The mammalian cochlea is a gem often overlooked by developmental biologists. Highly complex in function, it is nevertheless highly ordered in morphology. There are clear gradients along the principal axis of the organ: in length and number of the sensory hair cells; in height, orientation, and number of sensory cilia; and in width, thickness, and stiffness of the basilar membrane. These architectural features presumably reflect transient gradients of morphogenetic factors and may be sensitive assays for experimental manipulation of such factors.

Understanding development of the cochlea should also have a tremendous impact on public health. More than a third of us will have substantial hearing loss by old age, most of it resulting from the death of sensory hair cells. We are born with a complement of but 30,000 hair cells; like neurons, they do not regenerate, so most hearing loss is irreversible. A few laboratories are trying to understand the factors involved in hair-cell development in order to stimulate regeneration from stem cells or dedifferentiated supporting cells. Although no one underestimates the difficulty of the task, it may be the only possible strategy for recovery from most sensorineural deafness.

In understanding what makes a cell become a hair cell, much of the research is directed at expression and function of transcription factors in the inner ear. Receptors for retinoic acid and thyroid hormone have received most of the attention, both because these are well understood and because alterations in retinoic acid or thyroid hormone during development are known to cause inner ear defects. In this context, two recent papers take on particular interest. Bradley et al. (1) have used in situ hybridization to understand the spatial and temporal pattern of expression of the thyroid hormone receptors (TRs) in the developing ear. Both δ forms of TR are expressed only in the cochlear portion of the inner ear at embryonic day 12 (E12) in rat, whereas the α form appears in both cochlear and vestibular regions. In a study recently reported by Kelley et al. (2) cultured explants of developing cochlea from E13–E16 in mouse and found that addition of retinoic acid alters cell fate so as to produce more hair cells.

**Inner Ear Development**

The organs of the inner ear are derived from the otic placode, a region of surface and neural ectoderm on the lateral rhombencephalon that invaginates and pinches off to form a closed sack, the otic vesicle (3, 4). Elongations and distortions of the otic vesicle then create the six separate organs of the mammalian inner ear: the saccus, the utriculus, and the three semicircular canals of the vestibular system, and the snail-shaped cochlea responsible for auditory sensation (Fig. 1). The auditory and vestibular organs are similar in many respects beyond their common embryonic origin. Both use a single mechansensitve cell type—the hair cell—as the primary receptor cell. In both systems deflection of the hair cell’s ciliary bundle is the proximal stimulus; sensitivity to different physical stimuli (linear acceleration, angular velocity, or acoustic stimuli) is conferred by different accessory structures that convey these stimuli to hair cells in the different organs. Yet there are differences as well: the sensory epithelium in vestibular organs is roughly oval and contains hair cells of similar morphology; whereas in the cochlea the sensory organ of Corti is a spiraling, highly differentiated ribbon of hair cells and supporting cells. Moreover, the hair cells in the cochlea are of two forms: in addition to a single row of inner hair cells (IHCs), which are morphologically like vestibular hair cells and which send neural signals to the central nervous system, there are also three to five rows of outer hair cells (OHCs)—slender, motile cells that do not connect to afferent nerve fibers but are thought to provide mechanical amplification of acoustic signals.

The separation of inner and outer hair cells occurs fairly early in development (5). At E16 in mouse, the cochlea is little more than an elongated sack (Fig. 2). Yet the primordial sensory epithelium is longitudinally divided into the greater epithelial ridge, from which inner hair cells are derived, and the lesser epithelial ridge, which gives rise to outer hair cells. Over the next 20 days, hair cells differentiate, their ciliary bundles grow and organize, and Deiter’s supporting cells elongate to hold the tops and bottoms of outer hair cells. The tunnel of Corti appears between inner and outer hair cells, as pillar cells fill with filament bundles and separate from each other. A deeper layer of cells forms the mechanically tuned basilar membrane, on which the hair cells ride, and remnants of the greater epithelial ridge secrete the bulk of the tectorial membrane (which couples basilar membrane vibration to the hair-cell cilia). Spiral ganglion neurons developing below the greater epithelial ridge send dendrites to the inner hair cells and axons to the cochlear nucleus in the brainstem. It is not hard to imagine that the mammalian cochlea requires the synchronized action of many highly specific transcriptional factors to orchestrate its development.

