Their hair bundles did contain a single true cilium (kinocilium) (Fig. 3D), but their stereocilia were thin, with a relatively low density of microfilaments (Fig. 3, D and E). Many joined the cell body without any constriction at their proximal end or an obvious rootlet, and some arose in the absence of a well-differentiated underlying cuticular plate (Fig. 3E). Whereas most of the apparently maturing hair cells had no obvious synaptic contacts, some of those that possessed more mature stereocilia had a few fully developed synapses with afferent nerve boutons at their bases (Fig. 3, F and G).

These morphological observations demonstrate that replacement hair cells can develop after ototoxic damage to the utricle in guinea pigs. The replacements appeared in the regions from which the original hair cells were lost. Similar observations have been made in cristae and saccules, which suggests that these phenomena occur throughout the mammalian vestibular system. At least some of the new hair cells become innervated, making it likely that they could contribute to a recovery of sensory function. DNA labeling studies described by Warchol *et al.* (6) indicate that the new hair cells are produced after mitoses occurring in response to trauma and thus that regeneration of hair cells does occur in the mammalian inner ear.

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7. Initial studies were performed with albino animals (Dunkin-Hartley strain) and subsequent studies with a pigmented strain (Mill Hill strain 13). Qualitatively similar results were obtained. The animals were given subcutaneous injections of gentamicin (Cidomycin injection; Roussell) for ten successive days. The albinos received adjusted doses of 100 mg per kilogram of body weight each day, the pigmented animals, 125 mg kg^{-1}. These dosing regimes produce almost complete loss of outer hair cells in the basal coil of the cochlea in the respective strains [A. Forge, *Hearing Res.* **19**, 171 (1985); A. M. Brown, B. McDowell, A. Forge, *ibid.* **42**, 143 (1989)].
8. The bullae were removed and opened widely. The stapes was pulled out of the oval window, the round window of the cochlea was ruptured, the vestibule was widely exposed, small cuts were made through the bone covering the two exposed semicircular canals, and a small piece of bone was removed from the cochlear apex. The inner ear tissues were then perfused directly with fixative by gentle injection through these openings before the whole bulla was immersed in fixative. The fixative was 2.5% glutaraldehyde in 0.1 M sodium cacodylate (pH 7.3) with 3 mM $CaCl_2$; total osmolarity was 550 mosM. Fixation was continued for 1.5 hours with gentle rotation at room temperature, before postfixation in 1% OsO_4 in cacodylate buffer for 1.5 hours. The utricular maculae were then dissected out. Samples for SEM were processed by ligand binding of osmium with thiocarbohydrazide [S. J. Davies and A. Forge, *J. Microsc. (Oxford)* **147**, 89 (1987)] before critical point drying. Tissues for thin sectioning were en bloc stained in 1% aqueous uranyl acetate before dehydration and embedding. At least four ears were examined by each procedure at the chosen time points. Untreated animals selected at random from the populations available for study were used as controls.
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11. Quantitative analyses were made from scanning electron micrographs of the striolar regions of the utricles at a standard magnification that provided a field size of 2000 μm^2. In control utricles, the total density of hair cells estimated from these fields was 27.5 ± 1.92 per 1000 μm^2 (33 fields from 11 different animals). The vast majority of hair bundles showed the normal mature morphology; stereocilia occupied about half of the cell surface and were organized in typical staircase fashion. There was, however, in addition to the very small number of bundles that showed the distinctive morphology of the most immature forms (equivalent to those shown in Fig. 2, A and B), a subpopulation of bundles that were noticeably shorter than normal and with relatively small surface areas. In many of these, the kinocilium was as long as that of normal hair bundles and the stereocilia were organized in staircase fashion, but these (or at least some of them) may represent immature hair bundles. If these are included with those showing the most immature bundle form, then the density of immature hair cells in controls was 3 ± 1 per 1000 μm^2. At 4 weeks after treatment the density of hair cells was reduced to 16 ± 2 per 1000 μm^2 (35 fields from nine different animals). However, in addition to the bundles with the most immature morphology, there were numerous bundles in which stereocilia were erect but of equal height (equivalent to those shown in Fig. 2D) and which in terms of bundle height and stereocilia organization were clearly immature. If we include all these bundle forms and exclude bundles that showed the typical staircase form (regardless of relative height and cell surface size), then the total density of immature hair bundles at 4 weeks after treatment was 9 ± 1.5 per 1000 μm^2 (that is, 56% of the total number of hair cells present). These analyses not only confirm that mature hair cells are lost and replaced by immature cells after gentamicin treatment but also they suggest there may be ongoing development of hair cells in undamaged utricles of normal mature guinea pigs.
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Regenerative Proliferation in Inner Ear Sensory Epithelia from Adult Guinea Pigs and Humans

