific mRNAs. Ten thousand plaque-forming units of the auditory-epithelium cDNA library were plated at single-clone density and probed with an enriched probe that was generated by subtracting auditory-epithelium cDNAs with those derived from the retina, brain, and liver. One hundred ninety-six hybridization-positive clones were eluted, *in vivo*-excised, and partially sequenced. The expression patterns of the corresponding mRNAs were assessed by *in situ* hybridization on sections of embryonic chicken cochleae, heads, and bodies. The mRNAs represented by 120 of the clones were highly expressed in the inner ear, which was in some instances the exclusive site of expression.

Sequence analysis of these 120 isolates revealed clones encoding 12 proteins. Several of these, including β-tectorin (20 clones), calbindin (10 clones), and type II collagen (16 clones), had been described previously as specifically or highly expressed in the inner ear (16–19). In confirmation of the expression pattern observed earlier (20), β-tectorin mRNA was detected exclusively in the inner ear (Fig. 1*A*), where it occurred in supporting cells, clear cells, and cuboidal cells. Highly abundant in supporting cells, calbindin mRNA was also present in hair cells (Fig. 1*B*). Type II collagen mRNA was highly expressed in cartilage cells of the cochlea's longitudinal supports, the inferior and superior fibrocartilaginous plates (Fig. 1*C*). This mRNA was also detectable in spindle-shaped cells located between the auditory ganglion cells and the sensory epithelium. Although the strongest labeling for type II collagen was observed in the inner ear, its expression was not restricted to this organ: mRNA was detected in cartilaginous tissues throughout the body, with the eye cup a second site of relatively strong labeling (data not shown). Five additional collagen mRNAs were found to be expressed in the inner ear in a pattern similar to that of type II collagen; these included α-1 type IX collagen (five clones), α-1 type XI collagen (two clones), α-2 type I collagen (four clones), α-3 type IX collagen (11 clones; GenBank accession no. M83179), and short collagen (16 clones; accession no. K01702).

Coch-5B2. Twenty-seven clones encoded the chicken orthologue of the previously described cochlear protein Coch-5B2 (Fig. 2*A*; refs. 2 and 21), the mRNA for which appears to be expressed exclusively in mechanosensitive systems. In the basilar papilla, this mRNA was found in spindle-shaped cells located between the auditory ganglion and the sensory epithelium (Fig. 1*D*); it was also detectable in the inferior and superior fibrocartilaginous plates. The only other tissue with detectable expression of chicken Coch-5B2 was skeletal muscle, in which a few areas displayed very intense labeling. Labeling of alternate sections with Coch-5B2 antisense probe and with α-bungarotoxin demonstrated that hybridization did not occur at neuromuscular junctions (data not shown). Instead, all labeled cells were found to be surrounded by the capsules of muscle spindles (Fig. 1 *E* and *F*). Some capsules encircled intensely stained cytoplasm around chains of nuclei (Fig. 1*F*), demonstrating a strong expression of Coch-5B2 in nuclear-chain intrafusal muscle fibers.

Connexin 31. Our screening provided two independent isolates of a cDNA whose *in situ* pattern was confined to the basilar papilla (Fig. 1*G*). Strong labeling was detected in supporting cells, cells of the tegmentum vasculosum, cuboidal cells, and clear cells. No hybridization was detected in any other part of the body examined, including lung, liver, heart, stomach, muscle, skin, cartilage, midbrain, forebrain, spinal cord, and retina.

Sequence analysis revealed that this cDNA encodes a member of the connexin (Cx) family of gap-junction proteins. Conceptual translation of the coding sequence unveiled a 263-aa protein with a theoretical molecular mass of 31 kDa. The protein, hereby named chicken Cx31, displays 80% amino acid identity with murine Cx26 and 72% identity with murine Cx30 (Fig. 2*B*). Because these mammalian connexins are widely expressed, we performed Northern blot analysis by using a chicken Cx31 probe with total RNA derived from hatchling tissues, including those in which mammalian connexins have been found (22–26). A single mRNA species of 2.2 kb was detected in total RNA prepared from hatchling cochleae (Fig. 3*A*); no signals were found in total RNA prepared from any other organ. These data suggest that Cx31 is a member of the connexin gene family that is restricted to the inner ear.

