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Tailchaser (*Tlc*): A new mouse mutation affecting hair bundle differentiation and hair cell survival

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Summary

We have undertaken a phenotypic approach in the mouse to identifying molecules involved in inner ear function by *N*-ethyl-*N*-nitrosourea mutagenesis followed by screening for new dominant mutations affecting hearing or balance. The pathology and genetic mapping of the first of these new mutants, tailchaser (*Tlc*), is described here. *Tlc*/+ mutants display classic behavioural symptoms of a vestibular dysfunction, including head-shaking and circling. Behavioural testing of ageing mice revealed a gradual deterioration of both hearing and balance function, indicating that the pathology caused by the *Tlc* mutation is progressive, similar to many dominant nonsyndromic deafnesses in humans. Based on scanning electron microscopy (SEM) studies, *Tlc* clearly plays a developmental role in the hair cells of the cochlea since the stereocilia bundles fail to form the characteristic V-shape pattern around the time of birth. By young adult stages, *Tlc*/+ outer hair bundles are grossly disorganised although inner hair bundles appear relatively normal by SEM. Increased compound action potential thresholds revealed that the *Tlc*/+ cochlear hair cells were not functioning normally in young adults. Similar to inner hair cells, the hair bundles of the vestibular hair cells also do not appear grossly disordered. However, all types of hair cells in the *Tlc*/+ inner ear eventually degenerate, apparently regardless of the degree of organisation of their hair bundles. We have mapped the *Tlc* mutation to a 12 cM region of chromosome 2, between *D2Mit164* and *D2Mit423*. Based on the mode of inheritance and map location, *Tlc* appears to be a novel mouse mutation affecting both hair cell survival and stereocilia bundle development.

Introduction

The vertebrate hair cell displays a remarkable amount of organisation within a single cell and as such has been studied for years by those interested in how a cell controls its dimensions (for example see Tilney *et al.* (1992a)). The most striking feature of a hair cell is the array of actin-filled extensions called stereocilia which project from the apical surface. The stereocilia are key elements in the function of the hair cell, turning mechanical stimuli into electrical impulses (mechanotransduction). Mechanotransduction begins when the stereocilia are deflected as a result of fluid movement within the inner ear. The stereocilia are arranged in graded heights and a deflection in the direction of the tallest stereocilia causes cation channels to open, resulting in a depolarization of the hair cell and release of synaptic vesicles. The hair bundles in the mammalian inner ear have very precise dimensions depending on the type of hair cell and the region of the cochlea, suggesting that the development of the bundles is a complex process in which defects would be likely to lead to hair cell dysfunction. Already, three genes have been found to play a role in stereocilia genesis which, when mutated, lead to deafness (Avraham *et al.*, 1995; Gibson *et al.*, 1995; Probst *et al.*, 1998). Several more are likely to follow, considering there are a number of vertebrate models that show defects in bundle differentiation (Kitamura *et al.*, 1992; Nicolson *et al.*, 1998; Yonezawa *et al.*, 1996).

The genetic approach has thus far proven to be a successful method by which to identify molecules involved in hair cell function and deafness. The mouse has played an important role in this progress, as exemplified by a number of human deafness genes that have been found through the identification of mouse homologues, including *Pax3*, *Mitf*, *Myo7a*, *Pou4f3* and *Myo15* (reviewed in Probst and Camper (1999)). There are additional mouse mutants with hearing or balance problems, some cloned and some not, that may

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represent models for further forms of human deafness. However, deafness in humans is highly heterogeneous. There are to date over 50 different loci involved in non-syndromic deafness in humans and this number is growing rapidly, plus several hundred different human syndromes that include hearing impairment as a feature (Gorlin *et al.*, 1995; Online Mendelian Inheritance in Man, 1999; Van Camp & Smith, 1999). Most of these loci do not have an obvious mouse mutant as a candidate for homology, indicating a need for more mouse mutants.

To address this shortage of mouse models for human deafness, and to identify some of the molecules involved in normal hearing and balance, we have undertaken a phenotype-driven approach, using the mutagen *N*-ethyl-*N*-nitrosourea (ENU) to produce new mouse mutants (Hrabe de Angelis & Balling, 1998). ENU is a powerful mutagen in the mouse (Russell *et al.*, 1979) that induces primarily point mutations; consequently the mutation is likely to affect a single gene. Other mutagens such as irradiation, transgene insertions, or chlorambucil can cause chromosomal rearrangements and/or deletions and affect several genes (for reviews see Brown and Nolan (1998) and Flaherty (1998)). Male C3HeB/FeJ mice were mutagenised, mated to normal females and the offspring were screened for dominant defects indicative of a hearing or vestibular defect. One of the first mutants identified in the vestibular screen, tailchaser (*Tlc*), is described here.

In this paper we show that *Tlc* mutant mice display abnormal stereocilia development in the cochlea, leading to a disorganised pattern atop the outer hair cell. Specifically, the characteristic V-shape never forms in outer hair cells (OHCs), and, although their mature bundle morphology appears normal, the inner hair cells do not exhibit their normal transient V-shape during development. Young adult mutants show raised thresholds for cochlear responses, suggesting the hair cells are not functioning properly. Both cochlear and vestibular hair cells eventually degenerate, a situation that is reflected by progressive deafness and vestibular dysfunction. In addition, by localising the *Tlc* mutation to chromosome 2, we demonstrate that *Tlc* is a novel mouse mutant that could potentially serve as a mouse model for a form of human nonsyndromic deafness.

Methods

MICE

The founder mouse carrying the *Tlc* mutation was generated in a large-scale ENU mutagenesis program (Neuherberg, Germany; Hrabe de Angelis & Balling, 1998). Male C3HeB/FeJ mice were mutagenised and mated to normal C3HeB/FeJ females and the offspring were screened for a variety of defects, including deafness or vestibular dysfunction. The *Tlc* founder was identified because of head-shaking and an abnormal reaching response. For analysis of the phenotype, the colony was maintained on a C3HeB/FeJ background. Generally, heterozygotes were mated to wildtype animals, although some heterozygote $\times$ heterozygote matings were also set up and generated offspring. All animals described for the histological analysis resulted from heterozygote $\times$ wildtype matings, except one of the two litters studied by SEM at postnatal day (P) 20. However, no gross differences were observed between mutants from this litter. From around 3–4 weeks after birth, mutants could be distinguished from controls by a mild head-shaking behaviour, but before this time, whole litters were analysed blind to phenotype. All mice used for characterising the phenotype were on a C3HeB/FeJ genetic background, which includes the *rd* gene resulting in rapid onset of retinal degeneration, but both mutants and littermate controls carried the same visual impairment.

SCANNING ELECTRON MICROSCOPY

Samples were prepared using the osmium tetroxide-thiocarbohydrazide (OTOTO) method as described previously (Hunter-Duvar, 1978; Self *et al.*, 1998). Ears were dissected out in fixative (2.5% glutaraldehyde in 0.1 M phosphate buffer pH7.2), the windows and apex of the cochlea were pierced (only in samples P20 and older) or, for vestibular regions, the superior canal was pierced and samples were allowed to fix 4–5 hrs rotating at 4°C. To prepare vestibular samples at P3, these regions were dissected out in PBS, the otolithic membranes were removed using a bent tungsten needle and then immediately fixed for 4–5 hrs. Ears were washed overnight in 0.1 M phosphate buffer and, for preparation of the organ of Corti, the outer bony shell and stria vascularis was removed. Specimens were examined under a Phillips XL30 scanning electron microscope.

CLEARED EARS

3–4 month-old mice were decapitated, the heads were bisected and the brain was removed. Half heads were fixed in Bodian's fixative (75% ethanol, 5% formalin, 5% glacial acetic acid) overnight and washed (minimum 2 hrs for each wash), twice in 75% ethanol, twice in 95%, twice in 100% ethanol, and cleared overnight in methyl salicylate. The ears were then dissected out of the skull and photographed in methyl salicylate using an Olympus DP10 digital camera.

BEHAVIOURAL TESTING

Except the open field test, all tests were performed in the same afternoon and in the same order. The open field test was performed in the afternoon a day or two earlier than the rest of the tests. The equipment was cleaned with a solution of weak acetic acid (1.0 mM) to clear away any residual odours that may affect the behaviour. These tests have been previously described in Steel and Hardisty (1996).

Preyer reflex test

A custom built click-box (MRC Institute of Hearing Research) was held 30 cm above the mouse and a calibrated 20 kHz toneburst at an intensity of 90 dB SPL was delivered. The presence or absence of an ear flick response (Preyer reflex) was recorded. If the animal responded well (ears flat against the head) a score of 1.0 was given. If the animal did not respond at all, a score of 0.0 was given. If there was some response but it was not robust, a score of 0.5 was given.

Elevated platform

The mouse was placed on a platform measuring 7 × 7 cm at a height of 45 cm above a mound of sawdust. If the mouse remained on the platform for 2 min, the mouse received a score of 1.0. If the mouse fell before the 2 minutes were up, the mouse received a score of 0.0.

Contact righting

The mouse was placed in a perspex tube with a square cross section of 3 cm across. When the animal had crawled approximately halfway along the tube, the tube was rotated 180° so that the mouse was now crawling upside down. If the animal flipped back to its original upright position within 5 seconds the animal received a score of 1.0. If the animal continued crawling in its new upside-down position the animal received a score of 0.0. If the animal crawled in a sideways position it received a score of 0.5.

