A spontaneous mutation causing deafness and circling behavior was discovered in a C3H/HeJ colony of mice at the Jackson Laboratory. Pathological analysis of mutant mice revealed gross morphological abnormalities of the inner ear, and also dysmorphic or missing kidneys. The deafness and abnormal behavior were shown to be inherited as an autosomal recessive trait and mapped to mouse chromosome 1 near the position of the Eya1 gene. The human homolog of this gene, EYA1, has been shown to underly branchio-oto-renal (BOR) syndrome, an autosomal dominant disorder characterized by hearing loss with associated branchial and renal anomalies. Molecular analysis of the Eya1 gene in mutant mice revealed the insertion of an intracisternal A particle (IAP) element in intron 7. The presence of the IAP insertion was associated with reduced expression of the normal Eya1 message and formation of additional aberrant transcripts. The hypomorphic nature of the mutation may explain its recessive inheritance, if protein levels in homozygotes, but not heterozygotes, are below a critical threshold needed for normal developmental function. The new mouse mutation is designated Eya1bor to denote its similarity to human BOR syndrome, and will provide a valuable model for studying mutant gene expression and etiology.

INTRODUCTION

Hearing loss is the most common sensory deficit in humans and affects ~1 in 2000 live births (1). Tremendous progress has been made during the last 5 years in mapping and cloning of genes responsible for both syndromic and non-syndromic hereditary hearing loss (2,3). The mouse is an excellent animal model for the study of these human conditions because the anatomy, function and hereditary abnormalities of the inner ear have been shown to be similar in both humans and mice (4,5). Genetic and molecular analyses of mouse deafness mutations have aided the discovery of genes that underly several human hearing loss syndromes. For example, the mouse shaker-1 mutation (sh1) was shown to be a mutation of Myo7a (6), and the homologous gene in humans subsequently was shown to be responsible for both dominant (DFNA11) and recessive (DFNB2) forms of non-syndromic deafness (7,8), as well as for Usher syndrome type 1B (9). Recently, the mouse shaker-2 mutation (sh2) was shown to be a mutation of Myo15 (10), and the homologous gene in humans to be responsible for DFNB3 (11).

In mice, mutations affecting the vestibular system of the inner ear often result in a characteristic circling or head-bobbing phenotype; many of these mutations also affect the cochlea and cause deafness (4). As part of our research program at the Jackson Laboratory to identify genes causing deafness, we have been selecting and studying mutant mice that exhibit behavior characteristic of vestibular dysfunction. Pathological analysis of one such circling mutant revealed gross morphological abnormalities of the inner ear and also dysmorphic or missing kidneys.

Here, we describe the inner ear and kidney pathology of these mutant mice and our genetic mapping results that place the new mutation on proximal chromosome 1, near the position of the Eya1 gene (12). The human homolog of this gene, EYA1, has been shown to underly branchio-oto-renal (BOR) syndrome, an autosomal dominant disorder characterized by hearing loss with associated branchial and renal anomalies (13). Because of the coincident map position and the mutation’s phenotypic similarity to human BOR syndrome, we considered Eya1 to be a likely candidate gene for the new mouse mutation and undertook a molecular analysis of this gene in mutant mice. We show that Eya1 mRNA expression is altered in mice homozygous for the new mutation as compared with their normal, co-isogenic littermates, and demonstrate that this altered expression is caused by the spontaneous insertion of an intracisternal A particle (IAP) element into intron 7 of the Eya1 gene.

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RESULTS

Mice with circling and head-bobbing behavior were discovered in a C3H/HeJ colony at the Jackson Laboratory. The abnormal behavior was shown to be inherited as an autosomal recessive trait. Affected male homozygotes would sometimes breed; females did not. Auditory-evoked brainstem response (ABR) threshold measurements demonstrated that homozygous mutant mice are deaf (no evoked response to sound pressure levels >95 dB) at the earliest testable age (3–4 weeks), but heterozygotes hear normally. Mice homozygous for the mutation were also characterized by absent or dysmorphic kidneys.