Mark E. Warchol, Paul R. Lambert, Bradley J. Goldstein, Andrew Forge, Jeffrey T. Corwin

Supporting cells in the vestibular sensory epithelia from the ears of mature guinea pigs and adult humans proliferate in vitro after treatments with aminoglycoside antibiotics that cause sensory hair cells to die. After 4 weeks in culture, the epithelia contained new cells with some characteristics of immature hair cells. These findings are in contrast to expectations based on previous studies, which had suggested that hair cell loss is irreversible in mammals. The loss of hair cells is responsible for hearing and balance deficits that affect millions of people.

Sensory hair cells are essential for the transduction of mechanical stimuli into hearing and balance signals in the internal ear. Sound is transduced by hair cells in the cochlea, and movements of the head are transduced by hair cells in the five vestibular sensory epithelia. Tritiated thymidine labeling in mice in vivo has provided support for the belief that the cells of the sensory epithelia in the mammalian ear cease proliferation in the embryo or shortly after birth and do not proliferate in the adult (*1*).

Here we report that cells in the utricular sensory epithelia from adult guinea pigs and adult humans proliferate after treatment with ototoxic antibiotics when maintained in vitro and that some of the progeny of the proliferating cells begin to differentiate as

M. E. Warchol, P. R. Lambert, B. J. Goldstein, J. T. Corwin, Department of Otolaryngology–Head and Neck Surgery and Department of Neuroscience, University of Virginia School of Medicine, Charlottesville, VA 22908.
A. Forge, Institute of Laryngology and Otology, University College London, London WC1X 8EE, United Kingdom.

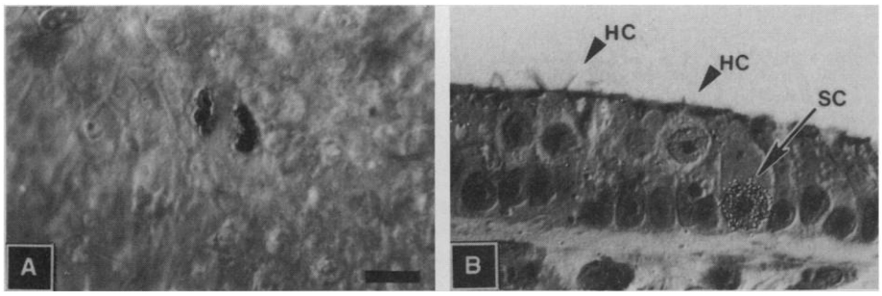

Fig. 1. Supporting cells begin to proliferate in sensory epithelia from the ears of juvenile and adult guinea pigs soon after the loss of sensory hair cells. The specimens in (A) and (B) were incubated for 24 hours in media that contained aminoglycoside antibiotics that are selectively toxic to the sensory transducing hair cells of the ear (see text). (**A**) A cell in the center of the sensory epithelium labeled with BrdU in the telophase stage of mitosis 2 days after the aminoglycoside treatment. (**B**) A transverse section through the center of a utricle cultured in medium that contained [^{3}H]methyl-thymidine for 6 days after hair cell lesioning. A supporting cell (SC) is heavily labeled by silver grains, which appear as small white spots over its nucleus, indicating that it replicated its DNA during the period during which the medium contained the radioactive tracer. Two surviving hair cells (HC) are to the left. This specimen contained 23 labeled supporting cells. Bar = 10 μm.