Reaching response

The mouse was held in the air by the tail. If the mouse stretched out with its forelimbs towards the sawdust it received a score of 1.0. If the mouse curled towards its belly the mouse received a score of 0.0. If the mouse curled only slightly it received a score of 0.5.

Swimming test

The mice were placed in tepid water (24–26°C) for no more than 2 minutes. If the mice exhibited any sinking behaviour they were immediately rescued. The mice that did not sink were submerged to determine whether the mouse could reorient and find the surface, a test that normal mice can easily perform. Mice were scored on a 3 point scale (Marshall & Berrios, 1979) depending on the amount of their body they kept above water and the vigour of their swimming. Scoring for swimming style was as follows: head and back above water, 3.0; head only above, 2.5; ears wet, 2.0; eyes at water level, 1.5; nose only above water, 1.0; head submerged, 0.5; whole body submerged, 0.0. Scores for swimming vigour were as follows: movement of all 4 limbs, 3.0; hindlimbs only, 2.5; periods of floating, 2.0; more floating than swimming; 1.5; occasional limb movements, 1.0; occasional hindlimb movements, 0.5; no movement, 0.0. Mice that could not recover from submersion were deducted 1.0 point.

Open field test

The animal was placed in a corner square of a 1 m^2 black perspex box divided into 25 equal squares by white tape. The mouse was videotaped for 10 min and later scored for the final 5 min for the following activities: ambulation, recorded by the number of times a mouse crossed into a new square, (what type of square, side, corner or center was also recorded); defecation (number of fecal boli), grooming, circling and backwards-walking behaviour.

PHYSIOLOGY

Mice were anaesthetised with urethane (20 mg/kg intraperitoneal), the middle ear cavity was opened, and a recording electrode was placed on the round window. Shaped toneburst stimuli of 15 ms duration, 1 ms rise/fall time with a 100 ms interstimulus interval, were delivered through a closed, calibrated sound system. Six mutants (*Tlc*/+) and six wild type littermate controls (+/+) aged 28 to 54 days old were used. Mutants were distinguished from controls by their mild head-tossing behaviour. Thresholds for detection of a compound action potential (CAP) in the averaged response waveform were obtained using 5 dB stimulus increments.

MAPPING

Tailchaser (*Tlc*) mice were maintained on a C3HeB/FeJ genetic background. *Tlc*/+ male mice were outcrossed to C57BL/6J mice and their mutant progeny were backcrossed to C57BL/6J females. A total of 126 offspring were obtained, of which 50 could be classified as *Tlc*/+ by their phenotype. All mutant mice were phenotyped independently by two people and only those with clearcut behavioural abnormalities were genotyped. Mutants on the mixed C3HeB/FeJ plus C57BL/6J genetic background were identified by head tossing, unstable gait, and an inability to swim properly. There were two slightly different phenotypes on this background, moderate and severe. The severe phenotype does not give a Preyer reflex in response to a 20 kHz, 90 dB SPL click from a click box from the age of 2–3 months. The moderate phenotype responds to the click box until the maximum age examined, 10 months. A normal mouse swims with head and tail tip above the water; the moderate phenotype swims with the head above water and the tail down in the water, while the severe phenotype is unable to swim at all. Both the moderate and severe phenotypes were genotyped. Only mutants, and not their littermate controls, were used for mapping, to avoid the possibility of reduced penetrance interfering with the analysis. Genomic DNA was analysed with simple sequence length polymorphism (SSLP) markers (Research Genetics). A total of fifty-nine markers, polymorphic between C57BL/6 and C3H, were used to scan the entire genome (complete list available from authors). The size difference between the two strains was at least 8 bp, so that they could be resolved on cold 4% Metaphor (FMC) gels. The coordinates were determined by referring to the MIT Genome Center genetic map. All sizes were as predicted on the MIT database, except for the C3H allele of *D2Mit257*. It should be noted that the Whitehead Institute/MIT Center for Genome Research report the same size alleles for C3H and C57BL/6 for marker *D2Mit257*, but our results indicate an approximately 20 bp size difference between the two alleles.

Results

ABNORMAL STEREOCILIA DEVELOPMENT IN THE COCHLEA

Differences in the stereocilia bundle between heterozygotes and wildtypes could be observed as early as birth (P0). The left and right cochleas of 7 animals were examined by SEM. Two animals displayed abnormal outer hair cell (OHC) bundles and were assumed to be mutants (Fig. 1). At this point in the development of the OHC in normal mice, the stereocilia in both the base and apex are arrayed in a distinctive V-shape with the kinocilium located at the point of this V (Fig. 1A, E

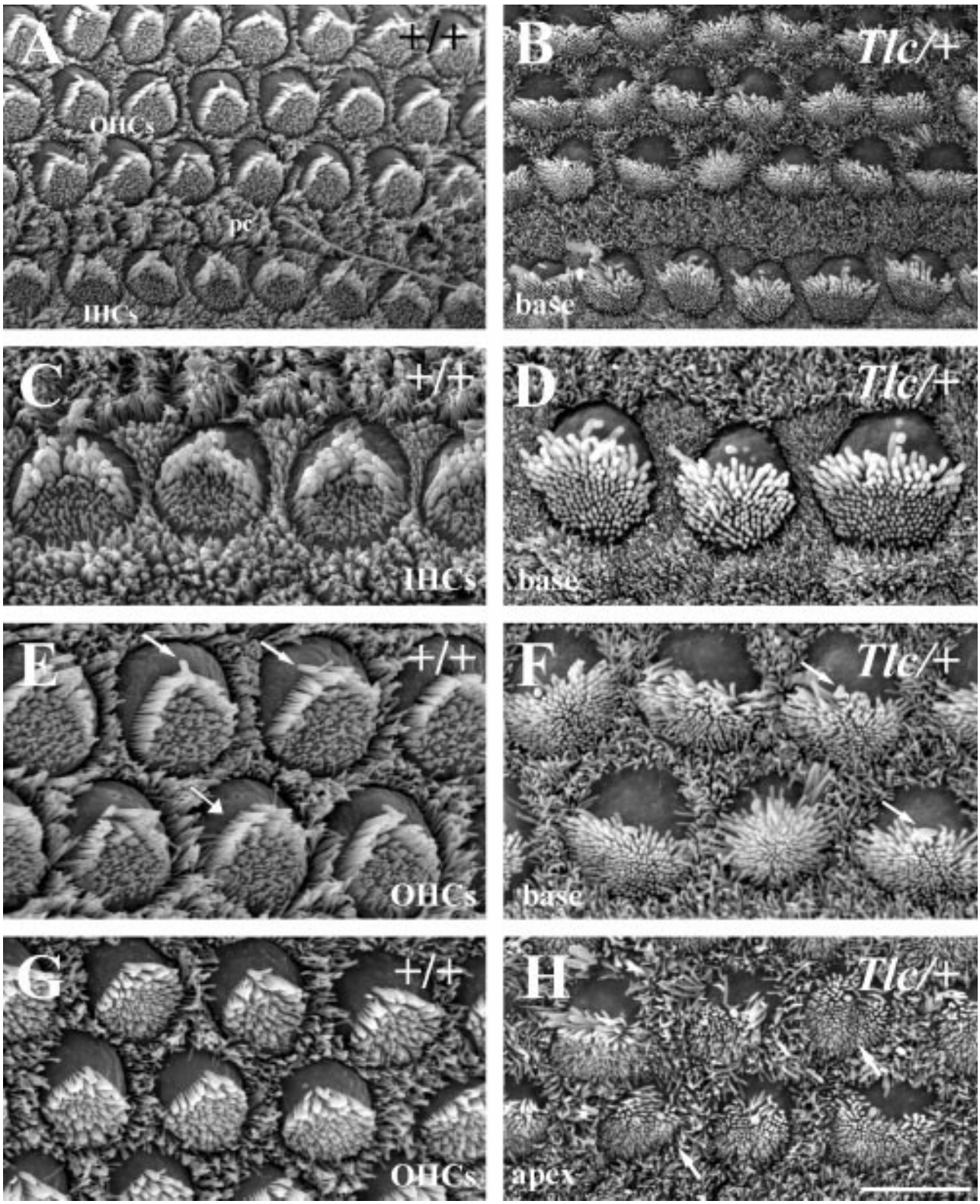

Fig. 1. Scanning electron micrographs of the organ of Corti in mutants and controls at P0. Genotypes are displayed in the upper right hand corner and the region of the cochlea (base or apex) is indicated. (A and B) Lower power views of the cochlea demonstrate the immature-looking bundles in the mutant. (C and D) Higher power views of the IHCs show little difference between mutants and controls except the V or crescent shape is not present. (E and F) Higher power views of the outer hair cells in the base show the lack of the V-shape in the mutants. Thick arrow in (E) indicates the elongating stereocilia, a feature that is not apparent in the mutant (F). Thin arrows in (E) and (F) indicate the position of the kinocilium, which looks roughly normal in the mutants. IHCs, inner hair cells; OHCs, outer hair cells; pc, pillar cells. Scale bar in (H) represents 10 microns for A and B and 5 microns for (C–H).

and G). Additionally, in the base of the cochlea where the hair cells mature earlier, what will be the tallest rows of stereocilia have already begun to elongate (Fig. 1E). In mutant cochleas, the stereocilia bundles showed no sign of the V-shape and the stereocilia all appeared to be of the same length (Fig. 1F), indicating the staircase had yet to begin to form. However, at least in the base, the OHCs seemed to be polarised correctly, with the hair bundles all grouped toward the inner sulcus region of the organ of Corti. In the apex, some hair bundles were not yet polarised and the stereocilia were spread across the entire surface of the cell (Fig. 1H; arrows). In the mutants, the kinocilium, although not present at the point of the V since there was none, generally appeared to be located in the normal position, on the correct side of the bundle and roughly in the center of the cell (Fig. 1B and F) The differences in the IHC stereocilia are more subtle, and signs of immaturity are not as apparent. One aspect of their development that does seem to be affected is the formation of a soft V or crescent shape. This shape can be observed at P0 in the wildtype hair cells (Fig. 1C) but the mutant hair bundles appear to lack this shape (Fig. 1C and D).