Homogenin. Six clones represented an mRNA that was extremely abundant in homogene cells of the basilar papilla (Fig. 1*H*). Because of this unique expression pattern, the name homogenin was assigned to the cognate protein. Sequencing revealed a protein with 75% identity to human gelsolin and 61% identity to bovine adseverin (Fig. 2*C*). Weak hybridization signals for homogenin mRNA also were observed in a subcutaneous layer of the skin (data not shown). No hybridization signals were detected in the other areas of the body examined, including lung, stomach, liver, skeletal muscle, cartilage, spinal cord, retina, and forebrain. Because members of the gelsolin gene family regulate the state of actin polymerization (27), we examined the distribution of filamentous actin in the chicken's cochlea by labeling with rhodamine-conjugated phalloidin. In addition to the signal from the actin-filled stereocilia of hair cells, intense labeling was observed in the cytoplasm of homogene cells, suggesting a high concentration of filamentous actin there (Fig. 1*I*).

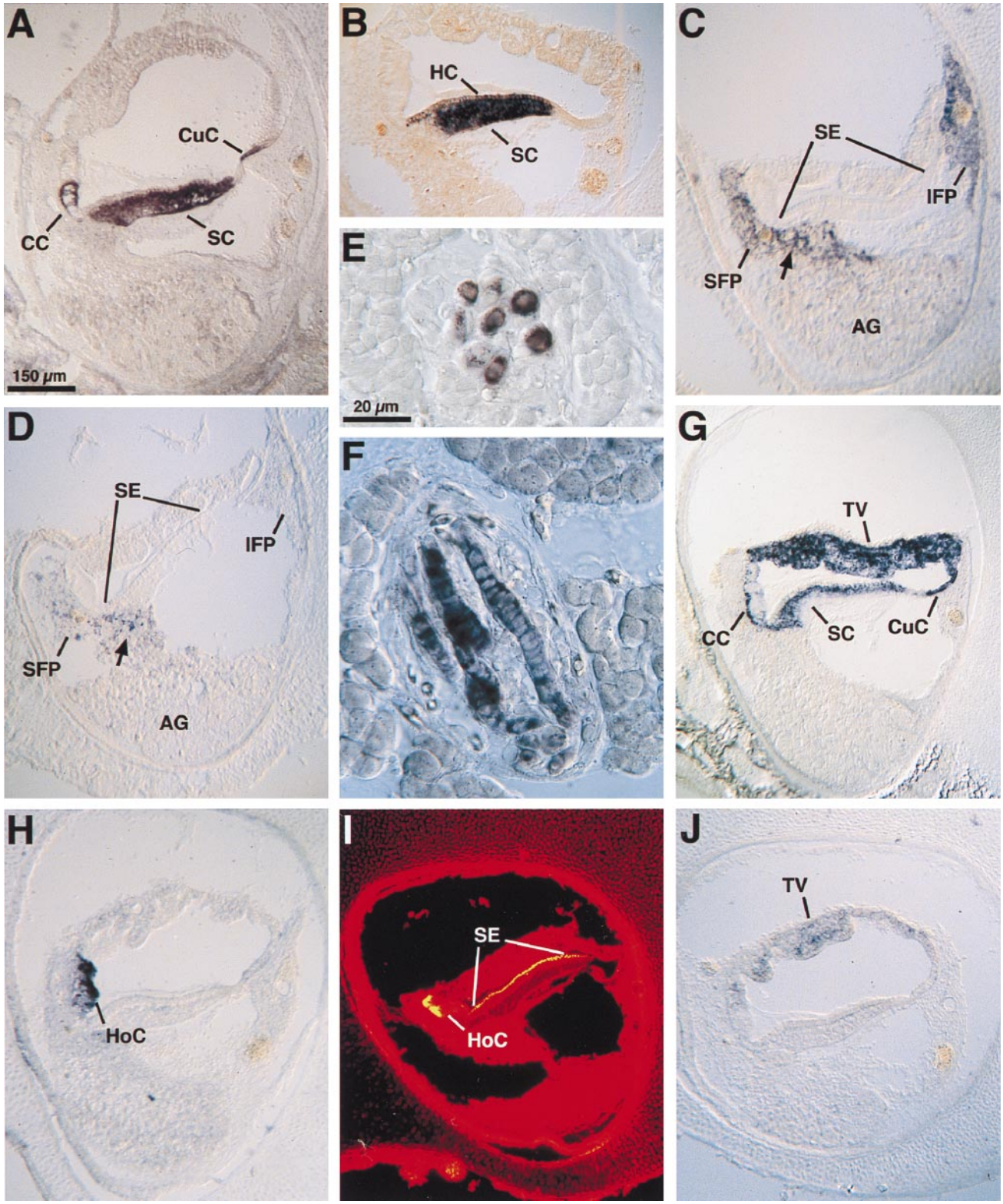

FIG. 1. *In situ* hybridization analysis of cDNAs expressed in the inner ear. (*A*) On a cross-section of the basilar papilla, β-tectorin mRNA occurs in clear cells (CC), supporting cells (SC), and cuboidal cells (CuC). (*B*) Calbindin mRNA is abundant in supporting cells (SC) and detectable in hair cells (HC). (*C*) Strong signals for type II collagen mRNA are visible in the superior fibrocartilaginous plate (SFP) and inferior fibrocartilaginous plate (IFP) and, as indicated by the arrow, between the auditory ganglion (AG) and sensory epithelium (SE). All collagen mRNAs found in the inner ear were expressed in a similar manner. (*D*) Coch-5B2 mRNA occurs in spindle-shaped cells (arrow) marking the path of neurites between the auditory ganglion (AG) and sensory epithelium (SE). Weaker expression is detectable in the superior and inferior fibrocartilaginous plates (SFP and IFP). (*E* and *F*) Coch-5B2 mRNA displays strong labeling of muscle spindles in the gastrocnemius muscle; note the encapsulation and the nuclear chain in (*F*). (*G*) Connexin 31 mRNA is expressed robustly in cells of the tegmentum vasculosum (TV), cuboidal cells (CuC), supporting cells (SC), and clear cells (CC). (*H*) Homogenin mRNA is detectable in homogene cells (HoC). (*I*) The intense yellow fluorescence of rhodamine-coupled phalloidin signals a high abundance of filamentous actin in homogene cells (HoC) as well as in hair bundles of the sensory epithelium (SE). (*J*) Otokeratin mRNA occurs in cells of the tegmentum vasculosum (TV).