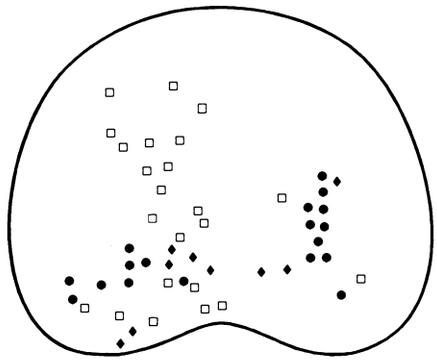

Fig. 2. Proliferating cells were present throughout the sensory epithelia after hair cell loss. The diagram shows the positions of proliferating cells in three utricular sensory epithelia oriented with their proximal (neural) ends at the bottom. Cells labeled during 2 days (●) and 4 days (◆) in culture with [^{3}H]methyl-thymidine after treatment with 1 mM neomycin. Cells labeled during 6 days (□) in culture with [^{3}H]methyl-thymidine after treatment with 1 mM gentamicin.

replacement hair cells. An accompanying report outlines ultrastructural evidence for recovery of hair cells in the vestibular organs of young guinea pigs after the loss of hair cells caused by administration of ototoxic antibiotics in vivo (*2*). Together, these findings suggest that a regenerative repair process analogous to that observed in nonmammalian hair cell epithelia (*3*) can occur in adult mammalian ears.

The sensory epithelia from the utricles of juvenile and adult guinea pigs (380 to 755 g, 6 to 14 weeks old) were dissected under sterile conditions and incubated in small volumes of culture media (*4*). Hair cells were intentionally killed in 18 cultures by incubation of the epithelia for 24 hours in media that contained 0.5 to 2 mM neomycin or gentamicin, aminoglycoside antibiotics that are selectively toxic to hair cells (*5*). Four control cultures were started in parallel and incubated in the basal medium without aminoglycoside antibiotics. After the first 24 hours, both groups were supplied with aminoglycoside-free media that contained [^{3}H]methyl-thymidine or bromo-deoxyuridine (BrdU) (*6*). These tracers are incorporated into replicating DNA, thereby identifying proliferating cells and their progeny. In initial experiments, the sensory epithelia were maintained in culture for 2 to 13 days in media that contained tracers. Half of the medium in each culture was replaced with fresh tracer-containing medium every 2 days. Cultures treated with BrdU were fixed and processed as whole mounts to examine cell proliferation through the use of immunocytochemistry (*7*). Cultures maintained in medium that contained [^{3}H]methyl-thymidine were fixed, sectioned, and processed for autoradiographic localization of labeled cells (*8*).

The 24-hour incubation in the aminoglycoside-containing medium resulted in dramatic losses of hair cells throughout the utricular sensory epithelia (*9*). Pyknotic cells with small, darkly stained nuclei were common in these epithelia and are presumed to have resulted from the aminoglycoside poisoning of the hair cells. Labeled cells were found in the sensory epithelia of all the specimens that were incubated with the mitotic tracers. The acid treatment necessary for the BrdU localization resulted in reduced histological preservation, but the method showed some cells in the sensory epithelia were labeled in anaphase and telophase (Fig. 1A).

Cell proliferation began soon after the loss of hair cells. Three sensory epithelia incubated in tracer-containing media for 2 days after the aminoglycoside treatment contained 3, 6, and 18 labeled cells. Two utricles incubated for 4 days after the treatment contained 9 and 23 labeled cells, and five utricles maintained in culture for 6 days after the treatment contained 4, 9, 21, 23, and 26 labeled cells. Most of the cells labeled with tritiated thymidine in those cultures had nuclei positioned just above the basal lamina. Both the positions of the nuclei and the overall shapes of the labeled cells were consistent with their identification as supporting cells (Fig. 1B).

Labeled supporting cells were distributed throughout the sensory epithelia (Fig. 2). A relatively large fraction of the labeled supporting cells was found in the proximal half of the sensory epithelium above the point at which the utricular branch of the statoacoustic nerve joins the organ. That site was most frequently damaged by the surgical manipulations involved in removing the sensory epithelium and by folding of the epithelium during the culture process.

In the four control utricles, normal hair cells were present at most locations after 5 days in culture without aminoglycosides (Fig. 3A). However, hair cells were killed unintentionally at a few sites in each of the controls during the surgical removal to culture. The four control utricles contained three, five, nine, and ten labeled supporting cells at locations where the sensory epithelia appeared to have been damaged (Fig. 3B). Labeled cells were not present in regions of the control epithelia that contained normal numbers of undamaged hair cells.