Wildtype and heterozygote cochleas were analysed by SEM at P3 ($n = 10$). Four of the cochleas showed a similar phenotype to that observed in presumed mutants at P0, except that the immaturity of the OHC stereocilia bundles was even more pronounced at this stage when compared to controls and many of the bundles appeared disorganised. At this point in development, wildtype outer hair bundles display a well-formed V-shape, the staircase is apparent, and most of the overproduced stereocilia have been resorbed (Lim & Anniko, 1985; (Figs. 2A and 3C). In contrast, the OHCs in the mutant cochleas still appeared very immature and the V-shape remained absent (Figs. 2B, C and 3D). Signs of disorganisation were apparent in the fact that on some hair cells, the stereocilia were no longer grouped into one bundle, but instead were organised in small clusters on the surface of the cell (Figs. 2B, C and 3D). In addition, some bundles lost their orientation and began to face sideways or backwards (Fig. 2B; arrows). However, some differentiation continued to take place; for example the beginnings of a staircase arrangement could be identified within groups of stereocilia (Fig. 2C). The developmental delay was apparent in that many of the excess stereocilia did not appear to be reabsorbed (Fig. 3D). The kinocilium showed more variation in position at this time point as compared to P0, and could be found at the side of the hair cell where it was not always aligned with the tallest stereocilia, as well as in its normal central position (Fig. 2C; arrows). The hair bundles in the apical portion of the cochlea looked much as they did at P0; very immature and signs of disorganisation were not as apparent as in the base (data not shown). Similar to P0, the inner hair bundles generally appeared normal except that the soft V-shape

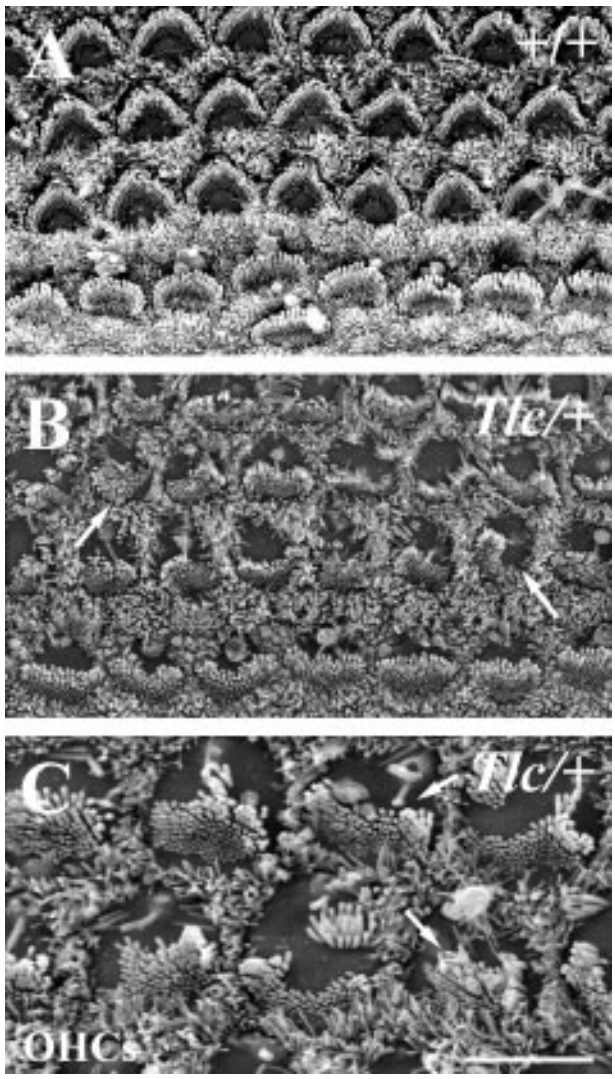

Fig. 2. Scanning electron micrographs of the organ of Corti in mutants and controls at P3. All micrographs are from the basal portion of the cochlea. (A–C) lower power views of the cochlea show the disorganisation that is beginning to become apparent in the mutant at this stage. Arrows in (B) demonstrate bundles that show a sideways or backward orientation, as interpreted from their staircase orientation. Arrows in (C) demonstrate the variable position of the kinocilium. IHCs, inner hair cells; OHCs, outer hair cells. Scale bar in (C) represents 10 microns for (A) and (B), and 5 microns for (C).

was not present and stray stereocilia were also sometimes observed (Figs. 2B and 3B).

By P20 the hair cells of normal mice are morphologically and functionally mature (Harvey & Steel, 1992; Shnerson & Pujol, 1981). The cochleas of 10 mice from 2 litters were examined at P20 by SEM, and 7 of the mice showed extreme disorganisation in the hair bundles of the cochlea. The cellular organisation of the organ of Corti appeared normal, with 3 rows of OHC separated by pillar cells from the IHC region (Fig. 4A and B). However, many of the OHC stereocilia were grouped into small clusters throughout the surface of

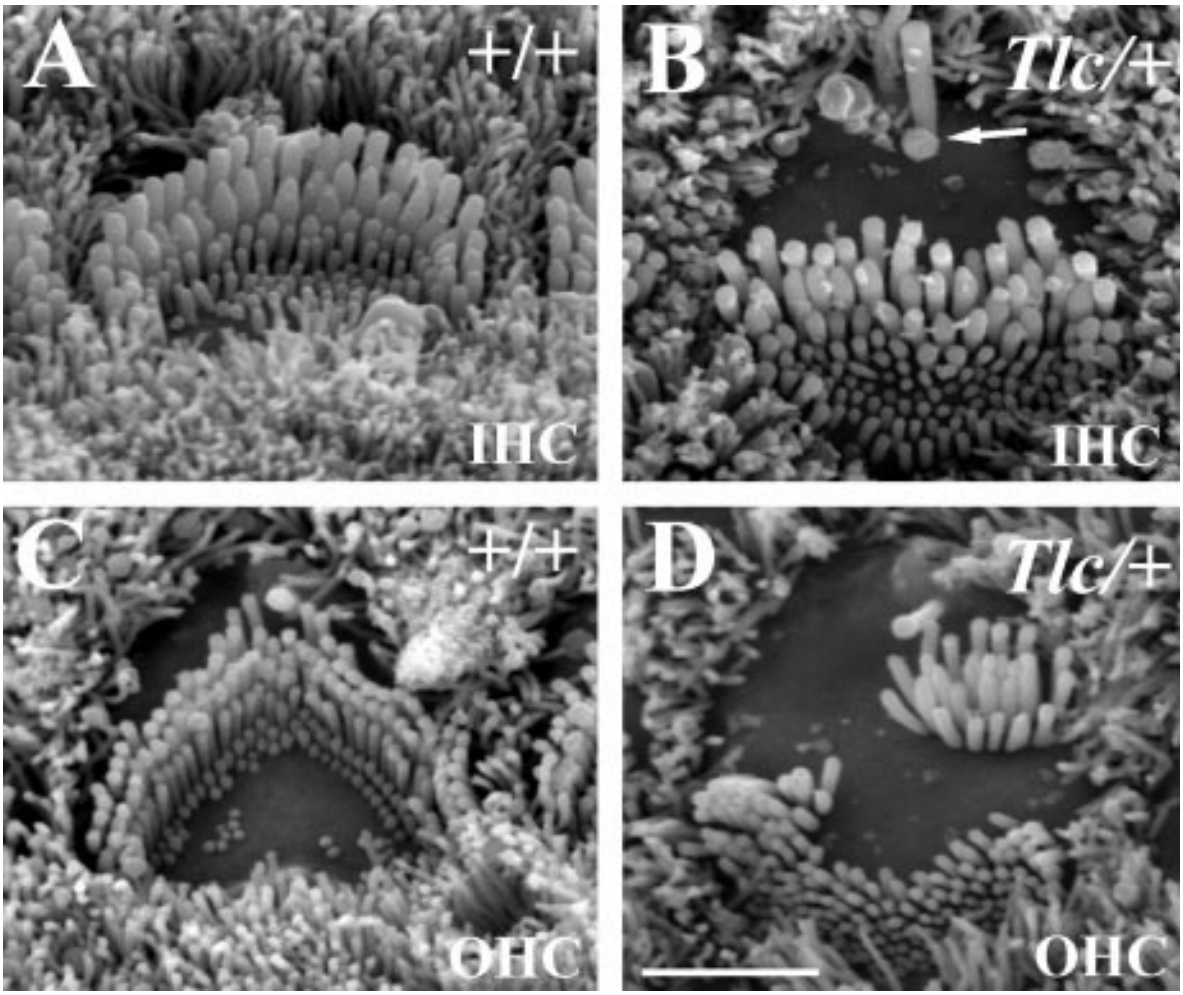

Fig. 3. High power micrographs of the IHCs and OHCs at P3. Note the absence of the crescent or V-shaped hair bundle in the mutant IHC (B). The arrow in (B) indicates the stray stereocilia that were sometimes observed on IHCs. (C and D) Clumps of stereocilia were often observed on some of the mutant OHCs at P3. IHCs, inner hair cells; OHCs, outer hair cells. Scale bar in (D) represents 2.5 microns for all panels.

the hair cell (Fig. 4B and D). In cases where the stereocilia were still in one bundle, there seemed to be no organisation that resembled the V or W-shape. Within most of the abnormal bundles there was some staircase formation (Fig. 4D; arrows), although the orientation of the staircase was not maintained correctly, and some bundles appeared to be facing a sideways or backwards direction (Fig. 4B and D). The kinocilium was absent as it should be at this time (Kikuchi & Hilding, 1965; Kimura, 1966). It was difficult to identify any abnormalities in most of the IHC stereocilia, although some of the bundles in the base appeared in two clusters (Fig. 4B; arrow). For the most part, the IHCs in the apex appeared normal from the surface (Fig. 4F).