**Transcription Factors**

The spatial and temporal enactment of developmental events depends to a large extent on selected patterns of transcriptional regulation. This is an extremely intricate process with many “dimensions of complexity” (6) that is only beginning to be understood. The two main components of transcriptional regulation are DNA-binding motifs (at least 12 distinct types) and protein transcriptional factors (perhaps up to 5000), which have a DNA-binding domain, a regulatory domain, and sometimes a ligand-binding domain (6). There seem to be almost limitless ways in which these components can be sorted and shuffled, including varying the spacing and array of DNA-binding sites, posttranslational modifications of transcription factors, and interactions between different factors. It is almost presumptuous at this point to define the transcriptional elements that are critical to the development of the inner ear, as most of the players have probably not even been described (e.g., ref. 7). Still, some elements have been identified based on congenital and genetic syndromes that affect ear development and on in situ hybridization studies that localize expression of certain transcriptional factor genes to the inner ear at critical times in development. Prominent among these are nuclear receptors for retinoic acid and thyroid hormone. Both are members of the steroid/thyroid hormone receptor superfamily that have
"zinc-finger" DNA-binding domains (for review, see refs. 7 and 8).

Components of the retinoic acid system include two ligands—all-trans- and 9-cis-retinoic acid; four ligand-binding proteins that are thought to control intracellular levels of free ligand—cytoplasmic retinol-binding protein (CRBP I and II) and cytoplasmic retinoic acid-binding protein (CRABP I and II); and two families of nuclear receptors—retinoic acid receptors (RARs) and retinoid "X" receptors (RXRs), each of which occurs in three forms (α, β, and γ) (for an overview see ref. 9). Moreover, these receptors can have different posttranslational modifications and can combine as homodimers or heterodimers with themselves or other receptors, notably the thyroid hormone receptors.

Components of the thyroid hormone system include the ligands T3-thyroxine (T3) and 3,5,3′-triiodothyronine (T3), and the TRs. After crossing the cell membrane, T3 is deiodinated to T3, which binds TRs. The receptors are encoded by two genes, c-erbAα and c-erbAβ, which in turn can undergo alternative splicing to yield three functional receptors, TRα1, TRβ1, and TRβ2 (10). A second splice form of c-erbAα produces a "receptor" sometimes referred to as TRα2, which does not bind thyroid hormone. Activation of transcription likely involves a heterodimer of TRs, each with thyroid hormone bound, binding to the DNA thyroid hormone response element (TRE) (11). Potential complexity arises in the different effects on transcription of TRα1, TRβ1, and TRβ2. Binding of thyroid hormone to TRs can in some cases reduce their binding to TREs. In addition to forming homodimers and heterodimers with each other (12), TRs can form heterodimers with other nuclear proteins, termed TR auxiliary proteins (TRAPs), which serve to enhance binding to TREs in the presence of thyroid hormone (13, 14). Among TRAPs are RXRα and β. This remarkable promiscuity of interactions allows tremendous diversity in the fine tuning of transcriptional regulation.

Retinoic Acid in the Inner Ear

Some insights into transcriptional factors that may be important in inner ear development have been provided by congenital syndromes in which hearing loss is a predominant feature. Several lines of evidence have implicated the intracellular receptors for retinoic acid and thyroid hormone in controlling critical steps in cochlear differentiation. When pregnant rodents are given retinoic acid or its analog isotretinoin, craniofacial development of embryos is disrupted, producing multiple defects in the inner and outer ear (15–17). In addition, exposure of embryonic otic vesicles to retinoic acid in culture induces a precocious differentiation of the sensory epithelium and the secretory and supporting epithelium (18). In situ hybridization analysis of mRNAs encoding the proteins involved in mediating the retinoic acid response reveal that RARβ is strongly expressed in the mesenchyme as well as in the sensory epithelium of the developing ear (9). Interestingly, the retinoic acid-binding protein CRABP II, which is thought to be involved in controlling the intracellular concentrations of free retinoids, is finely localized within the cochlear epithelium during development (19).