We examined the developmental fate of the progeny of the proliferating cells in three utricles that were maintained in culture for 27 days after antibiotic treatment (*10*). These utricles contained 15, 24, and 39 labeled cells, with 54 to 80% of the labeled nuclei located in the lumenal stratum that is usually occupied by hair cell nuclei. Some labeled cells appeared to develop structural characteristics of immature hair cells, including a surface projection that resembled a small ciliary bundle (Fig. 4). The percentage of labeled nuclei that were in the lumenal stratum was dramatically greater after 4 weeks than in those maintained for 2 weeks or less. Very few comparable cells were labeled in the short-term cultures (Fig. 3B). This apparent development of cells with labeled nuclei is consistent with evidence from other species in which laser microbeam surgery and time-lapse video microscopy have demonstrated that regenerated sensory hair cells can arise from supporting cell divisions at sites where hair cells are missing (*11*).

In order to test whether similar proliferation would occur in adult human ears, we

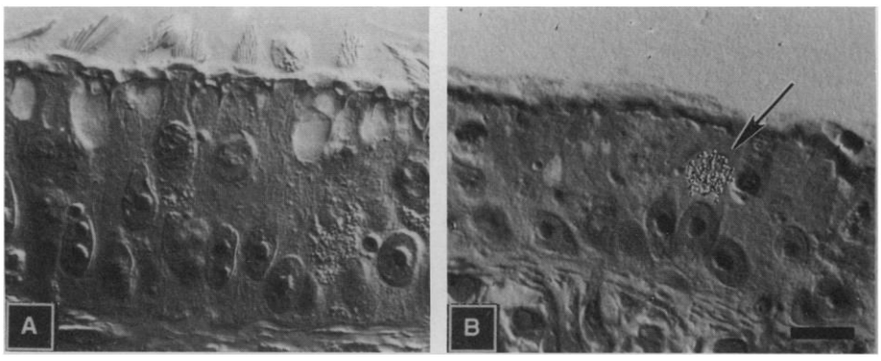

Fig. 3. (**A**) Hair cells appeared normal in the control utricles that were maintained in vitro for 5 days without aminoglycoside antibiotics. However, the supporting cells in the controls did begin to proliferate at sites where hair cells had been lost because of mechanical damage resulting from the culture method. (**B**) A labeled cell nucleus (arrow) in the apical stratum of a control sensory epithelium at a position where hair cells were lost. The nuclei of sensory hair cells normally occupy that stratum. Bar = 10 μm.

collected utricles from three patients during unilateral surgical labyrinthectomies (*12*). As before, each sensory epithelium was incubated in culture medium that contained 1 mM neomycin for 24 hours in order to kill hair cells. They were then incubated either for 6 days (two utricles) or for 24 days (one utricle) in aminoglycoside-free medium that contained [^{3}H]methyl-thymidine. The tissues were fixed and processed for autoradiography according to the protocol described above. The neomycin treatment and surgical damage resulted in the complete loss of hair cells from the sensory epithelia. Labeled supporting cells were present in all three specimens (Fig. 5, A and B). Counts were made of labeled cells in the two human utricles that were maintained in culture for 7 days. One specimen contained 127 labeled supporting cells and the other contained 159. The utricle that was in culture for 25 days contained some labeled nuclei in the lumenal stratum that is normally occupied by the nuclei of hair cells (Fig. 5B).

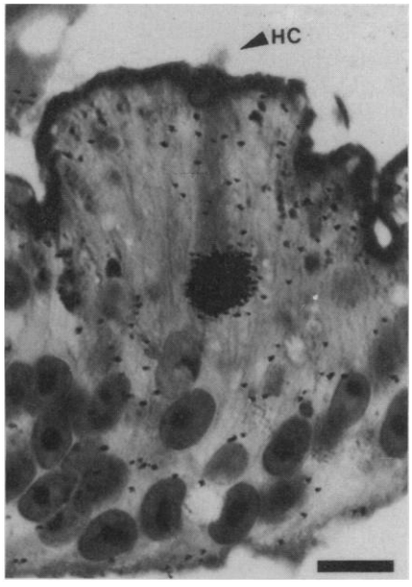

Fig. 4. A putative replacement hair cell in a utricle that was maintained in vitro for 4 weeks after hair cell loss. The dark spots over the nucleus of this cell are silver grains resulting from the autoradiographic exposure of nuclear track emulsion to [^{3}H]methyl-thymidine that was incorporated into the DNA during cell proliferation. Other labeled cells in these specimens also had morphological features characteristic of immature hair cells. A surface projection from the labeled cell resembles a developing ciliary bundle (arrowhead). Bar = 10 μm.