The adult inner ears of the mutant mice displayed a number of morphogenetic abnormalities (Fig. 1). Whole-mount preparations revealed subtle abnormalities of the pars superior, or vestibular portion of the inner ear. Specifically, the lateral semicircular canal, the last to appear developmentally, is foreshortened, with a much narrower diameter than that of the wild-type (not shown). Several of the postnatal inner ears studied also revealed an incomplete common crus, the region of the joined non-ampullated ends of the superior and posterior semicircular canals (not shown). The abnormalities of the pars inferior, or cochlear portion of the inner ear, were the most severe and constant. All but the most basal one-quarter of the cochlea was absent in the adult mutant inner ear (Fig. 1a and b). Histological analysis demonstrated the rudimentary basal portion was present in the mutant cochlea with a spiral ligament and no overlying stria vascularis. Additionally, there was complete absence of the organ of Corti, or sensory epithelium, in the mutant (Fig. 1c and d). No vascularis. Additionally, there was complete absence of the organ of Corti, or sensory epithelium, in the mutant cochlea with a spiral ligament and no overlying stria vascularis. Additionally, there was complete absence of the organ of Corti, or sensory epithelium, in the mutant cochlea with a spiral ligament and no overlying stria vascularis.

The overall size and exon–intron structure of the mouse Eya1 gene appeared similar to that described for the human EYA1 gene (14). Because of its large size (the human EYA1 gene consists of 16 coding exons and extends over 156 kb), the region of the EYA1 gene containing the mutation was narrowed by sequential hybridization of Southern blots with probes from increasingly restricted portions of the Eya1 cDNA (Fig. 3). The site of the mutation was thus refined to a 3 kb genomic region containing exon 8.

To compare DNA sequences in the mutated region of the Eya1 gene, genomic clones were identified by screening phage libraries from both mutant and wild-type mice with a 106 bp exon 8 probe. Restriction site mapping was then used to localize the mutation to intron 7 (Fig. 4), and ~3500 nucleotides of DNA from that region were sequenced (GenBank accession no. AF097544). PCR amplification with various combinations of primers designed from this sequence further narrowed the region of mutation to a 300 bp interval; primers spanning this interval failed to amplify a product with mutant genomic DNA. Sequence analysis of the mutant DNA clone in this region revealed an insertion, ~1480 bp upstream from the 3′ splice acceptor sequence of exon 8. A BLAST search of GenBank DNA sequences identified the insertion as an IAP transposon; 32 matches had alignment scores >200, including those IAP elements causing pale ear (ep) and agouti (A) mutations.

The IAP insertion was in the same transcriptional orientation as the Eya1 gene. The single mutant genomic clone did not contain the 5′ end of the IAP element; therefore, the DNA sequence of the cloned 3′ long terminal repeat (LTR) together with the Eya1 intron sequence 5′ to the IAP insertion site were used to amplify the uncloned 5′ LTR junction fragment from mutant genomic DNA. Sequence analysis of this PCR product revealed that the 5′ and 3′ LTR sequences were identical and that the IAP element was flanked by a 6 bp direct repeat (GATGAG), a characteristic of retrotransposition. The nucleotide sequences of the IAP junctions with intron 7 of the Eya1 gene have been deposited in GenBank under accession nos AF097545 (5′LTR) and AF097546 (3′LTR).

Northern blot analysis of total RNA from adult skeletal muscle hybridized with a 1.6 kb Eya1 cDNA probe showed an ~50% reduction in the level of wild-type message in homozygous mutant mice compared with +/+ controls (Fig. 5a). Furthermore, in RNA from mutant mice, two transcripts of ~7 and 10 kb were

