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Genetic modification of hearing in tubby mice: evidence for the existence of a major gene (*moth1*) which protects tubby mice from hearing loss

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Abstract

Quantitative trait locus (QTL) analysis of genetic crosses has proven to be a useful tool for identifying loci associated with specific phenotypes and for dissecting genetic components of complex traits. Inclusion of a mutation that interacts epistatically with QTLs in genetic crosses is a unique and potentially powerful method of revealing the function of novel genes and pathways. Although we know that a mutation within the novel *tub* gene leads to obesity and cochlear and retinal degeneration, the biological function of the gene and the mechanism by which it induces its phenotypes are not known. In the current study, a QTL analysis for auditory brainstem response (ABR) thresholds, which indicates hearing ability, was performed in tubby mice from F_2 intercrosses between C57BL/6J-*tub/tub* and AKR/J-+/+ F_1, hybrids (AKR intercross) and between C57BL/6J-*tub/tub* and CAST/Ei.B6-*tub/tub* F_1 hybrids (CAST intercross). A major QTL, designated as modifier of tubby hearing1 (*moth1*), was identified on chromosome 2 with a LOD

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score of 33.4 ($P <; 10^{-33}$) in the AKR intercross (181 mice) and of 6.0 ($P <; 10^{-6}$) in the CAST intercross (46 mice). This QTL is responsible for 57 and 43% of ABR threshold variance, respectively, in each strain combination. In addition, a C57BL/6J congenic line carrying a 129/Ola segment encompassing the described QTL region when made homozygous for tubby also exhibits normal hearing ability. We hypothesize that C57BL/6J carries a recessive mutation of the *moth1* gene which interacts with the *tub* mutation to cause hearing loss in *tub/tub* mice. A *moth1* allele from either AKR/J, CAST/Ei or 129/Ola is sufficient to protect C57BL/6J-*tub/tub* mice from hearing loss.

INTRODUCTION

Tubby mice have an autosomal recessive mutation which results in maturity onset obesity associated with insulin resistance and retinal and cochlear degeneration. The biological function of *tub* and the mechanisms by which it induces these phenotypes are still unclear. While obesity is not observed until 12–18 weeks of age, abnormal electroretinogram and auditory brainstem response (ABR) are observed by 3 weeks of age (1). Histological features of the sensorineural alterations are well studied (1,2). The retinal degeneration is characterized by progressive apoptotic loss of photoreceptor cells within the outer nuclear layer and the cochlear degeneration by loss of outer hair cells and afferent neurons (1 – 3).

Since the identification of the mutation as a splicing defect in a novel gene (4,5), we have determined that tubby is a member of a new gene family (6,7). The N-terminus of the four genes within this family (tubby and the tubby-like proteins *Tulp1, Tulp2* and *Tulp3*) is divergent whereas the C-terminus is highly conserved, suggesting that the C-terminus confers a common function to the family members. These genes, however, must have also evolutionarily acquired some unique functions and lost a part of some common function because *Tulp1, Tulp2* or *Tulp3* are unable to compensate for the *tub* mutation in tubby mice. In part, this divergence may have resulted in cell-specific expression (3). In addition to the mutation in the mouse *tub* gene, mutations within the human *TULP1* gene (8,9) have also been implicated in retinal degeneration, suggesting that these genes may share a similar function necessary for retinal cell survival. To understand the causes and the variability observed in the mutant phenotypes, it is important to investigate the function of the tubby gene family members.

Identification of genetic modifiers to reveal novel gene interactions is a well-established and powerful methodology in bacteria, yeast, nematodes and *Drosophila* research. Since the establishment of quantitative trait locus (QTL) analysis (10), the identification of such modifiers in mice has become practical and shown to be an extremely useful tool (discussed in ref. 11). In addition, with the development of genetic resources such as single-strand length polymorphism (SSLP) markers, expressed sequence tags (ESTs) and large insert genomic libraries, identification of genes which are responsible for QTLs is feasible. In the current study, QTL analysis is used to map genes affecting hearing in *tub/tub* mice. These genes are necessary for the generation of the *tub* phenotype and. therefore, may mediate its normal function. Identification of such genes will help to elucidate the pathways through which TUB functions.

RESULTS

Auditory brainstem response (ABR) analysis was performed to estimate the hearing ability of 6- to 8-week-old mice.

AKR intercross

A total of 181 F_2 mice homozygous for tubby were tested in the AKR/J × C57BL/6J-*tub/tub* intercross (AKR intercross). A bimodal distribution was observed for ABR thresholds for all four test stimuli; results for the broadband click stimulus are shown in Figure 1. Thirty-two percent of *tub/tub* mice showed abnormal click ABR thresholds (55 db SPL and higher), indicating the existence of one major recessive gene, designated as *moth*, which affected hearing ability in tubby mice (25% would be expected for a single recessive gene). In contrast, 7-week-old Fl *tub*/+ and F_2 +/+ mice did not show an elevation or a large variability in ABR thresholds (36.3 ± 3 and 36.7 ± 3 dB SPL, respectively, ranging from 35 to 40 dB SPL) in comparison with the values observed for inbred AKR/J, C57BL/6J and CAST/Ei mice at 22–33 weeks (42 ± 3, 39 ± 4 and 30 ± 0 dB SPL, respectively) (12). The large variation in ABR thresholds is observed only in mice that arc homozygous for the tubby mutation, suggesting the existence of genetic factors specifically interacting with the tubby mutation. All mice that were phenotype by ABR were also genotyped using 57 markers which were distributed across the entire genome. Additional markers were used to define the region encompassing the significantly linked QTL on chromosome 2. The results of the linkage analyses are shown in Table 1. In the AKR intercross, marker *D2Mit17* on chromosome 2 was highly associated with click ABR thresholds ($P < 4.22 \times 10^{-34}$, LOD = 33.4) (Fig. 2a and Table 1). The AKR/J allele of this QTL normalized ABR thresholds in tubby mice in a dominant fashion; a single copy prevented hearing loss (Table 1). This QTL is responsible for 57% of the click threshold variance observed in this intercross. Significant linkage to chromosome 2 was also observed with the other stimuli tested (8, 16 and 32 kHz). However, the LOD scores were lower than observed for the click stimulus (Table 1).