The differences in the number of labeled cells in the human and guinea pig utricles may be due to the more extensive loss of hair cells in the human tissue or to the fact that the adult human utricular epithelia were approximately four times the size of those from the adult guinea pigs. Rates of supporting cell labeling during regeneration are substantially higher in other species (*3*); the lower rates of regeneration observed in vitro in this study and in vivo in (*2*) suggest that the process occurs more slowly in mammals than in other species.

In rare cases the balance sensitivity of the human ear has been reported to recover after aminoglycoside antibiotics had abolished vestibular responses (*13*). Our results may explain those observations because they demonstrate that the supporting cells of the adult mammalian inner ear retain a capacity for proliferation that can be triggered in response to a loss of sensory hair cells. Forge *et al.* demonstrate that small cells with ultrastructural features that are characteristic of newly formed hair cells can appear in vivo several weeks after aminoglycoside treatments that cause hair cell loss in the utricles of guinea pigs (*2*). Together, these results indicate that the regenerative proliferation of supporting cells can lead to the formation of replacement hair cells in the balance organs of adult mammals. Additional investigations should determine whether this also occurs in the organ of Corti.

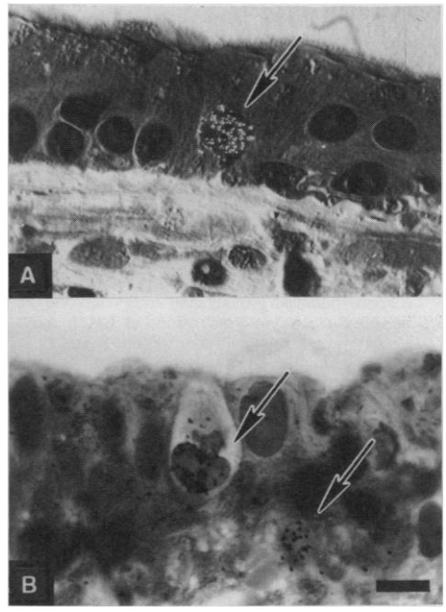

Fig. 5. Cells in utricular sensory epithelia from adult humans proliferated in culture after hair cell loss. (**A**) Exposed silver grains that appear as white dots over the nucleus (arrow) in this differential interference contrast view demonstrate that the radioactive thymidine in the culture medium was incorporated into the DNA of this supporting cell in the utricle from a 52-year-old patient. After 6 days in culture with [^{3}H]methyl-thymidine, this utricle contained 127 labeled cells. (**B**) The nuclei of two cells in this section of the utricle from a 63-year-old patient contain the radioactive DNA tracer revealed by black dots in this bright-field view (arrows). This utricle was in culture for 25 days, and the nucleus of one of the labeled cells is in the lumenal stratum that is normally occupied by hair cells. Bar = 10 μm.

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4. An in vitro protocol was used in order to limit the amount of isotope required and to reduce the time necessary for completion of the experiments. Albino Hartley guinea pigs were anesthetized by injection of ketamine (80 mg per kilogram of body weight, intramuscularly), euthanized by an intracardiac injection of 1 ml of pentobarbital (60 mg/ml), and decapitated. The heads were skinned and soaked in chilled 70% ethanol for 15