VESTIBULAR HAIR CELLS

To determine whether the vestibular hair cells bundles displayed a similar disorganisation to that displayed by the cochlear outer hair cells, utricles from left ears of the P3 animals described above were examined by SEM (4 presumed heterozygote and 6 wildtype utricles). At this stage of development most of the hair cells have reached their mature dimensions, although some new hair cells continue to appear in the epithelium until P6 (Lim & Anniko, 1985). The pattern of stereocilia atop the vestibular hair cells appeared normal; the stereocilia were arranged in their normal circular pattern and the staircase pattern was evident with the kinocilium located on the side of the bundle displaying the longest stereocilia (Fig. 5A–D). Additionally, the bundles appeared to have normal orientation, that is, the staircase of the bundles generally faced the same way except in the striolar region where the staircases were in a back-to-back orientation (Fig. 5A and B). In general however, the stereocilia in the mutant utricles did not appear as tightly bundled as controls and some hair cells displayed fused stereocilia.

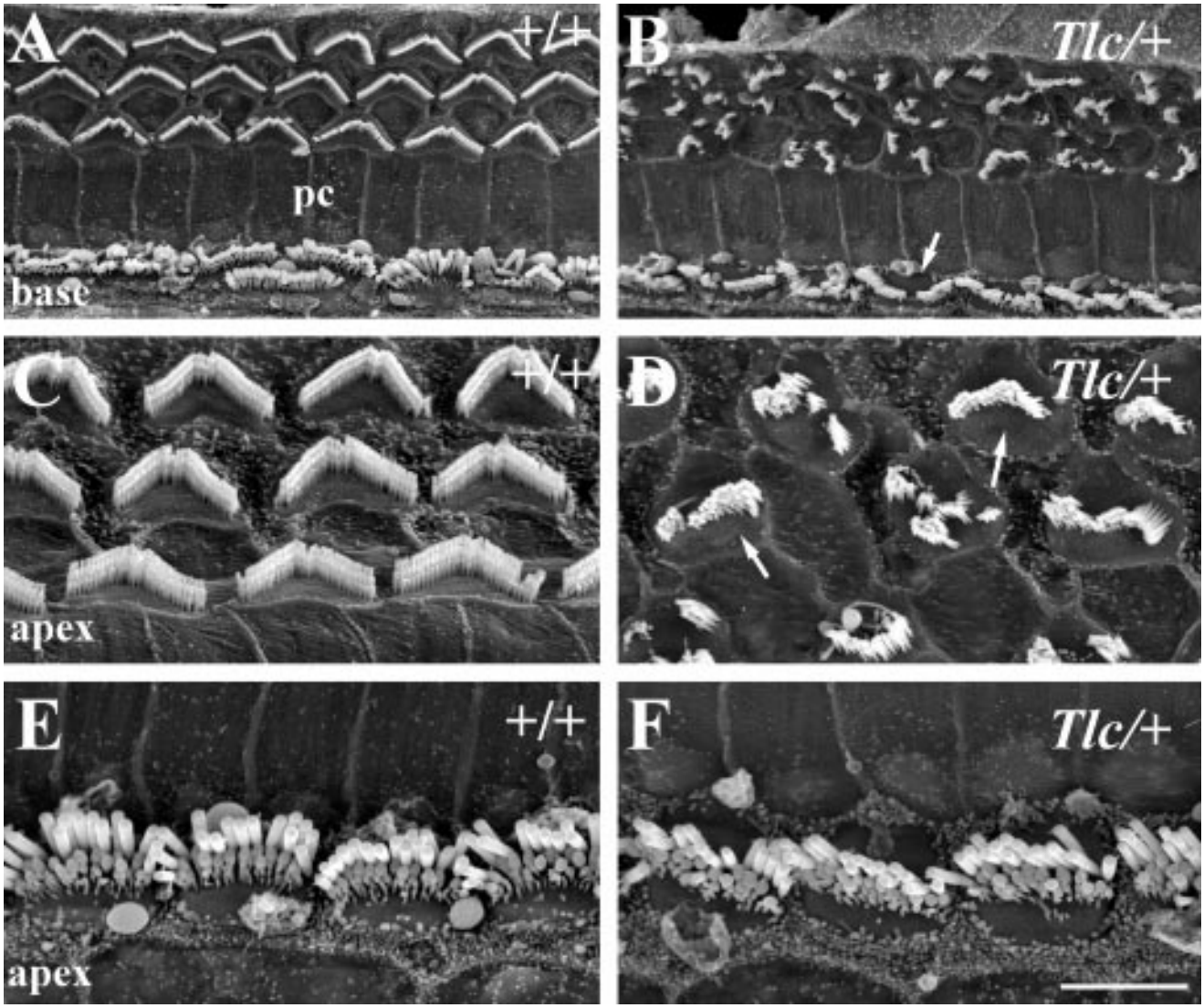

Fig. 4. Scanning electron micrographs of the organ of Corti in mutants and controls at P20. Genotypes and the region of the cochlea (base or apex) are indicated. (A and B) Low power views of the organ of Corti showing the severe disorganisation of the stereocilia of the mutant OHCs. Arrow in (B) indicates an IHC in the base in which the bundle is split in 2 halves. (C and D) Higher power views of the OHCs, arrows in D indicate some of the mutant bundles in which the staircase pattern can still be observed. (E and F) Higher power views of the IHCs in which little disorganisation was present. Scale bar in (F) is 10 microns for (A) and (B) and 5 microns for (C–F).

OTOLITHIC MEMBRANES ARE PRESENT IN THE *Tlc* VESTIBULE

Several mouse vestibular mutants, including pallid (*pa*) (Lyon, 1951, 1952), head-tilt (*het*) (Bergstrom *et al.*, 1998), and tilted (*tlt*) (Ornitz *et al.*, 1998), as well as numerous zebrafish ear mutants (Malicki *et al.*, 1996; Whitfield *et al.*, 1996) have been described as having absent or abnormal otolithic membranes, the extracellular material which lies above the macular hair cells. In order to establish whether *Tlc* exhibits a similar deficit we examined left and right wholemount cleared ears of wild-type ($n = 2$) and heterozygote ($n = 3$) animals from 2 litters aged between 4 and 5 months. Results showed the presence of the saccular and utricular otoliths in all cases (Fig. 6B; arrows), suggesting otolith formation is not affected in these mutants. In addition the gross morphology of the inner ear of *Tlc* mutants appeared normal (Fig. 6).

Tlc MICE HAVE PROGRESSIVE HEARING AND BALANCE DYSFUNCTION

It was observed that, upon weaning, the *Tlc*/+ mice showed only a subtle head- shaking behaviour. However, as the mice aged, the mutants began to display other behaviours indicative of a more severe vestibular defect, suggesting there was a progressive deterioration of inner ear function. In order to characterise

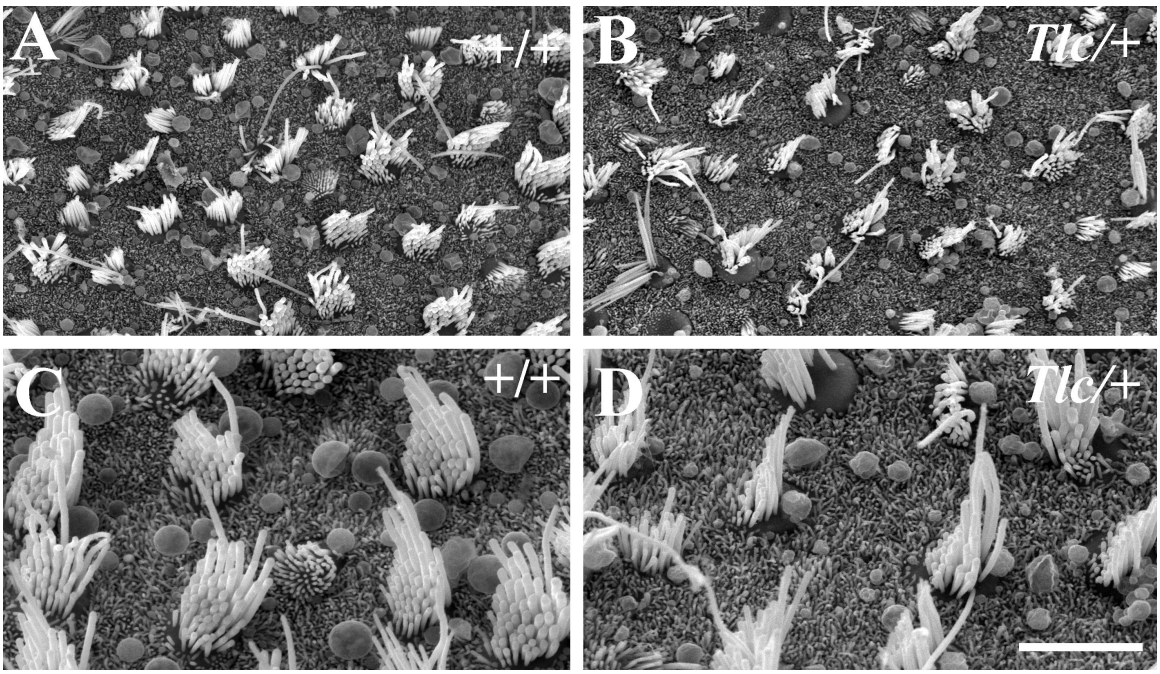

Fig. 5. Scanning electron micrographs of the utricular macula in mutants and controls at P3. Genotypes are displayed in the upper right hand corner. Micrographs are of utricular hair cells either in (A and B) or near (C and D) the striolar region, showing little difference between the mutants and controls. Scale bar in D represents 10 microns for (A) and (B) and 5 microns for (C) and (D).