CAST intercross

Congenic CAST/Ei.B6-*tub/tub* mice, generated in our laboratory, showed normal ABR thresholds (Fig. 3), suggesting that CAST/Ei, like *AKR/J*, had alleles of genes which protect tubby mice from hearing loss. To test whether CAST/Ei carried a protective allele of the *moth1* gene on chromosome 2, QTL analysis for ABR thresholds was performed using 46 F_2 mice homozygous for *tub* from a CAST/Ei.B6-*tub/tub* × C57BL/6J-*tub/tub* F_1 hybrid intercross (CAST intercross). A similar pattern of segregation was observed in the CAST intercross as in the AKR intercross (Fig. I b). Although a smaller number of mice was tested, a highly significant LOD score of 6.0 at *D2Mit17* was observed for click thresholds (Fig. 2b and Table 2). The LOD score distribution for click ABR thresholds in the CAST intercross is shown in Figure 2b. This QTL is responsible for 47% of click threshold variance (Table 2). A similar stimulus-specific effect was also observed in the CAST intercross as seen in the AKR intercross (Table 2).

129/Ola allele of *moth1*

To investigate other strain-specific alleles of *moth1* mice from a C57BL/6J stock (C57BL/6J.129-*B2m*$^{Tml/unc}$ congenic for a 129/Ola-derived donor segment including *moth1* were crossed to C57BL/6J-*tub/tub* to produce F_1 hybrids. Mice homozygous for tubby and heterozygous for *moth1*$^{129/Ola}$ from an intercross of the F_1 mice were phenotyped by ABR analysis. The 129/Ola congenic segment on chromosome 2 is ~11 cM in length, extending from *D2Mit442* to *D2Mit107* (Fig. 4). This region covers the majority of the 95% confidence interval identified for the click QTL in the AKR intercross (Fig. 4). The C57BL/6J-*tub/tub.moth1*$^{129/Ola}$ /*moth1*^{B6} mice showed normal ABR thresholds for all sound stimulations: click and 8, 16 and 32 kHz (Fig. 3).

Immunohistochemistry

To determine whether the TUB protein is indeed expressed in the ear and to identify the primary site of action of the tubby gene, immunohistochemical studies were performed. A polyclonal tubby antibody that specifically recognizes the N-terminal end of both normal and mutant TUB protein was used (3). TUB is localized in the outer and inner hair cells and spiral ganglion cells, as well as in the supporting cells of Deiters adjacent to the outer hair cells. A characteristic subcellular staining pattern could be detected, with a predominant cytoplasmic staining in outer and inner hair cells and in spiral ganglion cells and an intense nucleolar staining in the external phalangeal cells of Deiters (Fig. 5). Localization patterns of anti-TUB antibodies were the same in both C57BL6/J and CS7BL/6J-*tub/rub* mice. We could not find any significant morphological differences between the inner ear of normal and *tub/tub* mice at 8–10 weeks of age.

DISCUSSION

Genetic identification of *moth1* on chromosome 2, a major gene affecting hearing loss in tubby mice

In both the AKR and the CAST intercrosses, the same chromosomal locus was found to be linked to ABR thresholds by QTL analysis. The AKR/J and CAST/Ei alleles at this locus were able to protect *tub* mice from hearing loss. In addition, by congenic strain analysis we show that the 129/Ola al1ele of this locus also acts as a dominant protective allele. This gene, named *moth1* for modifier of *tub* hearing, may be directly involved in the hearing impairment cascade induced by the *tub* mutation. It is known that cochlear neuroepithelial degeneration, characterized by loss of hair and ganglion cells, is one possible cause of hearing loss in tubby mice (1,2). Although the function of TUB is still unknown, the tubby mutation is known to induce cell death in sensory neurons (1,3). Because a single *moth1* allele from the genetically unrelated strains AKR/J, CAST/Ei and 129/Ola is able to protect C57BL/6J-*tub/tub* mice against hearing loss, it is likely that B6 carries a recessive mutation at the *moth1* locus which is necessary to mediate the effects of the *tub* mutation on hearing. Because homozygosity for the *moth1* allele itself cannot induce bearing impairment in C57BL/6J mice, the allelic difference in the *moth1* gene between C57BL/6J and the other inbred strains used in this study may not be critical for maintaining the hair and spiral ganglion cells, but becomes significant only when the tubby gene is mutated and unable to function properly. A similar epistatic gene interaction has been described for the *dfw* and *mdfw* genes (13).

C57BL/6J mice carry the susceptibility allele (*ahl*) for late onset of hearing loss, which has been mapped to chromosome 10 (14). In the present study, all mice were tested at 6–8 weeks of age, well before the late onset of hearing loss attributable to *ahl.* It appears that the *tub* mutation is not interacting with the 66 *ahl* allele to accelerate the *ahl*-induced hearing loss, because we did not observe a QTL affecting hearing ability on chromosome 10. Thus, the abnormality in hearing observed in both crosses is not due to *ahl* and can be explained by the effect of the tubby mutation without the protective allele of the *moth1* gene.