A recent report by Corwin and colleagues (2) describes effects of retinoic acid on development of the cochlea in vitro. Cochleas were removed from mice at E13–E16 and maintained in explant culture, where they develop normally for 10–20 days (20). When retinoic acid was added to cultures (10 nM, at E14), the most striking effect was the development of extra hair cells; whereas there are normally 1 IHC and 3–5 OHCs in each cross section, treated cultures sometimes showed 2 IHCs and 6–10 OHCs. Overall, the number of hair cells was increased by 60%. By adding [3H]thymidine at E14, Kelley et al. (2) showed that most of the extra hair cells were born before E14, suggesting that retinoic acid increased hair cell number by altering the fate of existing cells, rather than by stimulating proliferation of precursor cells. The efficacy of retinoic acid in producing extra hair cells depended critically on age, dropping dramatically between E14 and E17. The dose–response curve suggests that retinoic acid is acting through an RAR rather than an RXR.

By culturing explants on the F9 reporter cell line, which responds to retinoic acid by expressing β-galactosidase, Kelley et al. (2) also showed that developing mouse cochleas contain retinoic acid, at least from E14 to P0. This parallels an increase in CRABP, detected by Western blot, from E14 to P1. Interestingly, adult cochleas did not show detectable levels of retinoic acid or CRABP, although adult vestibular organs did contain retinoic acid. This may be related to the ability of adult mice vestibular organs, but not cochleae, to regenerate hair cells following ototoxin treatment (21, 22).

In one instance, however, retinoic acid has been reported to stimulate regeneration of cochlear hair cells in vitro. Leefebvre et al. (23) cultured adult rat cochleas following ototoxin-induced hair-cell death. Addition of retinoic acid to these cultures caused appearance of new hair cells. Because of the relevance of the results to hair-cell regeneration in humans, it will be important to extend these studies.

Thyroid Hormone in the Inner Ear

Another critical transcriptional regulator for the inner ear appears to be the receptor for thyroid hormone. There are varying reports of the extent of hearing loss in humans with congenital hypothyroidism, with 20–80% having substantial hearing loss (24, 25). In cases of hypothyroidism produced by severe iodine deficiency, deaf-mutism is a predominant characteristic, in addition to other neurologic...
symptoms and disturbances in growth (26). When rat pups are made hypothyroid by administration of propylthiouracil, there is pronounced disruption of development of the organ of Corti that includes abnormalities in the sensory epithelium and the tectorial membrane (27, 28).

Genetic syndromes that disrupt signaling by thyroid hormone have both elucidated and confounded our understanding of this process. On the one hand, Pendred syndrome, an autosomal recessive condition associated with impaired thyroid hormone synthesis, has deafness as a prominent feature (29). However, the deafness may be caused by the defect in the synthetic enzyme. In addition, a submicroscopic chromosomal deletion removing essentially all of the gene for TRβ (and presumably other genes) has no apparent phenotype in the heterozygous state but causes deafness in the homozygous state (30). On the other hand, generalized resistance to thyroid hormone (GRTH), a more common, autosomal-dominant syndrome involving >38 different mutations in the gene for TRβ, manifests as hearing loss in <5% of patients (31). These mutations cluster in two regions of the T3-binding domain and act to reduce T3 binding to various degrees; they apparently act dominantly either by increased binding affinity of mutant homodimers for TRE combined with reduced affinity for thyroid hormone or by formation of inactive heterodimers (32). One of the mutations, a three-base deletion that eliminates one amino acid in the T3-binding domain, abolishes thyroid hormone responsiveness and produces GRTH but does not affect hearing in heterozygote carriers nor apparently in one homozygous individual severely affected with other neurological problems (33).