this deterioration a battery of behavioural tests known to assess vestibular or hearing function (reviewed in Steel and Hardisty (1996)) was performed at three different ages: i) 74 days (3 *Tlc*/+; 5 littermate controls), ii) 136–146 days (5 *Tlc*/+; 3 age-matched controls), and iii) 250–293 days (5 *Tlc*/+; 4 age-matched controls). The results of these tests are shown in Fig. 7A–G. In addition, cochlea and vestibular regions were taken for SEM soon after testing to confirm the phenotyping and to examine for signs of degeneration (see below).

Mutants lost their Preyer reflex, the ear flick response to sound, between 4.5 and 10 months of age (Fig. 7A), and some mice showed a reduced response at 4.5 months. All the mice, mutants and controls, demonstrated a robust reflex at 2.5 months. This was an interesting finding as it showed that *Tlc*/+ mice retain some level of hearing until they are quite old. Most of the vestibular tests also revealed a similar progressive dysfunction. For example, although most of the *Tlc* mutant mice stayed on the elevated platform at 2.5 months, all of the mice fell off the platform at 4.5 months of age, suggesting a significant deterioration in balance function (and/or increase in hyperactivity) in these two months (Fig. 7B). In the contact-righting test, in which the mouse must determine that it is upside down without tactile cues, the mice did not show a significant deterioration until 10 months (Fig. 7C). Interestingly, the *Tlc*/+ mice showed an abnormal reaching response at 2.5 months of age (Fig. 7D), suggesting this is one of the more sensitive tests of vestibular function, or is specific to a particular vestibular region(s) in which functionality is lost early in the *Tlc* mutants. Although variable, ambulation was shown to be increased in mutants across all age groups (Fig. 1E), a common finding in rodents with vestibular deficits (for example see Douglas *et al.* (1979); Ossenkopp *et al.* (1990); Porter *et al.* (1990)). In addition, increased bouts of backwards-walking were observed in all age groups of mutants compared to controls (Fig. 7F) as well as circling behaviour, which was never observed in controls and increased in frequency with age (data not shown). Although swimming ability rapidly decreased with increasing age, mutant mice did show some ability to stay afloat at 2.5 months, although the mice often swam in circles and could not reorient and surface after submersion (Fig. 7G). The mutants showed normal vigour during swimming (data not shown), demonstrating that the mice failed the test due to inability to orient and maintain themselves on the surface rather than because of motor problems. Measurement of defecation, which is thought to reflect emotionality which in turn may affect open field activity (Flint *et al.*, 1995), did not show a difference between mutants and controls (data not shown). *Tlc*/+ mice clearly show progressive hearing and balance dysfunction, as reflected in the increased failure rates of specific behavioural tests

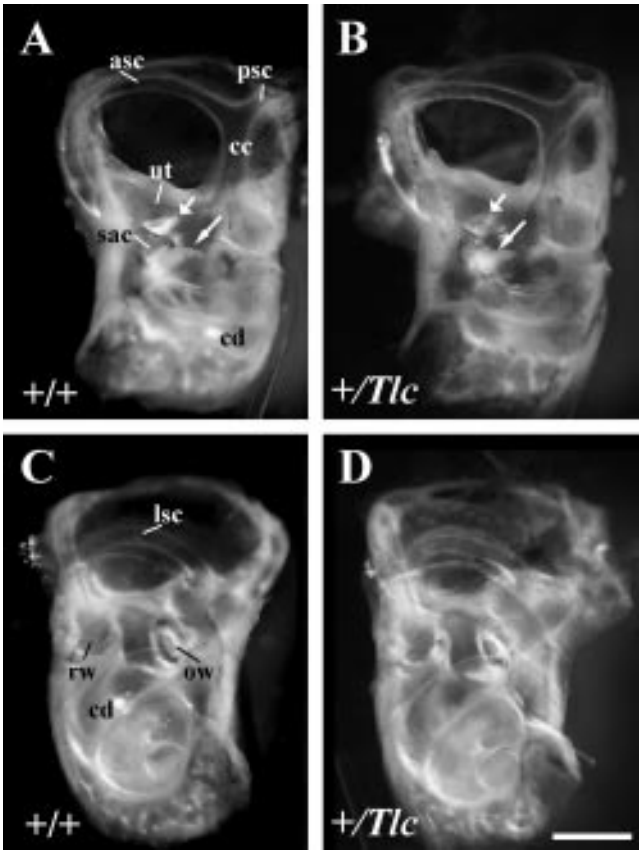

Fig. 6. Cleared wholemount ears from mutants and controls. Genotypes are indicated in the lower left of each panel. (A) and (B) are medial views of the cochlea in which the otolithic membranes can be observed in both mutants and controls (fat arrow, utricular otoliths; thin arrow, saccular otoliths). (C) and (D) are lateral views of the inner ear in which all the structures appear normal in the mutant. asc, anterior semicircular canal; cc, common crus; cd, cochlear duct; lsc, lateral semicircular canal; ow, oval window; rw, round window; sac, sacculus; ut, utriculus. Scale bar represents 1 mm.

such as the Preyer reflex test, the elevated platform test, the contact-righting test, and the swimming test.

DEGENERATION IN THE COCHLEA AND VESTIBULE

Cochleas and vestibular sensory regions from mice that were tested for behaviour were examined by SEM to determine if any changes could be observed in the sensory epithelia. Surprisingly, the cochlea showed little hair cell loss at 2.5 months. Many more changes were observed at 4.5 months, in which some IHCs were missing or appeared to be in the process of degenerating (Fig. 8A). OHCs appeared to be in a similar state of deterioration. By 10 months most of the IHCs in the mutants were missing or appeared to be in the process of dying in both the base and the apex of the cochlea. Interestingly, some cochleas at 10 months showed most of the OHCs still present in the apex of the cochlea, although many of the OHCs were missing in basal regions (Fig. 8B). One surprising result was that the OHCs did not appear to be dying preferentially when compared to the IHCs, despite having more abnormalities in their hair bundles.

The vestibular regions were examined at 2.5 months of age. It was difficult to preserve and analyse all the vestibular organs so in most cases the utricle and one of the crista were examined, usually the superior or lateral. At 2.5 months it appeared that the cristae of the *Tlc*/+ were virtually devoid of stereocilia (Fig. 9A and B). The mutant utricles at 2.5 months showed the presence of more stereocilia when compared to the cristae (Fig. 9C–F); however, the epithelium appeared much more sparsely populated by hair cells than controls, particularly in central regions, and many of the hair cells that were present displayed fused bundles (Fig. 9E and F).

COCHLEAR PHYSIOLOGY

The control mice showed normal thresholds, comparable with young adults from other mouse strains, with the most sensitive CAP responses obtained for higher frequencies (see Fig. 10). Mutants, however, showed raised thresholds, up to 50 dB higher than the controls at some frequencies. Summating potentials were seen in the response waveform (not shown), and these sometimes had a slightly lower threshold than the CAP thresholds shown in Fig. 10. All six mutants showed a Preyer reflex, albeit not so intense as the controls' response.