Although in the AKR intercross the most significant linkage for A6R thresholds was observed on chromosome 2 over all sound stimulations (click and 8, 16 and 32 kHz), a difference in the level of significance between each stimulus was observed. A similar pattern was observed in the CAST intercross as well. The lowest LOD scores were obtained with thresholds evoked from the 16 kHz stimulus; mice are most sensitive to frequencies of 12–24 kHz (15). It is also known that different strains exhibit different sensitivities to these particular stimuli (12), suggesting that there might be a genetic basis for the frequency-dependent sensitivities.

TUB protein is expressed in the cochlea

TUB protein was found to be expressed in the outer and inner hair cells and the spiral ganglion cells, regions which degenerate in tubby mice (1,2). In tubby mice, retinal degeneration has been shown to be caused by apoptosis (1,3). It is possible that the delayed cochlear degeneration may also occur via the same mechanism. However, while tubby mice show broadband hearing impairment at 3 weeks of age (1), cochlear degeneration is not observed (2). In this study, we also did not observe significant degeneration of the cells in the organ of Corti at 8–10 weeks of age. The cells may, therefore, lose their function prior to showing signs of degeneration.

A candidate gene for modifying tubby hearing

Genes expressed in neurons and associated with cell survival could be candidates for *moth1*. Brain-derived neurotrophic factor (BDNF), a gene known to play a role in neural cell survival (reviewed in ref. 16), is located on chromosome 2 at 62 cM (MGD), well within the 7.86 ± 1.39 cM region identified for *moth1*. Furthermore, it is known that BDNF and its high affinity receptor trkB are highly expressed in exactly the same subset of cells positive for the TUB protein (reviewed in ref. 17). This information, together with the fact that mice homozygous for a null allele for BDNF show sensory neural defects (18), suggested it as a good candidate gene for *moth1*. Sequence comparison of the BDNF coding region, however, showed no differences between C57BL6-*tub/tub* and AKR/J, thus making it less likely that BDNF is the *moth1* gene. Potential expression level differences will have to be explored using congenic lines that are currently being generated.

Chromosomal localization of *moth1*

The phenotypes of tubby mice resemble human diseases such as Alström syndrome and Bardet–Biedl syndromes, which are characterized by obesity, blindness and deafness. Interestingly, *moth1* maps to the region of mouse chromosome 2 which is homologous to the human chromosome 15q13–22, where one of the Bardet–Biedl genes (BBS4) maps (human chromosome 15q22.3–23) (19,20). Although only the hearing phenotype was characterized in the experiments described here, it would be interesting to see how the *moth1* gene affects the other tubby phenotypes, such as retinal degeneration and obesity.

Further studies are ongoing to identify the *moth1* gene. Identification of a gene which interacts with tubby may allow us to elucidate the function of tubby and, thereby, identify pathways that significantly correlate with the tubby phenotypes ill *vivo.*

MATERIALS AND METHODS

Congenic mice and crosses

All mice used in this study were originally obtained from the Jackson Laboratory. To generate the congenic strain CAST/Ei.B6-*tub/tub, tub/+* mice originally in the C57BL/6J background were sequentially backcrossed to strain CAST/Ei for 10 generations and intercrossed. The congenic strain B6.129-$B2m^{TMl/Unc}$/Dcr (N11) has been previously described (21). In its ~ 11 cM congenic 129/Ola-derived segment, it carries a null allele of the gene β_2-microglobulin *(B2m)* (which only expresses a detectable physiological phenotype when homozygous) and 129/Ola-derived markers including *D2Mit58* and *D2Mit107.* These mice were out-crossed to C57BL/6J-*tub/tub* and progeny were intercrossed to produce F_2 mice homozygous for *tub* but heterozygous for the 129/Ola congenic segment. An allele-specific PCR assay (22) was used to identify *tub/tub* mice and markers *D2Mit58* and *D2Mit107* were used to distinguish between the 129/Ola and C57BL/6J alleles of *moth1*. For the AKR intercross, F_1 progeny from a breeding pair of C57BL/6J-*tub/tub* and AKR/J-+/+ mice were intercrossed to produce F_2, progeny. The F_2 mice

homozygous for *tub* were selected by the allele-specific PCR assay as described above. For the CAST intercross, F_1 progeny from a breeding pair of C57BL/6J-*tub/tub* and CAST/Ei.B6-*tub/tub* mice were intercrossed to produce F_2 progeny. Phenotyping and the genome-wide scan of these F_2 progeny were carried out as described below.

ABR phenotyping

A computer-aided evoked potential system (Intelligent Hearing System, IHS; Miami, FL) was used to test mice for ABR thresholds as previously described (12). Mice were anesthetized with tribromoethanol (5.3 mg/10 g body wt i.p.). Subdermal needle electrodes were inserted at the vertex (active) and ventrolaterally to the right ear (reference) and to the left ear (ground). Specific acoustic stimuli were delivered binaurally through 1 cm plastic tubes channeled from high frequency transducers. Mice were tested with click stimuli and also with 8, 16 and 32 kHz tone pips at varying intensity, from low to high (10–90 dB SPL). An ABR threshold was determined for each stimulus frequency by identifying the lowest intensity which produced a recognizable ABR pattern (at least two consistent peaks) (12).