to 20 min. Utricles were explanted to chilled Medium-199 containing 25 mM Hepes buffer and Hanks' salts (Gibco). The sensory epithelia were isolated, and the otolithic membranes were removed with fine forceps. The culture chambers contained small wells made from cover glasses and Polyallomer rings 9 mm in diameter attached with Silastic adhesive. They were coated with Cell-Tak (Collaborative Research) before one or two utricles were placed in each well with 50 μl of medium. The medium consisted of Medium-199 with Earle's salts, 26 mM sodium bicarbonate, 25 mM Hepes, 0.69 mM L-glutamine (Gibco), supplemented with 20% fetal bovine serum (FBS) (Gibco), penicillin (10 units/ml) and Fungizone (25 ng/ml). Cultures were maintained at 37°C in a 5% CO_2 environment. Guinea pigs become sexually mature at 4 to 8 weeks of age [J. E. Wagner and P. J. Manning, *The Biology of the Guinea Pig* (Academic Press, New York, 1976), p. 9]. Seven of the specimens were older than 8 weeks, and all were at least 6 weeks old. The labeling observed was comparable in utricles from older and younger specimens. All protocols were in accordance with the University of Virginia's guidelines for use of animals in research.

5. Cultures were incubated for 24 hours in Medium-199 with 20% FBS that contained 0.5 to 1.0 mM neomycin or 1.0 to 2.0 mM gentamicin.
6. After 24 hours, cultures were rinsed twice with Medium-199, and fresh medium without any aminoglycoside antibiotics was added. The aminoglycoside-free media contained either of two mitotic tracers: [^{3}H]methyl-thymidine (0.8 μCi/ml, 65 Ci/mmol) or 5-bromo-2′-deoxyuridine (BrdU, 3 μg/ml) in solution with 5-fluoro-2′-deoxyuridine (0.27 μg/ml) from Amersham.
7. The epithelia that had been cultured with BrdU were rinsed in Medium-199 and fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB) for 30 min. Fixed epithelia were rinsed three times in phosphate-buffered saline (PBS), treated in 2 N HCl for 30 min, incubated in PBS with 0.5% Triton X-100 for 15 min, and incubated in PBS alone for an additional 15 min. The epithelia were then incubated overnight in a solution containing a monoclonal antibody to BrdU (Amersham) with 0.5% Triton X-100 at 4°C. After three rinses in PBS, specimens were incubated for 30 min with a secondary antibody conjugated to peroxidase-antiperoxidase. They were reacted with diaminobenzidine (0.5 mg/ml) in 0.05 tris buffer containing 0.01 M imidazole and 0.03% hydrogen peroxide, mounted in glycerol-PBS (9:1), and viewed as whole mounts.
8. The utricles that were cultured with [^{3}H]methyl-thymidine were fixed in 3% glutaraldehyde in 0.1 M PB (pH 7.4) and postfixed in 1% OsO_4. They were dehydrated in ethanol and embedded in methacrylate (Historesin, Leica). Serial sections were cut at 3-μm thickness, mounted on slides, and dipped in a 50% aqueous solution of nuclear track emulsion (Kodak NTB-2). Emulsion-coated slides were placed in light-tight boxes and stored at 4°C for 10 to 15 days. Slides were developed in Kodak D-19 and counterstained with thionine or toluidine blue.
9. Hair cells were counted in comparable regions through control and aminoglycoside-treated utricles that were maintained in culture for 5 days. Every third section through a 100-μm-wide band across the center of each sensory epithelium was counted. In two controls, the bands contained 458 and 615 hair cells, with 41.6 ± 5.1 and 55.9 ± 5.3 (mean ± SD) hair cells per section, respectively. In two utricles that had been treated for 24 hours with 1 mM neomycin and then cultured for 4 days in aminoglycoside-free medium, the bands contained 148 and 73 hair cells, with 13.5 ± 2.5 and 6.6 ± 2.9 hair cells per section, respectively.
10. All the utricles in this series were cultured for 24 hours in media that contained 1 mM neomycin. Then they were rinsed three times in fresh medium and mounted on cover glasses with the use of 20 to 25 μl of Matrigel (Collaborative Research). Cover glasses were placed in roller tubes that contained 2 ml of culture medium [Medium-199 supplemented with 20% FBS and containing [^{3}H]methyl-thymidine (0.8 μCi/ml)]. Cultures were maintained at 37°C and rolled through four revolutions per hour. At least half of the culture medium was replaced with fresh medium every 4 to 7 days. After 27 days in roller tube culture, the utricles were fixed in 3% glutaraldehyde (in 0.1 M PB) and processed for autoradiography as described (*8*).
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12. Utricles were obtained from three human patients, two females (40 and 52 years of age) and one male (63 years of age), during surgical intervention for the removal of acoustic neuromas. Written informed consent was given before surgery. Utricles were placed in sterile 0.9% NaCl, quickly transferred to chilled Medium-199, and transported to culture facilities in a chilled ice chest. After dissection to expose the sensory epithelium, utricles were placed in culture wells containing 50 μl of medium. Sixty to ninety minutes elapsed between surgical removal and placement in culture. Utricles were incubated in 1 mM neomycin in Medium-199 with 20% FBS for 24 hours and then cultured for either 6 or 24 days in aminoglycoside-free media that contained [^{3}H]methyl-thymidine (0.8 μCi/ml). At 2-day intervals, half of the culture medium was replaced with fresh medium that contained [^{3}H]methyl-thymidine. The utricles were fixed with 3% glutaraldehyde in 0.1 M PB and processed for autoradiography as outlined above.
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Effect of PU.1 Phosphorylation on Interaction with NF-EM5 and Transcriptional Activation