MAPPING

To identify the chromosomal location of the *Tlc* locus, we generated an intraspecific backcross and undertook a low resolution genome scanning approach. Mice segregating *Tlc* were used to localize the *Tlc* locus to an interval of approximately 12 centimorgans (cM) in the central region of chromosome 2, between markers *D2Mit164* and *D2Mit423* (Fig. 11). There was no evidence of any linkage elsewhere in the genome (data not shown). However, as shown on the haplotype diagram (Fig. 11A), there were three mice with anomalous typings (white boxes indicating a C57BL-like typing) within this interval, two with an apparent triple recombination and one with C57BL/6J typings down to and including *D2Mit280*. These typings were all repeated at least once and DNA resampled. The two mice with apparent triple recombinations could result from a mutation affecting primer binding for *D2Mit257* in the *Tlc*-bearing chromosome, which could lead to apparent homozygosity for the C57BL/6J allele in these mice because amplification would only occur from the chromosome derived entirely from the C57BL/6J parent of the backcross. Similar anomalous but repeatable typings have previously been reported from EUCIB mapping (Rhodes *et al.*, 1998). Alternatively, there may be

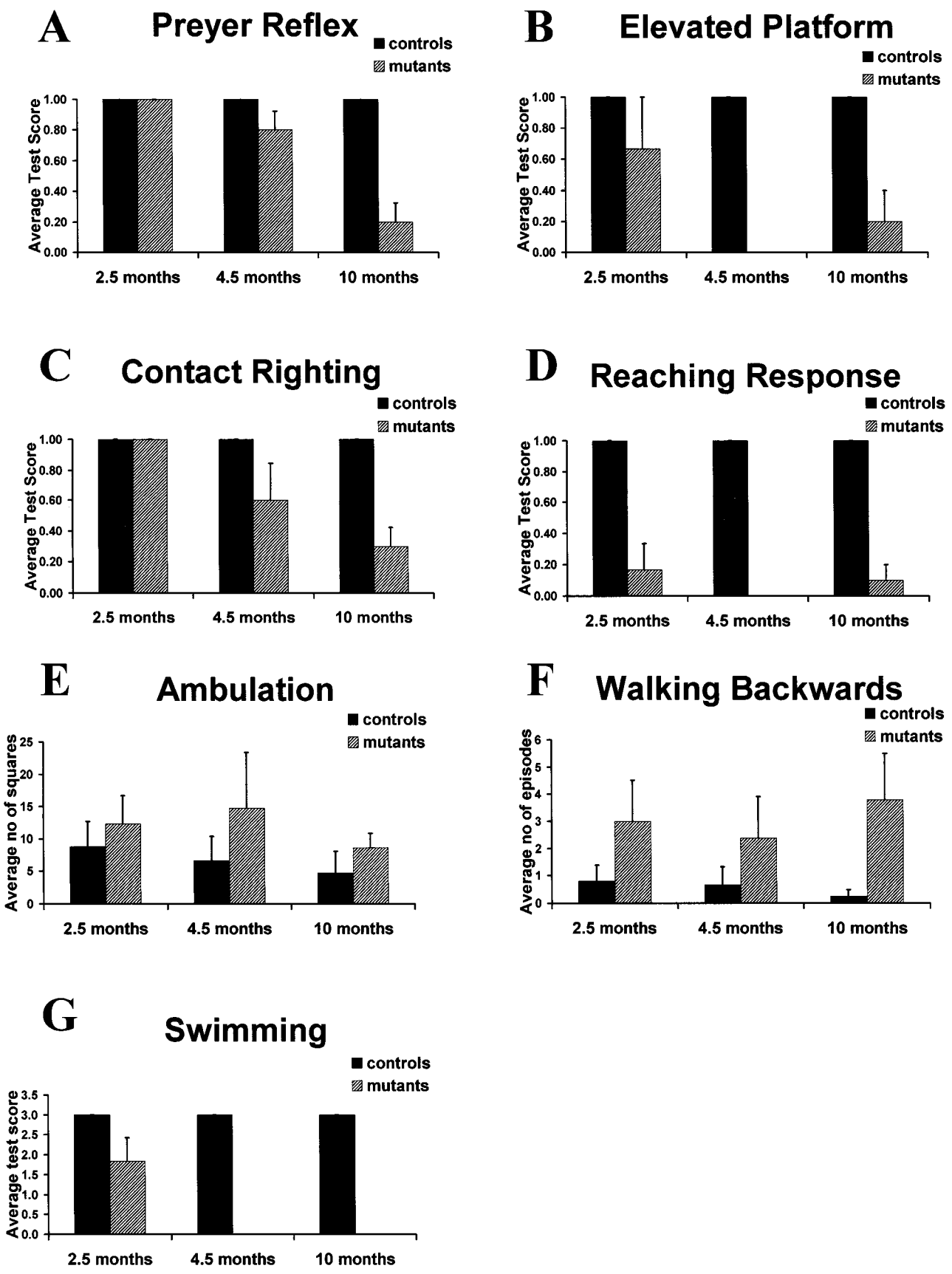

Fig. 7. Behavioural testing shows a progressive deterioration of inner ear function. Controls are represented by black bars and mutants (*Tlc*/+ in all cases) are represented by striped bars. Absence of a bar indicates that none of the mice scored above 0. Mutants perform less well over time for the Preyer reflex test, the elevated platform test, the contact righting test, and swimming (A–C and G). (D) Mutants showed an equally abnormal reaching response at all ages. (E and F) Ambulation and periods of walking backwards were increased in the mutants. Error bars represent standard error of the mean.

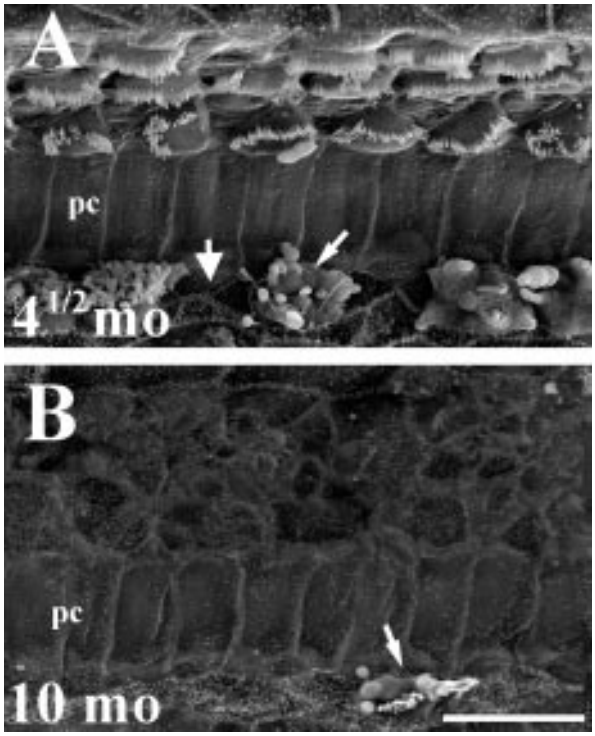

Fig. 8. Scanning electron micrographs of the organ of Corti in ageing mutants. The micrographs were taken from the basal portion of the cochlea. (A) Many inner hair cells are missing (thick arrow) or in the process of degenerating (thin arrow). (B). In this particular mutant most of the hair cells appeared to be missing by 10 months, and only a few degenerating hair cells could be observed (arrow). Scale bar in B represents 10 microns for both panels.

a region of genomic instability in this area leading to apparent multiple recombinations in a short stretch of chromosome, as has been suggested previously to account for anomalous typings in another chromosome (Rogers *et al.*, 1999). If these anomalous typings are due to a new mutation affecting *D2Mit257* or genomic instability, then the *Tlc* mutation could be within the interval *D2Mit280* to *D2Mit423*, a 2.2 cM interval according to the MIT mapping data for these markers, or a 10 cM interval according to our own backcross mapping. The order of all markers in our cross was the same as that reported by MIT for their 46 mouse intercross and is consistent with the order reported for EUCIB and all other published crosses using two or more of the markers we have used, but differs from that of the composite maps distributed by the Mouse Genome Database (MGD) which are derived from interpolating mapping information from many different sources.

Discussion

We report here the first of the new hearing-impaired mouse mutants generated by an ENU mutagenesis programme in Neuherberg designed to detect new dominant mutations affecting, among other functions, hearing and/or balance. Although the founder of this new mutant stock was initially detected because of behaviour indicative of a balance defect, we have found that hearing is also affected. The *Tlc* mutation was mapped to chromosome 2. Both hearing and balance defects appear to worsen with increasing age. Gross inner ear morphogenesis is normal, but cochlear hair cells, particularly OHCs, show an early delay in development of their bundles at P0, which appears to eventually lead to their disorganisation by P20. Interestingly, IHCs at P20 and vestibular hair cells at P3 do not seem particularly disorganised, although both eventually degenerate along with the OHCs. These data suggest the *Tlc* gene is most likely expressed within the hair cell and probably at prenatal stages of development.

Both compound action potential and summating potential responses were detected in the mutants, indicating that hair cells can depolarise and initiate action potentials despite the anomalies in stereocilia bundle development. Despite the finding of a Preyer reflex in young adult mutants at 2.5 and 4.5 months (e.g. Fig. 7A), thresholds for detection of a compound action potential response were raised to approximately 80 to 90 dB SPL (see Fig. 10), emphasising the fact that this reflex is a high intensity response and cannot detect mild, moderate, or even moderately severe (in the case of the *Tlc* mutants) hearing impairments.

The homozygote phenotype is unknown. Some matings were set up that would potentially produce homozygotes; however very few offspring resulted from these matings and it was therefore not possible to calculate whether it was likely that the homozygotes were dying prenatally. In litters that were produced from heterozygote parents, no difference in behaviour among mutants was observed.

Tlc MAPS TO CHROMOSOME 2

An intraspecific backcross of 50 mice allowed us to position the *Tlc* locus to the central region of mouse chromosome 2. *Tlc* maps between *D2Mit164* and *D2Mit423*, a region spanning approximately 12 cM. In order to determine which mouse mutants and genes might be candidates for *Tlc*, we examined reports of markers mapping to this region of chromosome 2.