Genotyping

Tail DNA was isolated according to Buffone (23). SSLP markers polymorphic between strains AKR/J and C57BL/6J or CAST/Ei and C57BL/6J were selected at ~35 cM intervals across the mouse genome. A total of 57 SSLP markers were tested for the initial genome-wide screen. Additional markers were tested around loci that showed significant or suggestive linkage. For PCR amplification, 100 ng of DNA were used in a 10 µl volume containing 50 mM KCl, 10 mM Tris–HCl, pH 8.3, 2.5 mM $MgCl_2$, 0.2 mM oligonucleotides, 200 µM dNTP and 0.02 U AmpliTaq DNA polymerase. The reactions were subjected to the following temperature cycling program: initial denaturation for 2 min at 95°C; 20 s at 94°C, 30 s at 50°C, 40 s at 72°C for 49 cycles: followed by a 7 min extension at 72°C. PCR products were separated by electrophoresis on a 4% MetaPhor (FMC, Rockland, ME) agarose gel and visualized under UV light after staining with ethidium bromide.

Light microscopy and immunofluorescence analysis

C57BL/6J (n = 4) and C57BL/6J-*tub/tub* (n = 4) mice at 8–10 weeks of age were deeply anesthetized and transcardially perfused with phosphate-buffered saline (PBS) followed by 4% parafonnaldehyde (PFA) in PBS. The cochlea was removed and stored in 4% PFA for 4 h. For decalcification, the cochlea was incubated in 7% EDTA–PBS for 4 days before embedding in paraffin. Serial sections (7 µm) of the cochlea were stained with Mayer's hematoxylin using standard procedures. Adjacent sect ions were subjected to immunohistochemical analysis using rabbit polyclonal antibody specific for an N-terminal portion of the tubby protein (3). Briefly, after blocking with 2% goat serum in PBS, sections were incubated with the primary antibody (N2, 1:1000) at 4°C overnight. Binding was detected using biotinylated goat anti-rabbit IgG (1:200; Vector. Burlingame, CA) followed by FITC–avidin D (1:200; Vector) or Cy3-conjugated donkey anti-rabbit IgG (1:150; Jackson Immunoresearch, West Grove, CA). A nuclear counterstain was performed with 4,6-diamidine 2-phenylindoldihydrochloride (DAPI) at a final concentration of 5 µg/ml. Images were collected on a Leica DMRXE fluorescent microscope equipped with a SPOT CCD camera using appropriate bandpass filters for each fluorochrome.

QTL and statistical analysis

QTL analysis was performed using MapManager QT (v.3.0b26; Manly). LOD scores were calculated by dividing the F scores by 4.6 (24). To evaluate the significance of the results, a permutation test (25) with 1000 replications was used.

The data generated from the entire set of F_2, progeny were also subjected to analysis of variance (ANOVA) between groups defined by genotype for selected genetic loci. Analysis of variance was performed using the Statview v4.5 program for the Macintosh Computer (Abacus Concepts, Berkeley, CA).

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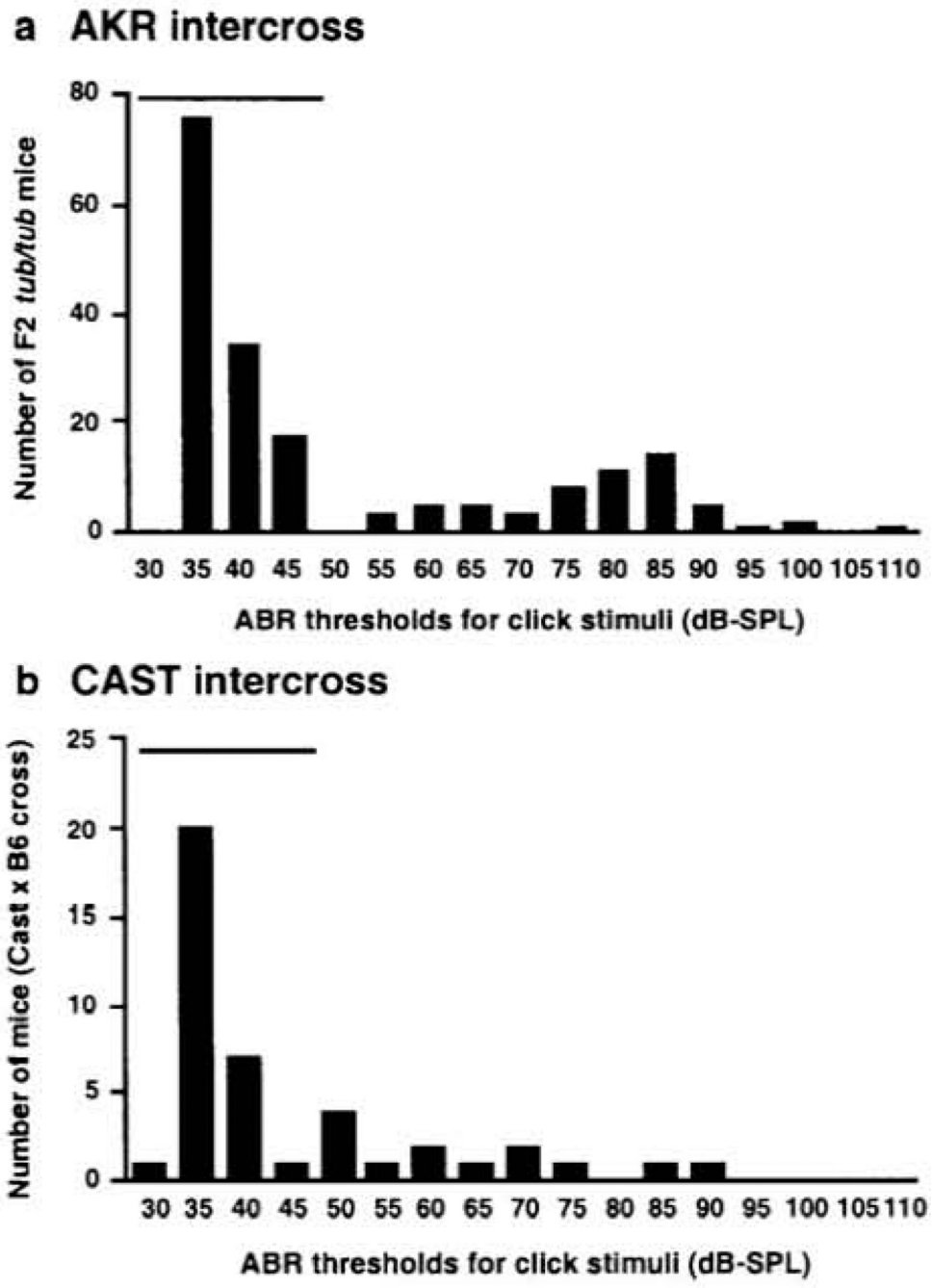