Jagan M. R. Pongubala,* Charles Van Beveren, Sujatha Nagulapalli,* Michael J. Klemsz, Scott R. McKercher, Richard A. Maki, Michael L. Atchison†

PU.1 recruits the binding of a second B cell–restricted nuclear factor, NF-EM5, to a DNA site in the immunoglobulin κ 3′ enhancer. DNA binding by NF-EM5 requires a protein-protein interaction with PU.1 and specific DNA contacts. Dephosphorylated PU.1 bound to DNA but did not interact with NF-EM5. Analysis of serine-to-alanine mutations in PU.1 indicated that serine 148 (Ser^{148}) is required for protein-protein interaction. PU.1 produced in bacteria did not interact with NF-EM5. Phosphorylation of bacterially produced PU.1 by purified casein kinase II modified it to a form that interacted with NF-EM5 and that recruited NF-EM5 to bind to DNA. Phosphopeptide analysis of bacterially produced PU.1 suggested that Ser^{148} is phosphorylated by casein kinase II. This site is also phosphorylated in vivo. Expression of wild-type PU.1 increased expression of a reporter construct containing the PU.1 and NF-EM5 binding sites nearly sixfold, whereas the Ser^{148} mutant form only weakly activated transcription. These results demonstrate that phosphorylation of PU.1 at Ser^{148} is necessary for interaction with NF-EM5 and suggest that this phosphorylation can regulate transcriptional activity.

Modification by phosphorylation can influence either the binding to DNA or the functional activity of a number of transcription factors including SRF, Myb, Max, c-Jun, and cyclic adenosine monophosphate response element–binding protein (CREB) (*1*). Phosphorylation of IκB-like proteins can also control the nuclear localization of Rel-related proteins such as NF-κB (*2*). PU.1 is an Ets-related transcription factor (*3*) that is implicated in the genesis of erythroleukemia (*4*). PU.1 expression is restricted to macrophages and B cells. Many Ets family members are expressed in a tissue-restricted fashion and therefore may control the expression of tissue-specific genes (*5*). PU.1 recruits the binding of another B cell–restricted nuclear factor, NF-EM5, to a specific DNA sequence in the immunoglobulin κ 3′ enhancer (κE3′) (*6*). NF-EM5 requires a protein-protein interaction with PU.1 and specific protein-DNA interactions in order to bind to DNA. The DNA binding sites for PU.1 and NF-EM5 lie adjacent to one another in the κE3′ enhancer. These two sites appear to be important for κE3′ enhancer activity because mutation of either site reduces transcriptional activity (*6*). Recruitment of NF-EM5 to DNA requires a 43–amino acid

J. M. R. Pongubala, S. Nagulapalli, M. L. Atchison, Department of Animal Biology, University of Pennsylvania, School of Veterinary Medicine, Philadelphia, PA 19104.
C. Van Beveren, M. J. Klemsz, S. R. McKercher, R. A. Maki, La Jolla Cancer Research Foundation, La Jolla, CA 92037.

*These authors contributed equally to this work.
†To whom correspondence should be addressed.