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**Figure 1.** Effect of the *Eya1* mutation on adult inner ear morphology. Cleared whole-mount preparations (a and b) compared at equal magnification, and histological sections (c and d, 80× magnification, and e and f, 200× magnification) of inner ears from C3H/Hej +/+ control (a, c and e) and C3H/HeJ-Eya1bor/Eya1bor mutant (b, d and f) mice. Asterisks indicate the distinct interscalar septi of a normal cochlea (a); arrowheads designate a normal cochlea in the +/+ control (a) and its absence in the mutant inner ear (b). The stria vascularis (small arrow) and organ of Corti (large arrow) shown in the +/+ control (e) are absent in the mutant inner ear (f). sl designates the spiral ligament (e and f).

seen in addition to the 4 kb wild-type message. Additional transcripts were also seen in RNA from eye and brain tissue of mutant mice (data not shown); however, *Eya1* expression is much reduced and more difficult to quantify in these tissues compared with skeletal muscle expression. RT–PCR analysis with primers flanking exon 8 also produced additional products from mutant RNA, larger in size than the product expected from the wild-type *Eya1* transcript (Fig. 5b).
Figure 2. Effect of the Eya1 mutation on adult kidney morphology. Top center: autopsy of a 4-month-old male mutant showing complete absence of the left kidney. The right kidney, indicated by an arrow, shows normal morphology. The intestinal tract was excised intentionally and the left ureter is partially obscured by the pancreas. Both testes were present. Bottom panels: the right kidney from a 7.5-month-old female +/- control (a, d and g) is compared with the left (b, e and h) and right (c, f and i) kidneys of a 7.5-month-old female sibling mutant: kidney whole-mounts 10x (a, b and c); longitudinal sections, 10x, H&E stained (d, e and f); and cortex sections, bar = 100 µm, PAS (g, h and i). Although hypoplasia is obvious in both mutant kidneys, their cellular morphology is similar to that of the control kidney. However, functional stress is indicated by elevated plasma urea nitrogen (32 mg/dl for the mutant versus 18 mg/dl for control).
DISCUSSION

Both the new mouse mutation and human BOR syndrome are caused by alterations of homologs of the *Drosophila* eyes absent gene (mouse *Eya1*, human *EYA1*). These disorders are consistent with the role of this gene in early embryonic development. BOR syndrome is characterized by developmental anomalies of the branchial arches (pre-auricular pits, branchial cysts, outer and middle ear malformations), the inner ear (absent or undeveloped cochlea and semicircular canals) and the kidneys (kidney hypoplasia to bilateral agenesis) (13,15). The prominent features of the mouse *Eya1* mutation, including cochlear and kidney hypoplasia, are strikingly similar to those of BOR syndrome.

The reduced, but still present, expression of normal *Eya1* transcripts in affected homozygotes is evidence that the mouse mutation is hypomorphic, which may explain its recessive nature compared with the dominant inheritance of human BOR syndrome. The ∼50% level of *Eya1* expression observed in homozygous mutant mice (Fig. 5) might be below a critical physiological threshold necessary for normal function, but the expression level in heterozygous mice may be high enough to allow normal development. The *EYA1* mutations reported for human BOR syndrome alter protein-coding sequences in exons or disrupt splice sites (13,14,16), and thus are likely to be more severe than the inserational disruption of an intron reported here for the mouse. Consequently, human homozygotes may have gene expression levels similar to those of homozygous mice. The additional transcripts produced by the mouse mutation are unlikely to cause a gain of function or have dominant-negative effects because no phenotypic abnormalities were seen in heterozygotes even though aberrant transcripts were detected in these mice.

The mouse genome contains ∼2000 copies of retroviral-like IAPs (17). IAP retrotranspositions have been shown to underly several inherited mutations in the mouse, including agouti, *A* (18); pale ear, *ep* (19); vibrator, *Pitpmv* (20); *Lamb3* (21); reeler, *Reln* (22); albino, *Tyr* (23); fused, *AxinF* (24); and β-glucuronidase, *Gusmps–2J* (25). IAP element insertions into non-coding introns have been shown to cause reduced expression and/or formation of chimeric transcripts in the *Pitpmv* (20), *AxinF* (24) and *Gusmps–2J* (25) mutations, similar to our findings for the *Eya1bor* mutation. It is thought that IAP element insertions introduce cryptic splice and polyadenylation sites that disrupt normal transcript processing or stability and consequently reduce gene expression. In the *Eya1bor* mutation, the junction of the 6 bp direct repeat with the 5′ LTR of the IAP created a consensus 5′ splice site (AGGTGTG; Fig. 4c), which, along with two LTR polyadenylation sites, may contribute to the aberrant RNA expression observed in northern blots and RT–PCR (Fig. 5).