Figure 1.

Frequency distribution of click ABR threshold values from F_2 mice homozygous for tubby. (**a**) F_2 mice from the AKR/J × C57BL/6J-*tub/tub* intercross. (**b**) F_2 mice from the CAST.B6-*tub/tub* × C57BL/6J-*tub/tub* intercross. The horizontal bar indicates the range of click ABR thresholds observed in mice from the parental strains C57BL/6J, AKR/J and CAST/Ei tested at the same age.

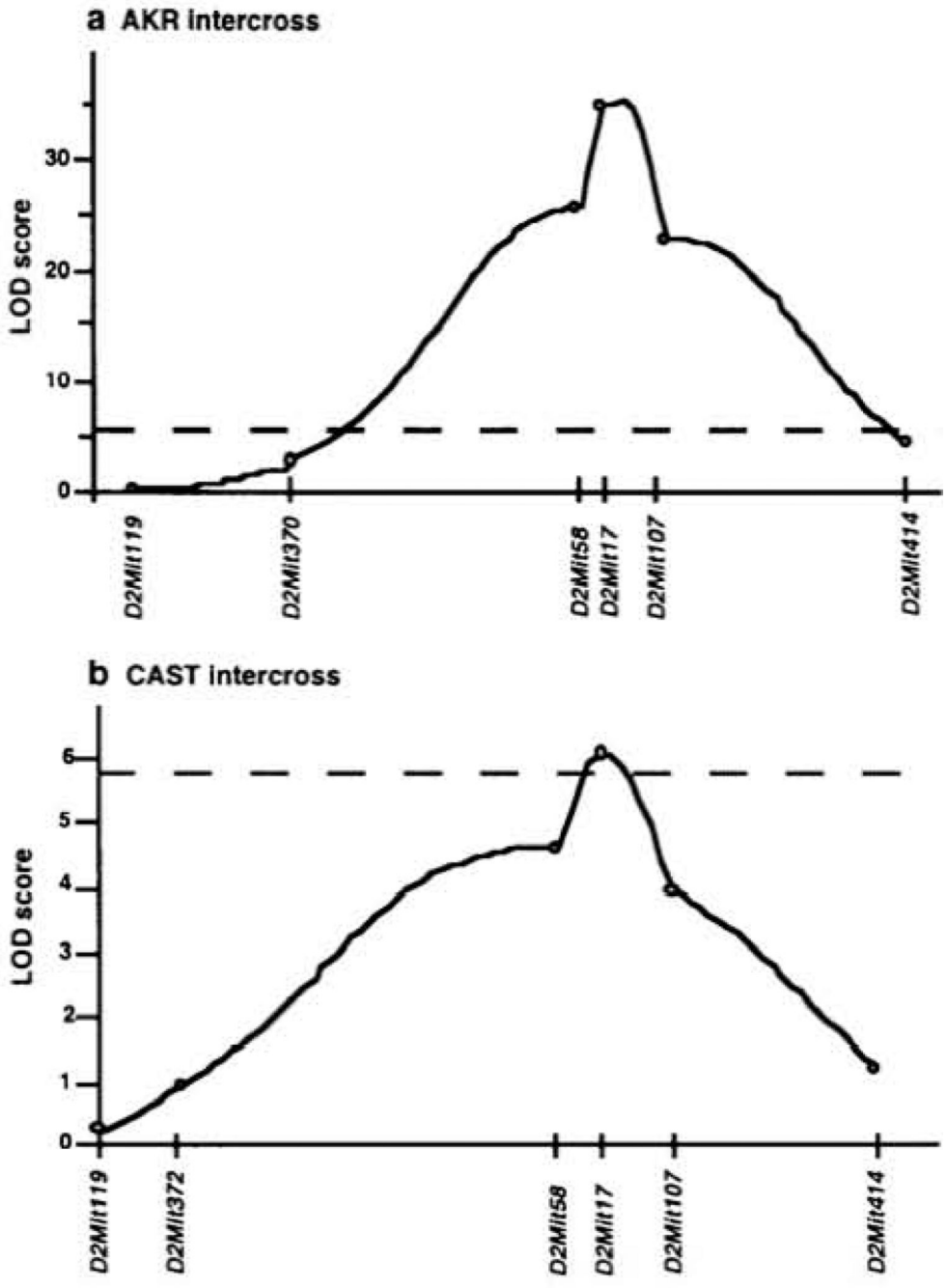

Figure 2.

