Multiple mutations in mouse Chd7 provide models for CHARGE syndrome

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Received August 10, 2005; Revised and Accepted September 29, 2005

Mouse ENU mutagenesis programmes have yielded a series of independent mutations on proximal chromosome 4 leading to dominant head-bobbing and circling behaviour due to truncations of the lateral semicircular canal of the inner ear. Here, we report the identification of mutations in the Chd7 gene in nine of these mutant alleles including six nonsense and three splice site mutations. The human CHD7 gene is known to be involved in CHARGE syndrome, which also shows inner ear malformations and a variety of other features with varying penetrance and appears to be due to frequent de novo mutation. We found widespread expression of Chd7 in early development of the mouse in organs affected in CHARGE syndrome including eye, olfactory epithelium, inner ear and vascular system. Closer inspection of heterozygous mutant mice revealed a range of defects with reduced penetrance, such as cleft palate, choanal atresia, septal defects of the heart, haemorrhages, prenatal death, vulva and clitoral defects and keratoconjunctivitis sicca. Many of these defects mimic the features of CHARGE syndrome. There were no obvious features of the gene that might make it more mutable than other genes. We conclude that the large number of mouse mutants and human de novo mutations may be due to the combination of the Chd7 gene being a large target and the fact that many heterozygous carriers of the mutations are viable individuals with a readily detectable phenotype.

INTRODUCTION

Large-scale ENU mutagenesis programmes have provided us with large numbers of new mouse mutants to use in the analysis of gene function, and a number of these provide good models for human diseases. We have previously described a series of 12 independent semidominantly inherited mutations which lead to hyperactivity, circling and headshaking behaviours in heterozygotes and which map to the proximal part of chromosome 4, suggesting that these lines may have a mutation in the same gene (1–4).

The behavioural phenotype of the heterozygotes can be attributed to a characteristic inner ear malformation, involving truncation of the lateral semicircular canal and variable truncation or reduction in size of the posterior semicircular canal. These truncations arise early in development and can be detected by a reduced size of the pouch that extends from the developing vestibular part of the inner ear at embryonic day (E) 12.5, and a delay in fusion of the opposing sides of the pouch which would normally leave the canal intact around its rim (1,2). In addition to the inner ear malformation, we have also reported variable middle ear ossicle anomalies and small body size (2). The first of these mutants to be detected, Wheels, was found to have an abnormally extended circadian period and abnormal behavioural response to light. In addition, homozygous Wheels mutants die with vascular defects at mid-gestation (1,5,6).

We were interested in establishing why so many independent mutations appear to have occurred at this locus following ENU mutagenesis, so we resequenced most known exons in the smallest genomic region defined by genetic mapping (between D4Mit104 and D4Mit181) (1). Here we describe a series of different mutations in the Chd7 gene in nine of these mutants.
The human CHD7 gene has been implicated in CHARGE syndrome (7–10). Children affected by CHARGE syndrome have a variable association of features including coloboma of the eye, heart defects, atresia of the choanae, retarded growth, genital anomalies and ear malformations (11–13). Further features have also been described such as olfactory defects, semicircular canal malformation, hyperactivity, recurring otitis media, cranial nerve palsies, hypothalamo-hypophyseal dysfunction and mental retardation (14–19).

All these characteristics have variable degrees of penetrance, with some being present in virtually all CHARGE patients, whereas others are more infrequently observed. Although some of these features were clearly present in the series of mutant mice, such as the semicircular canal defects and retarded growth described previously, other features had not been investigated; therefore, we carried out a more detailed study of the phenotypic characteristics. We found evidence of many of the features of CHARGE syndrome present in the mutant mice with varying levels of penetrance, including eye defects, genital anomalies, cardiovascular defects, cleft palate and choanal atresia. Furthermore, the Chd7 gene was expressed widely during development, including in many of the structures affected in CHARGE syndrome.

RESULTS

Chromosome 4 mutant mice have mutations in the Chd7 gene

Fine-mapping of the Wheels mutation has previously shown that this mutation lies in a 1.1 Mb region between D4Mit104 and D4Mit181 (1). This region contains the Rub2, Asph and Chd7 genes and two predicted genes, ENSMUSG00000057725 and ENSMUSG00000041216, with a total of 69 exons predicted by Ensembl version 31. We carried out exon resequencing of these exons and discovered a series of mutations in the Chd7 gene. While resequencing was in progress, the human CHD7 gene was reported to underlie CHARGE syndrome, which, like the mouse mutants, features semicircular canal truncations of the inner ear (7,13).