The DNA sequence of the *Eya1* IAP LTR (Fig. 4) indicates that the U3 region was derived from the LS-type and the R-region from the T-type of IAP elements; this composite LTR structure is characteristic of other IAP insertions causing germline mutations, most of which have occurred in the C3H/He inbred strain (25). The *Eya1* IAP LTR sequence is very similar to the LTRs of other IAP elements isolated from C3H/He mice. It is identical to the LTR of the IAP causing the pale ear mutation (GenBank AF003867), except that 30 bp are deleted at the position indicated in Figure 4, possibly the result of unequal crossing-over in this region of imperfect CT-rich repeats.
In summary, we believe that retrotransposition of an IAP element into intron 7 of the Eya1 gene is responsible for the phenotype of the new mouse mutation for the following reasons. (i) The inner ear and kidney abnormalities of mutant mice are similar to the characteristic traits of human BOR syndrome, known to be caused by mutations of the human EYA1 gene. (ii) The new mouse mutation and the Eya1 gene co-segregated in 116 tested meioses from the linkage cross. (iii) Northern blot and RT–PCR analyses showed that Eya1 gene expression is altered in mutant mice compared with controls. (iv) Southern blot and DNA sequence analysis revealed that an IAP element was present in intron 7 of the Eya1 gene of mutant mice but not in co-isogenic controls. (v) PCR genotyping (Fig. 4c) has confirmed that all mutant mice are homozygous for this IAP element insertion. (vi) Other inherited mouse mutations have been shown to be caused by similar IAP element insertions into introns.

This new mouse model will permit studies of Eya1 gene expression and etiology not possible in humans with BOR syndrome. For example, linkage backcross mice homozygous for the Eya1 mutation, with varying genetic backgrounds derived from parental C3H/HeJ and CAST/Ei genomes, displayed variable phenotypes for both inner ears and kidneys. Analysis of such genetically well-defined mice will permit identification of genes that modify the phenotypic manifestation of the Eya1 mutation and may provide insight into causes of the incomplete penetrance and variable expressivity characteristic of human BOR syndrome (15). The mouse mutation also provides a means to study mutant Eya1 gene expression during embryonic development. Other developmental control genes such as Pax2 are also known to contribute to inner ear and kidney patterning (26). Studies can now be undertaken to examine the role of Eya1 in molecular pathways leading to these common morphogenetic events.

MATERIALS AND METHODS

Genetic mapping

PCR primer pairs (MapPairs) for microsatellite markers distributed throughout the mouse genome were purchased from Research Genetics (Huntsville, AL) and typing was performed as
Previously described (27), except that PCR reactions were carried out for 30 cycles and products were separated on 3% agarose gels (Metaphor; FMC BioProducts, Rockland, ME) and visualized by ethidium bromide staining. Gene order, determined by minimizing the number of obligate cross-over events, and recombination frequency estimates were calculated with the aid of the Map Manager computer program (28).

Histological and clinical analyses

Adult inner ears were harvested after transcardial perfusion with 1% paraformaldehyde, 1% glutaraldehyde in phosphate buffer, pH 7.2. For light microscopy, inner ears were dissected and post-fixed in buffered 4% OsO4, dehydrated in ethanol and propylene oxide, and embedded in SPURR resin (Polysciences, Warrington, PA). Semithin sections (1–2 µm) were cut with a diamond knife and stained with 0.5% toluidine blue or periodic acid–Schiff (PAS). Histological and clinical analyses

ABR threshold determinations

Mice were tested at the University of Cincinnati for ABR thresholds. A computer-assisted evoked potential system (Intelligent Hearing Systems, Miami, FL) was used to obtain ABR thresholds for clicks and for pure-tone pips at 8, 16 and 32 kHz, as described previously (29).