LOD score distributions for associations of click ABR threshhold with loci on chromosome 2 in the AKR intercross (**a**) and in the CAST intercross (**b**). The dotted line represents highly significant linkage as assessed by permutation testing ($P < 0.001$).

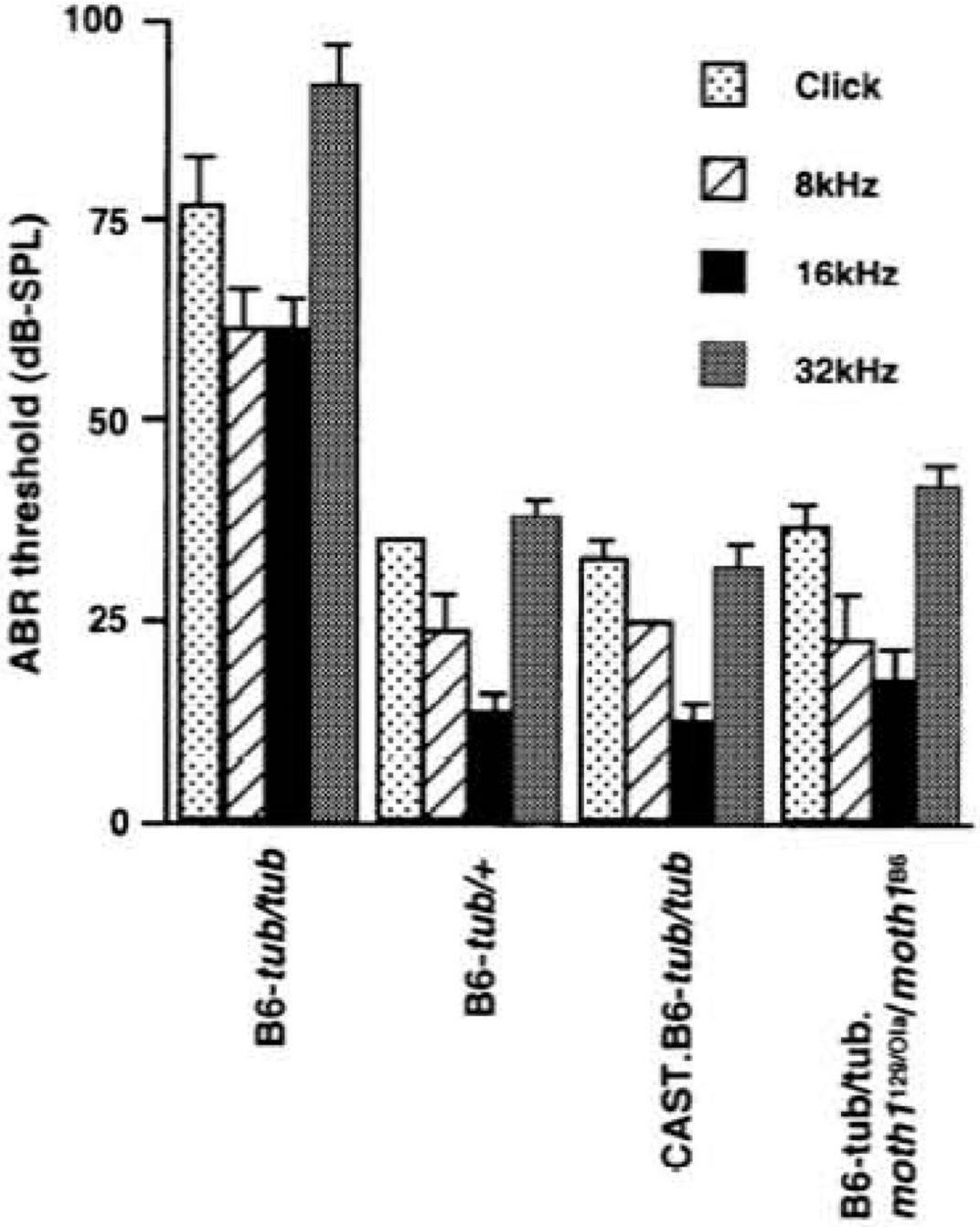

Figure 3.

The ABR thresholds in C57BL/6J-*tub/tub.moth1*$^{129/Ola}$/*moth1*^{B6} and CAST/Ei.B6-*tub/tub* mice. The mean ± SD of the sound pressure threshold is represented by a bar and shown for each genotype and stimulus as indicated. Five to six mice at 6–8 weeks of age were tested for each column. C57BL/6J-*tub/tub* and C57BL/6J-*tub/+* strains were used as an abnormal and normal control, respectively. C57BL/6J-*tub/tub.moth1*$^{129/Ola}$/*moth1*^{B6} and CAST/Ei.B6-*tub/tub* mice were generated as described in Materials and Methods.