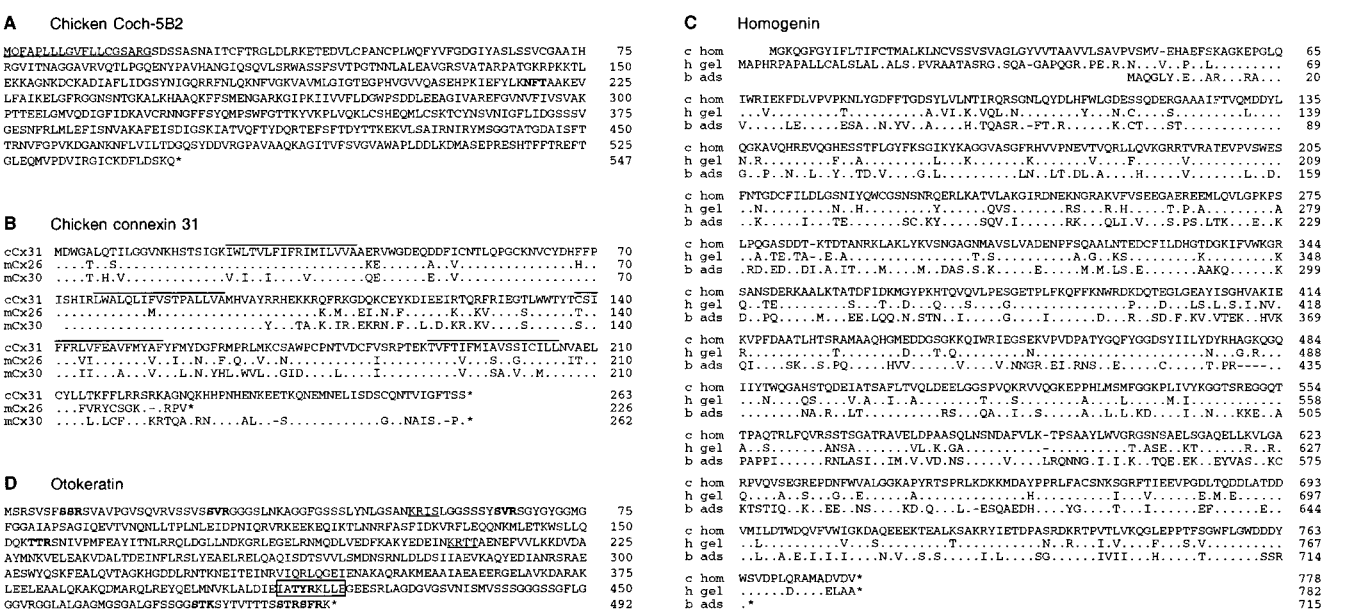

FIG. 2. (*A*) Amino acid sequence of chicken Coch-5B2. A predicted signal peptide at the amino terminus is underlined; a putative *N*-glycosylation site (N-X-[S/T]) at amino acid positions 218–220 is printed in bold. (*B*) Comparison of the deduced chicken connexin 31 (cCx31) amino acid sequence with those of murine connexins 26 (mCx26) and 30 (mCx30). Identical amino acid residues are marked with dots, and gaps are indicated by dashes. Potential transmembrane domains of chicken connexin 31 at amino acid positions 23–39, 76–92, 138–154, and 189–205 are indicated by lines. (*C*) Comparison of the chicken homogenin amino acid sequence with those of human gelsolin and bovine adseverin. Identical residues are marked with dots, and gaps are indicated by dashes. Note that the similarities of homogenin to gelsolin and to adseverin are distributed nearly equally throughout the protein. (*D*) Amino acid sequence of chicken otokeratin. Two putative cAMP- and cGMP-dependent protein kinase phosphorylation sites ([R/K]-[R/K]-X-[S/T]) are underlined. Eight putative protein kinase C phosphorylation sites ([S/T]-X-[R/K]) are printed in bold. The intermediate-filament signature motif ([I/V]-X-[T/A/C/I]-Y-[R/K/H]-X-[L/M]-L-[D/E]) is boxed.

Northern blot analysis with total RNA prepared from several hatchling tissues revealed a high abundance of a single mRNA species of 3.1 kb in total RNA prepared from the cochlea; heart and skin yielded moderate signals (Fig. 3*B*). Lower expression of homogenin mRNA was observed in total RNA derived from eye, forebrain, cerebellum, and muscle.

Otokeratin. The riboprobe derived from one clone labeled cells of the tegmentum vasculosum (Fig. 1*J*), the vascularized tissue that covers the scala media. Except for weak signals in the endothelial layer of the heart, hybridization was undetectable in all other organs examined. Analysis of the cDNA insert revealed a partial sequence of a member of the cytokeratin gene family, which suggested the name otokeratin. Screening of the auditory-epithelium cDNA library with this fragment led to the isolation of a