Bradley et al. (1) have now used in situ hybridization to find exactly where the different TR subtypes are expressed in the developing rat inner ear. They had previously found that TRα1 is widely expressed in rat brain, while TRβ1 occurs in some cortical regions, and TRβ2 is seen predominantly in pituitary, as expected (34, 35). In the ear, they find a similarly restrictive pattern of the TRβ types. As early as E13, TRβ1 and TRβ2 mRNAs are found only in the ventral otic vesicle, which gives rise to the cochlea, and not in the dorsal vesicle, which becomes the vestibular organs. At P0, when the sensory epithelium is still developing, TRα1 and TRα2 hybridize throughout the inner ear, whereas TRβ1 and TRβ2 occur only in the cochlea, and only in the region of the cochlea that forms the sensory epithelium. At P4, TRβ2 hybridizes in the sensory epithelium but especially in the greater epithelial ridge (Fig. 2), which has given rise by this time to IHCs in the sensory epithelium and the tectorial membrane. Reverse transcriptase amplification of inner ear RNA and nested polymerase chain reactions (RT-PCRs) indicated that the transcript to which the TRβ2 probe hybridizes in inner ear is most likely authentic TRβ2.

The localization of TRβ mRNA to specific regions of the cochlea is intriguing and indicates one performer in the detailed orchestration of cochlear development. The finding of TRβ especially in the greater epithelial ridge around P0 fits nicely with the observation (27, 36) that in rats and mice made hypothyroid at birth organ of Corti differentiation is greatly slowed. However, understanding the different roles of TRα and TRβ, and the specific relation of TRβ to congenital deafness, may be less straightforward considering the complex interactions of the TRs and the mixed phenotype of patients with TRβ mutations.

**Additional Transcription Factors**

Several other genes have been identified that appear to have a role in the development of the inner ear (37). As might be anticipated from their critical role throughout developmental processes, the homeobox genes are also implicated here. These genes (termed Hox in the mouse) encode transcriptional factors that bind to DNA homeodomains and have been implicated in a number of developmental processes involving segmentation and neurogenesis (38). Early in mouse embryogenesis (E9–E13) the homeobox genes Hox1.6 and 2.9, as well as the zinc-finger DNA-binding protein Krox 20, are expressed in the fifth and sixth rhombomeres involved in formation of the otocyst (39). "Knock-out" mice lacking the *Hox* 1.6 gene manifest numerous defects in the developmental progeny of these rhombomeres with disruption of inner ear development as a predominant feature (40–42). A promising development is the finding of three homeobox genes, Dlx-3, *Msh*-C, and *Msh*-D, expressed in the zebrifish otocyst (43). Although lower vertebrates do not have organs with the highly specialized morphology of the mammalian cochlea, early aspects of inner ear morphogenesis are likely to be the same.
Other transcriptional factors important in inner ear development include members of the Pax family, which recognize a paired box DNA motif (38). Mutations in the human homolog of Pax-3 cause an inherited deafness in humans, Waardenburg syndrome, which accounts for 2–5% of congenital deafness (44–48). Pax-2 is also expressed in regions of the mouse otic vesicle that form neuronal components (49). Recently, a novel member of the basic helix-loop-helix leucine zipper transcriptional family has been identified, through a mutation in the mi gene causing small eye size and early onset deafness in mice (50). Not to be left out, the transcription factor c-fos also shows transient and stage-dependent expression in inner ear (18).

Timed expression of these transcriptional factors is regulated in part by their ability to turn each other on and off. Retinoic acid, for example, induces sequential expression of c-fos, Krox, and Hox genes (18, 51). Transcription factors also respond to metabolic changes instigated by growth factors. Some that appear to have a role in early inner ear development include basic fibroblast growth factor (bFGF) (52, 53) and the bFGF-related signal, int2 (54), the FGF receptor 3 (55), and the neurotrophins BDNF, NT3, and NGF (refs. 56–60; for review see ref. 61).

At this point in our understanding of inner ear development this vast array of transcriptional factors and growth factors strikes one more as a tempest than an order in inner ear. Elucidating the coordination and synchronization of these factors in development of the inner ear provides an enormous challenge for the future.