Alignment of the mouse Chd7 gene (http://vega.sanger.ac.uk/Mus_musculus/contigview?chr=4&vc_start=8617617&vc_end=8794806&highlight=OTTMUSG0000004417) to human CHD7 (unpublished data, annotated by Wellcome Trust Sanger Institute Havana group) showed that the intron–exon structure of these genes is highly conserved (Fig. 1A). On the basis of Ensembl annotation, we identified a zebrafish gene on chromosome 2 that is highly similar to the human CHD7 gene. Alignment of the zebrafish genomic sequence (unpublished data, annotated at WTSI) with mouse Chd7 and human CHD7 shows the gene structure is conserved across species (Fig. 1A).

We analysed the coding sequence and protein similarity of the mouse Chd7 gene and protein and compared it to the human CHD7 gene and protein (Fig. 1B). The putative mouse Chd7 protein has a very high identity and similarity to human CHD7 (94.9 and 97.0%, respectively), and contains, like other CHD family members, a unique combination of predicted functional domains (Fig. 1B). Two chromo domains, involved in binding to methylated histones, are located at the N-terminal part. Centrally, it has SNF2-like ATPase and helicase domains, which have been implicated in DNA-unwinding. Two domains involved in binding to histone tail (SANT) and DNA (BRK) are located at the C-terminal part of the protein. Finally, mouse Chd7 has four nuclear localization signals (NLS) spread across the protein. This suggests that the mouse Chd7 is a nuclear protein involved in chromatin remodelling, as has been shown in other Chd members (20).

We resequenced most of the 38 exons of Chd7 in a series of mouse mutant lines mapping to chromosome 4 (Cyn, Dz, Edy, Flo, Lda, Mt, Oht, Todo, Whi) and found nine mutations in the coding sequence (Table 1 and Fig. 1B) (Supplementary Material, Fig. S1). We identified six nonsense mutations in six different exons of the Cyn, Dz, Edy, Lda, Oht and Whi mutants. In addition, we found two mutations in donor splice sites (Flo, Todo) and one in an acceptor splice site (Mt). For all three splice site mutants, we predict that these would lead to exon skipping and premature stop codons (Table 1). The mutations identified in the chromosome 4 mutant mice were predominantly located in A or T residues (six out of nine; Table 1), which is consistent with previous reports that ENU mainly targets A or T residues (21). The Edy and Todo mutations are situated in the upstream part of the coding sequence so are likely to result in a complete truncation of all known functional domains and may be null alleles. The Whi and Lda mutations are located in exons 11 and 13, respectively. These mutations would lead to proteins lacking a large part of the SNF2 and all other more C-terminal located domains. The Oht mutation is located in exon 16, C-terminal of the SNF2 domain. The Cyn, Dz, Mt and Flo mutations would probably result in proteins lacking only the most C-terminal NLS, SANT and BRK domains.

Some of the more N-terminally located mutations (including Edy and Todo) are most likely to be functional null alleles. However, all other identified mutations are expected to lead to proteins lacking presumed essential domains. Previous analysis of the inner ear of these mutant mice showed that all have lateral canal truncations, and the similarity in phenotype suggests to us that all identified mutations are functional null alleles.

The mutations we identified lead to premature stop codons in several parts of the protein. Interestingly, most of the mutations we identified lie in similar positions to mutations found in CHARGE syndrome patients (Fig. 1B) (7), and therefore, the mutant mice could represent a valuable model to study this complex syndrome.

Chd7 is expressed in the developing inner ear

As it was previously reported that these chromosome 4 mutant mice and CHARGE syndrome patients have inner ear malformations, we analysed the expression during crucial stages of semicircular canal and cochlear duct formation. Chd7 expression can be detected in the otocyst from E9.5 (Supplementary Material, Fig. S3). The results shown are obtained using the Chd7-3 probe, but similar results were obtained with Chd7-1 and Chd7-2 probes (data not shown). Tissues incubated with a sense Chd7-3 probe (negative control) gave no staining in the cochlea or any other tissues at E12.5 and E14.5 (Fig. 2A) (data not shown). At E16.5, the sense
control probes gave non-specific staining in the submandibular glands, but other tissues were negative (data not shown).