Genomic DNA and RNA, and cDNA preparation

Genomic DNA was prepared from mouse spleens by standard phenol–chloroform extraction and ethanol precipitation methods. Total RNA was purified from mouse brain, eye and skeletal muscle tissues with TRIzol reagent, according to the manufacturer’s protocol (Gibco BRL, Gaithersburg, MD). DNA and RNA concentrations were estimated by spectrophotometric measurements of absorbance. cDNA was prepared from total RNA from adult mouse skeletal muscle with the SuperScript Pre-amplification System for First Strand cDNA Synthesis (Gibco BRL).

Southern and northern blots

Blotting, probe labeling and hybridization procedures used for both Southern and northern blots were as previously described (30). A 1.6 kb fragment of the mouse Eya1 cDNA, containing most of the protein-coding sequence, was used as a probe for initial Southern and northern blot analyses. Additional probes for Southern analysis were produced by PCR amplification with the primers described below. For northern analysis, 15 µg of total RNA was loaded per lane on a 0.8% agarose gel with 2.2 M formaldehyde, electrophoresed in 1× MOPS buffer at 2 V/cm for 4 h, vacuum blotted in 10× SSC onto a positively charged nylon membrane, and UV cross-linked. For quantitative estimation of transcript abundance, densitometry measurements were made on developed X-ray film using the Computing Densitometer and ImageQuant software from Molecular Dynamics (Sunnyvale, CA).

Genomic clones

The wild-type Eya1 clone was isolated by standard filter hybridization screening methods from a commercially available lambda phage library prepared with 129/SvJ mouse genomic DNA (Lambda FIX II, Stratagene, La Jolla, CA). The mutant Eya1 clone was isolated from a custom-made phage library (Lambda FIX II) prepared with genomic DNA from homozygous mutant mice.

PCR primers

PCR primers used to amplify portions of the mouse Eya1 cDNA for sequence comparisons and as probes for Southern blot analysis are given in Table 1. Primers used for sequence comparisons of genomic DNA are given in Table 2; their approximate positions are shown in Figure 4a. Primers ex8F and ex8R were used to amplify the genomic region around exon 8 (nucleotides 592–1097). Primers gF5 and IAPR1 were used to amplify the junction of intron 7 with the IAP 5′ LTR. Primers gF5 and gR5 were used to amplify wild-type, but not mutant DNA. Primers IAPF1 and gR5 were used to amplify mutant, but not wild-type DNA. All three primers (gF5, IAPF1 and gR5) were used simultaneously to distinguish genotypes (Fig. 4c).
PCR reactions

The following reaction conditions were used for PCR amplifications: 20–50 ng of template DNA, 50 mM KCl, 10 mM Tris–HCl, 0.01% Triton X-100, 2.25 mM MgCl₂, 100 nM of each primer, 100 µM of each of four deoxyribonucleoside triphosphates and 0.5 µM of Taq DNA polymerase. Amplification consisted of one cycle of denaturation at 94°C for 3 min followed by 35 cycles, each consisting of 94°C for 15 s denaturation, 60°C for 2 min of annealing and 72°C for 2 min of extension. After the 35 cycles, the final product was extended for 7 min at 72°C. PCR reaction products were separated on 2.5% Metaphor agarose gels, stained with ethidium bromide and visualized with UV light.

DNA sequencing

PCR-amplified products from cDNA and genomic DNA templates were excised from gels and purified with QIAquick Gel Extraction kit (Qiagen, Valencia, CA). DNA was sequenced using an Applied Biosystems 373A DNA Sequencer (Perkin Elmer, Norwalk, CT) and an optimized DyeDeoxy Terminator Cycle Sequencing method. The same primers used for PCR amplification were also used for cycle sequencing.

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