Although there are several mouse mutations in this region, based on phenotype the only likely potential allele of *Tlc* is the dominantly-inherited mouse mutation coloboma (*Cm*). Heterozygotes have a specific eye defect called a coloboma, and additionally exhibit hyperactivity and behaviour indicative of a vestibular defect including head bobbing and circling (e.g. Hess *et al.*, 1992). The *Cm* mutation has been defined as a 1–2 cM deletion including several genomic markers, and the *Plcb*, *Jag1*, and *Snap25* genes (Hess *et al.*, 1994; Xue *et al.*, 1999). Jagged1 (*Jag1*) is a ligand in the Notch

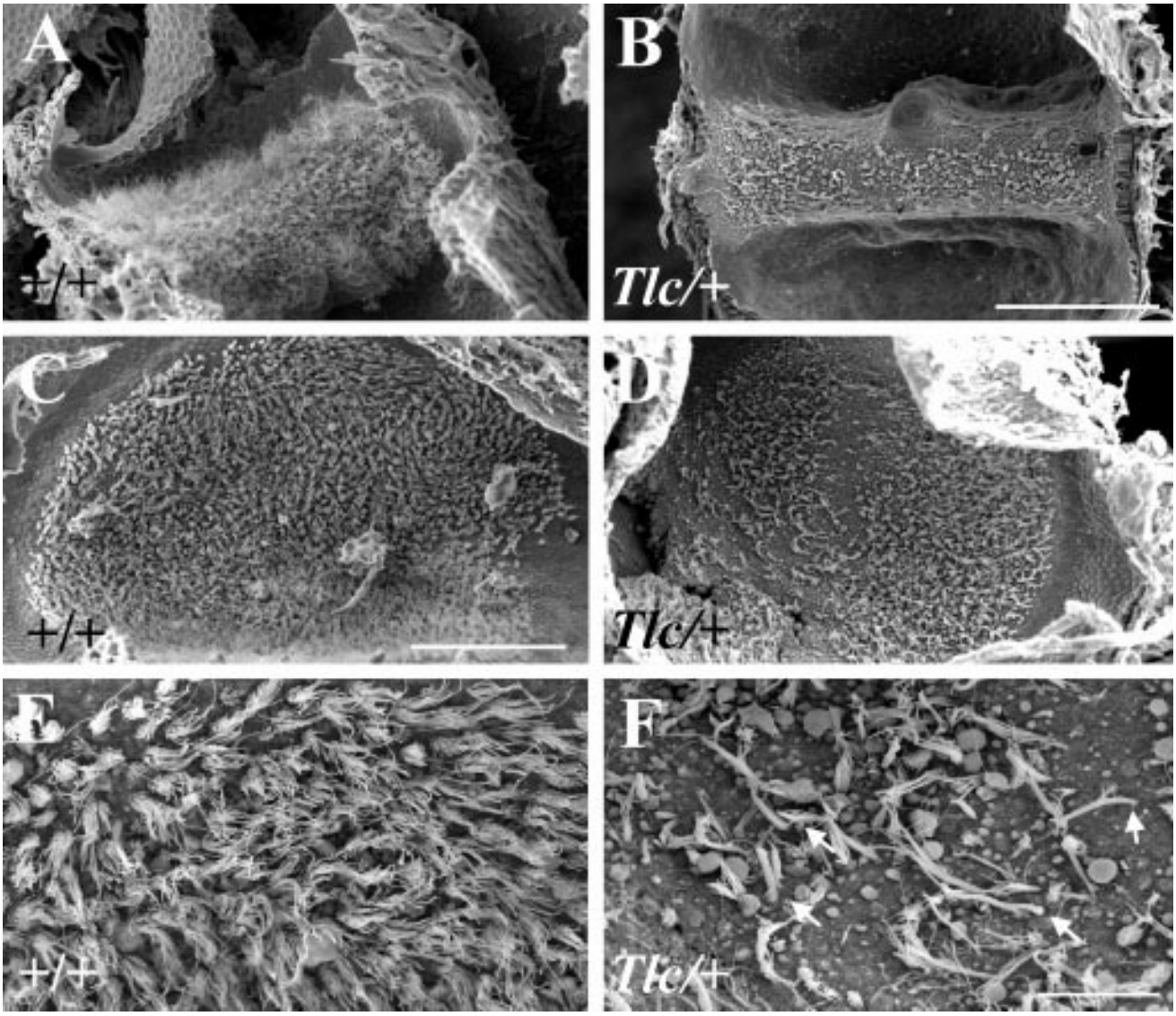

Fig. 9. Scanning electron micrographs of the vestibular regions at 2.5 months. Genotypes are indicated in the lower left of each panel. (A and B) Low power views of the lateral crista show very few hair cells, as demonstrated by the bald appearance when compared to wildtype. (C and D) low power views of the utricular macula, which has a sparse appearance in the mutant indicating fewer hair cells, particularly in central regions. (E and F) Higher power micrographs of the utricles shown in (C) and (D), demonstrating fewer hair cells in the mutant and many with fused stereocilia (arrows). Scale bar in (B) is 100 microns for (A) and (B). The scale bar in (C) is 100 microns for (C) and (D) and the scale bar in (F) is 20 microns for (E) and (F).

signalling pathway and is expressed in the developing mammalian inner ear (Lewis *et al.*, 1998) making it a potential candidate for *Tlc*. However, mice carrying one copy of a *Jag1* mutation show eye defects but no behavioural anomalies, which suggests that deletion of the *Jag1* gene in *Cm*/+ mice is responsible for the eye phenotype but not for the hyperactivity or head bobbing (Xue *et al.*, 1999). Another deleted gene in the *Cm* mutant, synaptosomal-associated protein, 25 kDa (*Snap25*), is a molecule implicated in directing vesicular fusion mechanisms in certain populations of neurons (Bark *et al.*, 1995). *Snap25* is also unlikely to be the *Tlc* gene because transgene rescue experiments using the *Snap25* gene can rescue the hyperactivity of *Cm*/+ mutants, but not the head bobbing feature of the behaviour (Hess *et al.*, 1996). Thus it appears there is a deleted gene in the *Cm* region that causes a dominant vestibular phenotype that is not *Jag1* or *Snap25* and may be the *Tlc* gene.

Another interesting gene recently reported in the region is *moth1*, modifier of tubby hearing 1, which appears to have a C57BL/6J-derived allele that interacts with the tubby, *tub*, mutation to produce hearing loss in double homozygotes (Ikeda *et al.*, 1999). Although the C57BL/6J allele of *moth1* is recessive and has not been shown to have any effect in the absence of *tub*, a more severe mutation in the same gene might have a more severe effect on the phenotype, as in tailchaser.

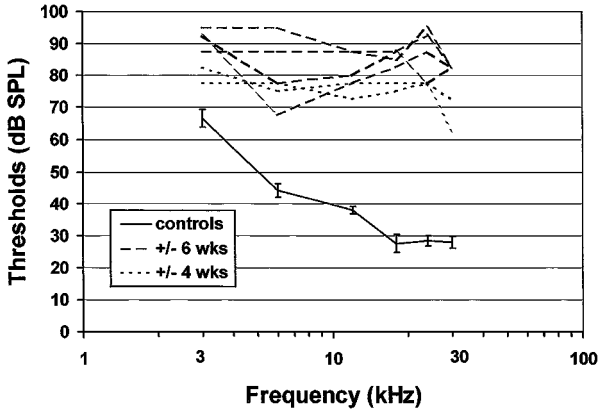

Fig. 10. CAP thresholds from the six littermate controls (solid line ± SEM) and the six mutants (plotted individually above, dashed lines), showing raised thresholds in mutants compared with controls. Mice were aged 28 to 54 days old. The two youngest mutants (28 days) are represented by shorter dashed lines, and had slightly better thresholds at high frequencies than the older mutants (47 to 54 days), suggesting there may be some progression of impairment.

The mouse chromosome 2 *Tlc* region shares homology with several human chromosomes, including portions of chromosome 2, 11, 15 and 20 (Online Mendelian Inheritance in Man, 1999). *D2Mit423* maps to a region with homology to 15q15-q22, and DFNB16, a recessive form of hearing loss, also maps to this region at 15q21-q22 (Campbell *et al.*, 1997). The human orthologues for genes flanking *D2Mit257* lie on human chromosome 20pter-p12, but there are no human deafness loci yet mapped to human chromosome 20. In addition, two loci, DFNB9 (encoding otoferlin; Yasunaga *et al.*, 1999) and DFNA16 (Fukushima *et al.*, 1999), reside on a portion of human chromosome 2 which appears to lie outside the homologous region. However, since the DFNA16 locus is positioned relative to markers other than the ones used in this study, and both this locus and *Tlc* are dominantly-inherited and associated with a progressive hearing loss, further mapping would be helpful to establish if the two loci are homologues. Another recessive locus, DFNB18, lies on human 11p14-15.1, in a region homologous to a portion of mouse chromosome 2 (Jain *et al.*, 1998). Furthermore it has been estimated that there are over 100 loci for non-syndromic sensorineural hearing impairment in addition to the syndromic deafness loci not yet mapped (Morton, 1991). Therefore, additional loci mapping to the *Tlc* human homologous region may yet be discovered.