At E12.5, Chd7 was expressed in the developing canal pouches of the vestibular system, whereas expression in other areas including the endolymphatic sac was absent (Fig. 2B). In addition, strong expression in the developing vestibulo-cochlear ganglion was detected (Fig. 2B and C). Chd7 expression in the cochlea was lower, but above background (Fig. 2C). At E14.5, Chd7 was detected strongly in the developing sensory patches in the vestibular system, especially the cristae (Fig. 2D). In the cochlea, expression appeared stronger than at E12.5, especially in the developing organ of Corti (Fig. 2D and E). The expression in the vestibulo-cochlear neurons remained strong (Fig. 2D) (data not shown).

Chd7 is expressed in tissues affected in CHARGE syndrome patients

Epithelial–mesenchymal interactions underlie the development of most of the organs affected in CHARGE syndrome patients. It was hypothesised that CHARGE syndrome affects these epithelial–mesenchymal interactions (22). Previously, CHD7 expression was detected in several organs by RT–PCR, including organs affected in CHARGE syndrome patients (7). Due to technical limitations of the technique used, it is unknown whether CHD7 is expressed in epithelial, mesenchymal or both tissue layers. Therefore, we analysed the expression pattern of Chd7 at E12.5 and E14.5 using probe Chd7-3.

At E12.5, Chd7 was expressed in the developing head in a wide range of tissues at a low level, but at a significantly higher level in specific areas (Fig. 3A). Chd7 was expressed at low levels in the tongue, facial mesenchyme, the ganglionic eminence and Rathke’s pouch (future pituitary). A striking graded expression was detected in several brain regions, including the neopallial (future frontal) cortex, tectum and the ventricular zone of the medulla, with expression highest in proximity to the ventricles (Fig. 3A and B). Lower levels were detected in the choroid plexus and the roof of the fourth ventricle (Fig. 3B). We detected strong Chd7 expression in the developing olfactory epithelium, whereas the levels in the surrounding mesenchyme were much lower (Fig. 3A and C). In addition, Chd7 expression was detected in the olfactory and facial ganglia (Fig. 3C) (data not shown). Chd7 mRNA was strongly expressed in several regions of the body, especially the dorsal root ganglia and
lung epithelium (Fig. 3D). *Chd7* expression was high in the gut and stomach epithelium, the Meissner and Auerbach’s plexi (future enteric neurons) and kidneys (Fig. 3E). *Chd7* expression was not above background in the myocardium and conotruncal region of the heart (data not shown). *Chd7* expression was detected in the lens vesicle and the neurectoderm layer during the development of the olfactory and other types (olfactory, lung and gut), ganglion (vestibulo-cochlear, facial, olfactory and dorsal root) and several specific areas (medial ganglionic eminence and the ventricular layer). 

At E12.5, we detected weak expression of *Chd7* in blood vessels (Fig. 3F). Because it was reported that homozygous *Wheels* mutant embryos die around midgestation due to severe angiogenesis defects in yolk sac and the embryo proper, we analysed *Chd7* expression during earlier steps of blood vessel development. At E7, *Chd7* expression was not detected in the yolk sac, consistent with an absence of blood vessels at this stage (data not shown). However, *Chd7* was found to be expressed in the developing vascular plexus in the yolk sac at E8–8.5 (Fig. 3G), supporting a role for *Chd7* in blood vessel formation and/or remodelling.

At E14.5, expression persisted in several areas of the brain, including the medial ganglionic eminence and the ventricular zone of the medulla. In addition, *Chd7* expression was detected in the external (granular cell) layer of the cerebellum (Fig. 3H). *Chd7* remained strongly expressed in the developing olfactory epithelium and the eye (Fig. 3I) (data not shown). In addition, *Chd7* was expressed in the oral ectoderm and the tooth primordial (Fig. 3J). Figure 3J shows the sense control.

To summarize, *Chd7* is expressed in organs affected in CHARGE syndrome patients (eye, olfactory epithelium, ear, kidney and vascular system). *Chd7* expression is widespread during foetal development, with high levels in epithelial cell types (olfactory, lung and gut), ganglion (vestibulo-cochlear, facial, olfactory and dorsal root) and several specific areas in the brain. Lower levels of expression were detected in mesenchymal cell types. The high levels in epithelial cell layers imply that *Chd7* has an important function in this cell layer during the development of the olfactory and other systems.