cDNA clone that encompassed the presumptively complete coding sequence of chicken otokeratin, a protein with 492 amino acid residues and a theoretical molecular mass of 54 kDa (Fig. 2*D*). A comparison of the otokeratin sequence with those of other cytokeratins (28) suggests that otokeratin is most closely related to type II cytokeratins numbers 6 and 8. A partial cDNA encoding chicken's otokeratin has been described as a transiently expressed early marker for trunk ectoderm (28). Although this partial sequence and the otokeratin sequence are largely identical at the nucleotide level and thus likely stem from transcription of a common gene, there are two significant differences. In otokeratin, the amino acid sequence at positions 254–257 is ELRE; the corresponding embryonic form is DVSEG. At a second site, positions 321–330, the otokeratin sequence of DDLRNTKNEI includes a potential casein kinase II phosphorylation site (consensus sequence [S/T]-X-X-[D/E]) absent from the embryonic cytokeratin sequence GELG.

Northern blot analysis of otokeratin mRNA expression with total RNA derived from several hatchling organs revealed a single, 2.9-kb mRNA species that was abundant in the cochlea (Fig. 3*B*); prolonged exposure of the blot (14 days) disclosed weak signals from the heart and forebrain. These data suggest that otokeratin expression in the hatchling is relatively restricted to the tegmentum vasculosum of the inner ear.

DISCUSSION

A detailed understanding of hearing and equilibrium requires the identification of inner ear-specific proteins and the assignment of their functions. In addition to providing insight into normal aural development and functioning, these proteins are candidates to underlie the various forms of heritable hearing loss in humans. Here we report the initial results from a screen for cDNAs encoding proteins that are specifically expressed at late developmental stages in the chicken's cochlea. Because our analysis was confined to E16 and E17 embryos and hatchlings, we cannot assess whether the restricted expression patterns observed at these stages prevail at other times during development.