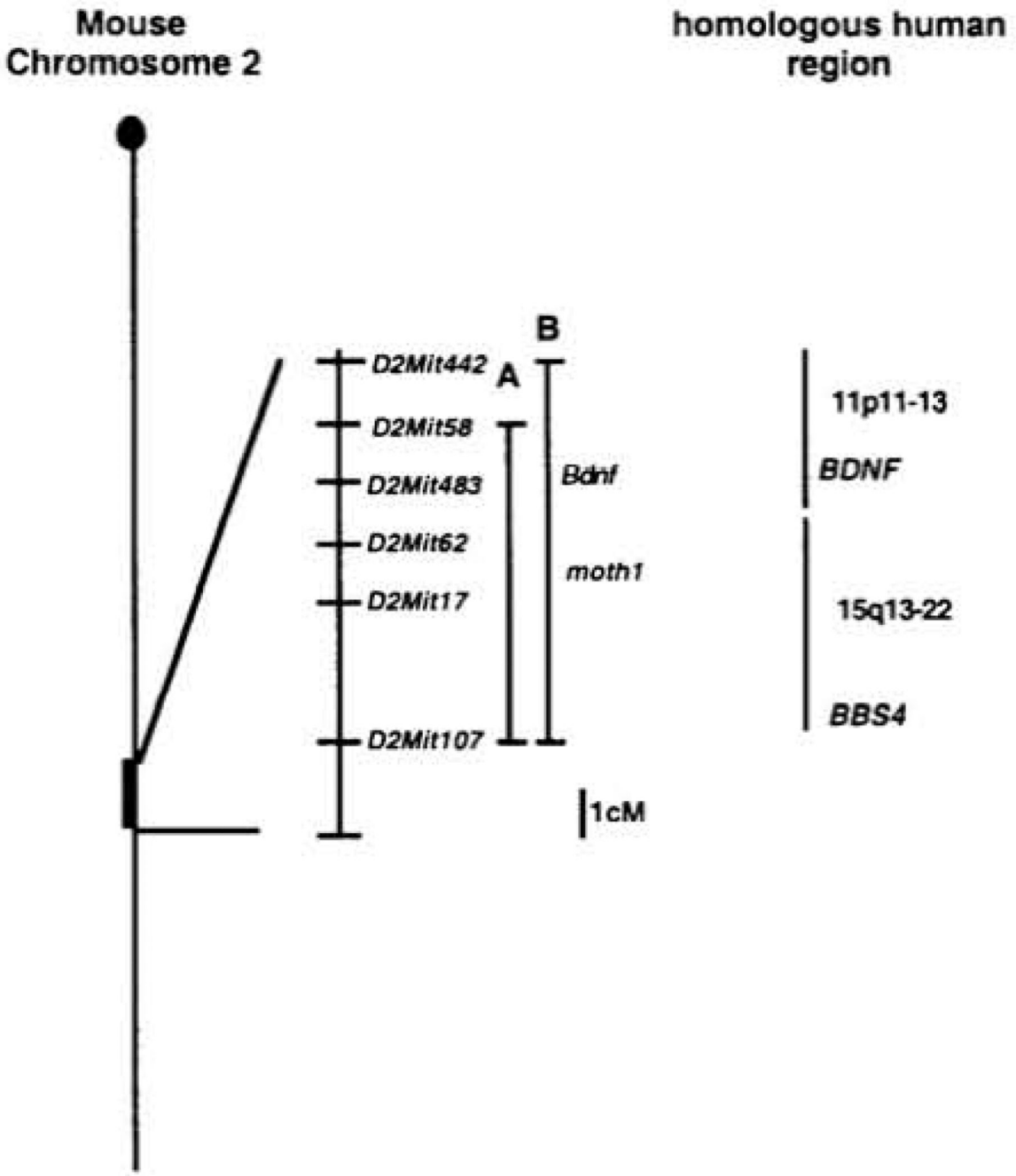

Figure 4.
Map position of the *moth1* gene and the syntenic human genetic regions. Bar A represents the confidence intervel including the *moth1* gene. Bar B represents the 129/Ola segment which the C57BL/6J.129/Ola congenic line carries.