Tlc IS A NOVEL MOUSE MUTANT

Based on the mode of inheritance (dominant), localisation in the mouse genome, and behavioural and histological defects, *Tlc* appears to be a novel mutation and may represent a new locus. It is possible that it may

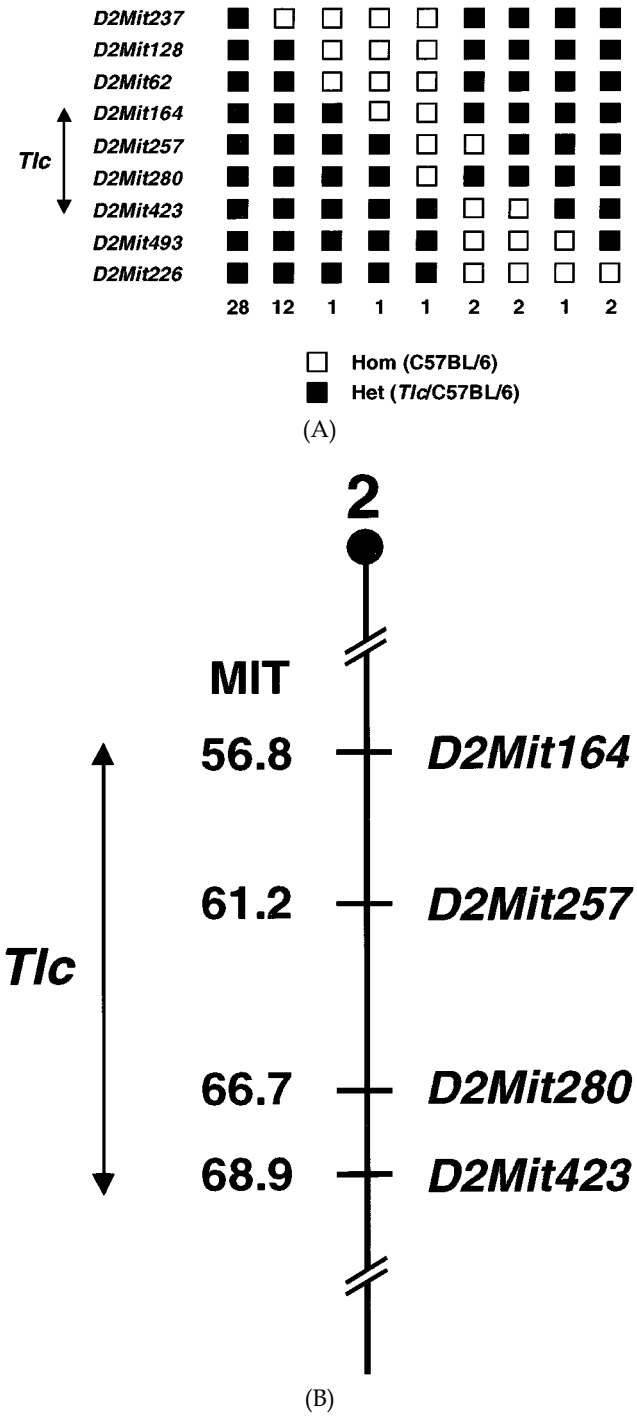

Fig. 11. (A) Haplotype analysis of 50 intraspecific backcross mutant mice for chromosome 2. Markers are shown to the left. Each column represents the chromosome identified in the backcross progeny that was inherited from the (C57BL/6J × C3HeB/FeJ-*Tlc*/+)F_1 parent. The shaded boxes represent the presence of a C3HeB/FeJ allele and white boxes represent the presence of a C57BL/6J allele. The number of offspring inheriting each type of chromosome is listed at the bottom of each column. (B) Partial chromosome 2 linkage map showing the location of *Tlc* relative to linked markers. The distances given on the left are derived from the MIT map, given in cM from the centromere (Whitehead Institute for Biomedical Research/MIT Center for Genome Research, 1999).

involve the same gene causing the head bobbing in the coloboma mouse mutant, which has a 1–2 cM deletion covering several genes, but it could represent a different locus in the same region; more detailed mapping will help distinguish these possibilities. Very few deaf mouse mutants have been identified that show a dominant mode of inheritance and, of these, none has so far been reported to show abnormalities in stereocilia development. Human deafnesses often show a dominant mode of inheritance and many human loci of this type have been mapped (see Van Camp and Smith (1999) for the latest number). Interestingly, dominant deafness is most frequently progressive, a feature that the *Tlc* mutant also shares, making it a good potential model for one or more of these dominant loci.

THE ROLE OF THE *Tlc* GENE PRODUCT IN STEREOCILIA

The *Tlc* mutation has a severe affect on the stereocilia development of the OHCs. The aspect of bundle development that seems most affected is the formation of the V-shape. Other aspects of stereocilia development, such as the staircase formation and resorption of the excess stereocilia, appear delayed but seem to occur by P20, an adult-like stage. Little is known about how the stereocilia attain their adult dimensions (reviewed in Kollmar (1999)) and what is known has been studied most extensively in the chicken cochlea. These studies have shown that the stereocilia bundles reach their mature dimensions through discrete steps that are separated in time (Tilney *et al.*, 1992a). However, studies in the hamster have suggested that the situation in mammals may be different, as these discrete steps of growth were not observed (Kaltenbach *et al.*, 1994). One aspect of stereocilia development that has not been addressed in great detail, particularly in mammals, is the pattern that the stereocilia form on the surface of the hair cell. In mammals the stereocilia form vastly different patterns depending on the type of hair cell. As previously discussed, the most distinctive pattern is displayed by the OHC in which the stereocilia form a V-shape in the apex of the cochlea which widens to a W-shape in the basal portion. The stereocilia on the IHCs are arrayed in a fairly straight line whereas both types of vestibular hair cells, type I and type II, are organised in a roughly circular pattern. In the chicken cochlea, the stereocilia all display a similar rectangular pattern. This arrangement is achieved when the staircase begins to form and the shape changes from a circular one to a rectangular one; an event that has been associated with the appearance of tip links (Tilney *et al.*, 1992b).

One hypothesis that may account for the lack of the V-shape and the delay of other aspects of bundle development is that the *Tlc* gene is specifically involved in the V formation and other aspects of stereocilia growth are delayed due to its failure to form. Alternatively, the *Tlc* gene may have a more general role in stereocilia development, causing a general delay in the mutants, and V-shape formation must occur within a specific time window. A third hypothesis is that the *Tlc* gene is involved in multiple aspects of stereocilia development, and certain aspects of differentiation are delayed (such as staircase formation) or absent (such as V-shape) in animals bearing a mutation in *Tlc*.

Interestingly, *Tlc* does not seem to play a general role in all types of hair bundle patterning since the IHCs and the vestibular hair cells do not look grossly disorganised from the surface. During development the mutant IHCs do not form their normal soft V or crescent shape, although it is unclear exactly why they form this shape since, in the mature hair cell, the stereocilia are arrayed in a straight line. Perhaps this transient shape is important for the correct functional development of the IHC, or may result from the appearance of a functionally important feature, such as tip links. It has previously been suggested that the rectangular pattern of the cochlear bundles in the chicken may in part result from the formation of tip-links (Tilney *et al.*, 1992b).

Based on the fact that the kinocilium is always located in a highly specific region of the hair cell bundle (the point of the V), and is lost prior to normal adult hearing function (Kikuchi & Hilding, 1965; Kimura, 1966), it has been speculated to play a role in the development and organisation of the stereocilia (Sobkowicz *et al.*, 1995). In *Tlc* mutant hair cells at P0, the kinocilium appears to be in roughly its normal position, although because the V-shape does not exist it is difficult to be definitive about this. Thus it appears that the product of the *Tlc* mutant gene does not affect bundle organisation via disruption of the location of the kinocilium. By P3 the kinocilium position is variable and not always associated with the tallest stereocilia as it is normally, suggesting that some later aspects of stereocilia development can proceed without the normal position of the kinocilium.

WHAT TYPE OF PROTEIN MIGHT THE *Tlc* LOCUS ENCODE?

Thus far, the molecules that have been found to affect stereocilia differentiation have all been unconventional myosin isozymes (Avraham *et al.*, 1995; Gibson *et al.*, 1995; Probst *et al.*, 1998). Mutations in myosin VIIA, myosin VI, and most recently, myosin XV in mice have been shown to give rise to a variety of abnormal stereocilia bundle phenotypes including (respectively): disorganised bundles; fused stereocilia, and short, stubby stereocilia (Self *et al.*, 1998, 1999; Probst *et al.*, 1998). The disorganisation seen in *Tlc* heterozygote hair cell bundles resembles the phenotype observed in the myosin VIIA (shaker1) mutants, suggesting that *Tlc* may have a role in the same pathway or structure as the *Myo7A* gene or protein. Unfortunately, at present the exact function of the myosin VIIA protein is unknown, although many

roles have been proposed including apical membrane turnover, stereocilia crosslinking, cuticular plate cohesion, or stereocilia anchoring.

Several mouse mutations have been described in the literature as specifically affecting hair bundle formation and all have been localised to distinct regions of the genome, indicating several different genes are involved. The Jackson shaker mouse mutant (*js*) and the bustling mouse mutant (v^{bus}), both of which show a recessive mode of inheritance, also display disorganised stereocilia in the cochlea (Moriyama *et al.*, 1997; Yonezawa *et al.*, 1996; Kitamura *et al.*, 1992; Kitamura *et al.*, 1991). Several features of the js defects are similar to the *Tlc* mutant including primarily OHC defects rather than IHC, but leading to a degeneration of both types of hair cells. The dominant dancer mutation (*Dc*) also causes degeneration of the hair cells (Wenngren & Anniko, 1988; Wenngren & Anniko, 1990), although this degeneration may be secondary to the morphogenetic defect also observed in these mutants. In addition to these mouse mutants, several zebrafish mutants have been found to display abnormal hair bundle morphology (Nicolson *et al.*, 1998).

Clearly, based on the number of vertebrate mutants that show a defect in hair bundle integrity, a number of genes appear to be involved. As yet there have been no reports of a delay in bundle morphogenesis, suggesting *Tlc* may be unique in this regard, although early developmental studies have not been performed in many mutants.

Acknowledgment

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