The most stringent requirement for successful cloning of inner ear-specific cDNAs is a library of high quality. This need was met by the construction of a library from late embryonic cochlear tissue, which is relatively easy to dissect because of incomplete ossification of the temporal bone before hatching. Utilization of subtractive enrichment to generate a probe for screening of a small portion of the auditory-epithelium cDNA library resulted in the identification of 120 clones whose corresponding mRNAs are highly or exclusively expressed in the chicken's cochlea. Sequence analysis revealed that these 120 clones corresponded to 12 different proteins. The identification of β-tectorin, calbindin, and type II collagen, proteins that had been described as exclusively or highly expressed in the inner ear (16, 18, 20, 29, 30), proved that the assay system based on *in situ* hybridization was effective in the detection of inner ear-specific mRNAs.

The identification of several collagen isoforms that are highly expressed in the inner ear confirms that collagens are major components of the cartilaginous extracellular matrix of the inner ear. The different collagens might be particularly

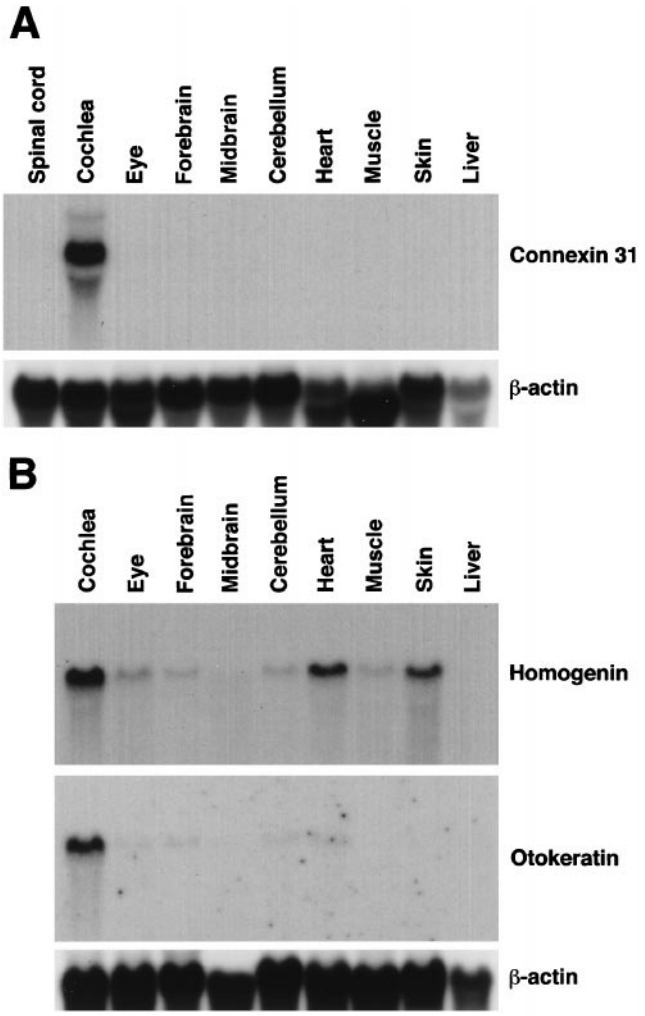

FIG. 3. Northern blot analysis of the expression of connexin 31 (*A*) and homogenin and otokeratin (*B*) transcripts in hatchling tissues. Total RNA isolated from the tissues indicated was hybridized to ^{32}P-labeled cDNA fragments covering the complete coding sequences and the 3′ untranslated regions of connexin 31, homogenin, and otokeratin. As a control for RNA loading, the blots were rehybridized to a chicken β-actin probe.

important for processes that sculpt the elaborate compartments of the inner ear during morphogenesis. This speculation is corroborated by the observation that perturbations of collagen metabolism lead to abnormal aural structure (31) and that autoimmune responses against type II and IX collagens culminate in progressive hearing loss (32, 33).

Candidate Genes for Hearing Disorders. The mammalian equivalent of chicken Coch-5B2 has been advanced as a candidate gene for the autosomal dominant human hearing disorder DFNA9 (21). Temporal-bone histopathology samples from patients with this postlingual sensorineural hearing loss reveal the deposition of mucopolysaccharide-laden substance in the extracellular space surrounding cochlear and vestibular nerve fibers. This material is believed to lead to degeneration of the sensory dendrites that carry signals from hair cells to the cochlear and vestibular ganglia (34, 35). The human gene occurs on chromosome 14 (36), where distinct missense mutations in three kindreds of DFNA9 patients were found recently to segregate with hearing impairment (N. G. Robertson and C. C. Morton, personal communication). The expression of chicken Coch-5B2 by spindle-shaped cells along nerve fibers in the basilar papilla (Fig. 1*D*) supports the hypothesis of a causal relation between Coch-5B2 and the deafness disorder DFNA9 (21). The strong expression of Coch-5B2 in muscle spindles indicates that some molecular features are shared by these mechanoreceptive organs and the cochlea.