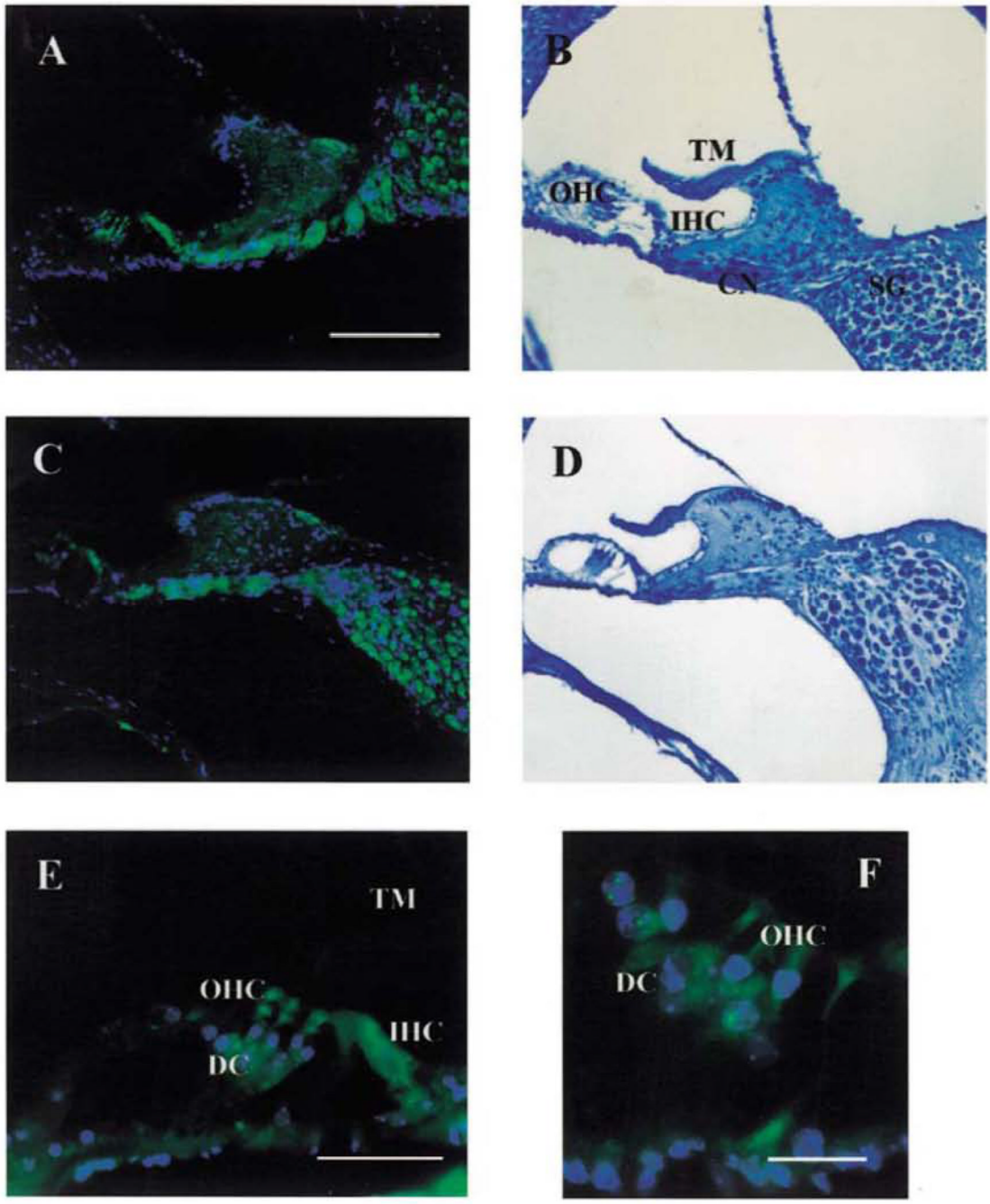

Figure 5.

Distribution of tubby immunoreactivity (**A, C, E** and **F**) and light microscopic morphology (**B** and **D**) of the cochlea of C57BL/6J (wt) and C57BL/6J-*tub/tub* (*tub*) mice at 10 weeks of age. The TUB protein is strongly expressed (green) in the outer and inner hair cells (OHC/IHC), the adjacent external phalangeal cells of Deiters (DC), cochlear nerve fibers (CN) and in the spiral ganglion cells (SC). Note that there are no obvious differences in distribution or expression of the tubby protein between normal and mutant mice (A and C). At this 8–10 weeks time point, no histopathological changes in the organ of Corti could be detected (B and D). Higher magnifications of representative sections of the Corti organ of C57BL/6J mice reveal the subcellular distribution of TUB. A cytoplasmic localization pattern can be

observed in the outer and inner hair cells, depicted by arrowheads (E and F), whereas there is a distinct nucleolar immunoreactivity detectable in the external phalangeal cells (marked by arrows in F). TM, tectorial membrane, Scale bars: (A–D) 100 μm; (E) 5 μm; (F) 20 μm.

Table 1

A major QTL and genotypic effect at *D2Mit17* in the AKR intercross

Sound stimuli	Genotype at locus[a]	ABR threshold (db SPL)[b]	LOD score	Variance explained (%)
Click	AA	41.2 ± 2.15[b] (47)	33.4	57
	AB	41.1 ± 1.19 (89)		
	BB	76.2 ± 2.35 (45)		
8 kHz	AA	27.3 ± 1.98 (47)	23.7	45
	AB	26.2 ± 1.00 (89)		
	BB	50.9 ± 2.06 (45)		
16 kHz	AA	25.2 ± 2.40 (47)	17.8	36
	AB	24.0 ± 1.64 (89)		
	BB	50.6 ± 2.08 (45)		
32 kHz	AA	48.2 ± 1.98 (47)	24.4	46
	AB	46.2 ± 1.20 (89)		
	BB	73.4 ± 2.09 (45)		

[a] AA, homozygous for AKR/J; AB, heterozygous; BB, homozygous for C57BL/6J alleles at *D2Mit17*.

[b] Mean ± SEM (number of mice in parentheses).

Table 2

A major QTL and genotypic effect at *D2Mit17* in the CAST intercross

Sound stimuli	Genotype at locus[a]	ABR threshold (db SPL)[b]	LOD score	Variance explained (%)
Click	CC	35.4 ± 0.89 (13)	6.0	43
	CB	38.8 ± 2.08 (20)		
	BB	59.2 ± 4.90 (13)		
8 kHz	CC	25.0 ± 1.70 (13)	3.5	26
	CB	24.8 ± 1.90 (20)		
	BB	37.3 ± 3.23 (13)		
16 kHz	CC	16.7 ± 3.06 (13)	2.0	14
	CB	16.8 ± 2.03 (20)		
	BB	34.3 ± 7.75 (13)		
32 kHz	CC	40.0 ± 1.96 (13)	5.1	37
	CB	42.3 ± 2.42 (20)		
	BB	61.9 ± 4.50 (13)		

[a] CC, homozygous for CAST/Ei; CB, heterozygous; BB, homozygous for C57BL/6J alleles at *D2Mil17*.

[b] Mean ± SEM (number of mice in parentheses).