From sequence alignments of the ear-specific Cx31 with mammalian Cx26 and Cx30 (Fig. 2*B*), it is unclear whether the avian protein represents an orthologue of one of the mammalian connexins or is a heretofore unknown member of the connexin family of gap-junction proteins (for review see ref. 37). Most of the avian protein, amino acid positions 1–182, aligns better with murine Cx26 (84% identity) than with Cx30 (74% identity). However, a comparison of the carboxyl termini from amino acid position 183 to the respective stop codons indicates a greater similarity of Cx31 to murine Cx30 (68% identity) than to Cx26 (64% identity). This relation is emphasized by the absence from murine Cx26 of the protracted carboxyl termini found in the chicken Cx31 and murine Cx30 sequences.

The expression data for Cx31 demonstrated no mRNA in brain, liver, or skin, organs that have been reported to contain high levels of mammalian Cx26 and Cx30 mRNA (22–26). Chicken Cx31 therefore may be a member of the connexin gene family that is characterized by exclusive expression in the inner ear. An alternative possibility is that the unique expression pattern of Cx31 is a species-specific trait and that Cx31 is the avian equivalent of either mammalian Cx26 or Cx30.

Mutations in human Cx26 have been identified recently as the potential cause of the nonsyndromal hearing disorders DFNB1 and DFNA3 (38). The expression pattern in the inner ear (Fig. 1*G*) suggests a high degree of intercellular communication through Cx31-bearing connexons between most of the cell types encircling the endolymph-filled scala media. It should be noted that hair cells appear to be excluded from this network, because they were not labeled with the Cx31 antisense probe. Although tight and intermediate junctions and desmosomes are found in hair cells, these cells generally do not possess gap junctions (but see ref. 39). This exclusion emphasizes the exceptional status of the hair cell within the organ of Corti and raises the question of why all other cell types are coupled to such a high degree.

Markers for Homogene Cells and the Tegmentum Vasculosum. Homogenin is a member of the gelsolin family of proteins. In view of our current lack of knowledge regarding cDNA sequences of the avian gelsolin family, we cannot designate unequivocally the protein encoded by the cDNA that we cloned from the inner ear as the chicken's ortholog of gelsolin, adseverin, or another related molecule. The *in situ* hybridization data suggest that homogene cells express very high levels of homogenin mRNA, whereas the expression in other tissues consists instead of low-level expression in many cell types.

Proteins of the gelsolin class are regulators of actin polymerization (for review see ref. 40). The high expression levels of homogenin and filamentous actin in homogene cells suggest that these cells participate in a mechanical process. Each downward movement of the basilar membrane that occurs during acoustical stimulation exerts an extensile force on the tectorial membrane; the homogene-cell cytoskeleton may counter this force. Because members of the gelsolin family regulate the extent of actin polymerization in response to Ca^{2+} and other cytoplasmic signals (27, 40), homogenin may participate in a system that adjusts the tension in the tectorial membrane.

Otokeratin mRNA was found in cells of the tegmentum vasculosum. Because all tissues that separate the different compartments of the inner ear must provide mechanical barriers that withstand pressure changes, one might expect specializations of the intermediate filament network. That otokeratin contributes to mechanical stability is suggested by the molecule's expression in the endothelial cell layer of the cardiac ventricles, a tissue that is exposed to constant mechanical stress by deformation and pressure changes.

The expression of otokeratin mRNA in 16-day-old chicken embryos and in hatchlings was restricted largely to the ear and heart. In early chicken embryos, however, otokeratin is expressed in the simple epithelium that is formed by the trunk ectoderm (28). This early form of otokeratin differs from that in the tegmentum vasculosum at two positions that probably reflect alternative splicing.

Implications. Screening of 10,000 clones from an auditory-epithelium cDNA library with a probe enriched by subtractive hybridization led to the identification of cDNAs representing 12 mRNAs that are highly expressed in the inner ear. Because only a relatively small number of clones have been analyzed so far, our future efforts lie in the identification of additional mRNAs that are highly specific to the chicken's auditory system. The chances of identifying rare mRNAs might be increased by further enrichment of the screening probe, for example by subtracting the cDNAs that have been identified in this study.

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