**Adult Whirligig mice have eye and genital abnormalities**

To determine whether *Chd7* mutant mice are a good model for studying the mechanisms underlying CHARGE syndrome, Whirligig heterozygotes (*Whi/+*) were examined at postnatal stages for anatomical abnormalities.

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**Table 1. Mutations identified in nine Chd7 mutant mouse lines**

<table>
<thead>
<tr>
<th>Mutant</th>
<th>DNA mutation</th>
<th>Exon</th>
<th>Protein consequence</th>
</tr>
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<tbody>
<tr>
<td>Edy</td>
<td>307C–T</td>
<td>2</td>
<td>Q103X</td>
</tr>
<tr>
<td>Todo</td>
<td>IVS3 + 2T–C</td>
<td>3</td>
<td>Donor splice site—(H549X)</td>
</tr>
<tr>
<td>Whi</td>
<td>2918G–A</td>
<td>11</td>
<td>W973X</td>
</tr>
<tr>
<td>Lda</td>
<td>3195T–A</td>
<td>13</td>
<td>Y1066X</td>
</tr>
<tr>
<td>Oht</td>
<td>3945T–A</td>
<td>16</td>
<td>Y1315X</td>
</tr>
<tr>
<td>Cycn</td>
<td>4266T–A</td>
<td>18</td>
<td>L1429X</td>
</tr>
<tr>
<td>Mt</td>
<td>IVS22-2A–G</td>
<td>22</td>
<td>Acceptor splice site—(V1688X)</td>
</tr>
<tr>
<td>Dz</td>
<td>5536G–T</td>
<td>27</td>
<td>E1846X</td>
</tr>
<tr>
<td>Flo</td>
<td>IVS27 + 2T–C</td>
<td>27</td>
<td>Donor splice site—(S1864X)</td>
</tr>
</tbody>
</table>

It has been reported that CHARGE syndrome patients have a normal birth weight, but have retarded growth and remain small even after puberty (23). Some reports of CHARGE syndrome previously suggested that growth hormone levels are the underlying cause of the growth delay, although other studies have not confirmed this (24). As in *Chd7* mutant mice, hyperactivity has recently been reported in boys with CHARGE syndrome, and this behaviour might be the cause of lower growth rates (17,25). *Whi/+* and control wild-type littermates (+/+) were weighed at several ages. No significant difference could be detected at postnatal day (P) 2 ([+]/+ (n = 17): mean = 2.12 g, SEM = 0.1669; *Whi/+* (n = 8): mean = 2.05 g, SEM = 0.1973). After weaning, both male and female *Whi/+* animals had a significantly lower body weight than control animals at all ages analysed (Fig. 4A). However, the rate of weight gain in *Whi/+* animals appeared not to be affected. When *Whi/+* and control littersmates were sacrificed after P50, we observed that *Whi/+* animals appeared to have less body fat.

One major feature of CHARGE syndrome is coloboma due to a normal birth weight, but have retarded growth and remain small even after puberty (23). Some reports of CHARGE syndrome previously suggested that growth hormone levels are the underlying cause of the growth delay, although other studies have not confirmed this (24). As in *Chd7* mutant mice, hyperactivity has recently been reported in boys with CHARGE syndrome, and this behaviour might be the cause of lower growth rates (17,25). *Whi/+* and control wild-type littermates (+/+) were weighed at several ages. No significant difference could be detected at postnatal day (P) 2 ([+]/+ (n = 17): mean = 2.12 g, SEM = 0.1669; *Whi/+* (n = 8): mean = 2.05 g, SEM = 0.1973). After weaning, both male and female *Whi/+* animals had a significantly lower body weight than control animals at all ages analysed (Fig. 4A). However, the rate of weight gain in *Whi/+* animals appeared not to be affected. When *Whi/+* and control littersmates were sacrificed after P50, we observed that *Whi/+* animals appeared to have less body fat.
circling mice in the Crsl (Carousel) and Flo (Flouncer) lines. Mutations in the Chd7 gene may affect the stability of the tear film.

A variety of minor abnormalities are associated with CHARGE syndrome, including skeletal and renal abnormalities. We did not find any overt defects in the skeleton and internal organs of P2 and adult Whi/+ mice (data not shown). Genital hypoplasia, including micropenis and clitoral abnormalities, occurs in half of the patients with a confirmed mutation in CHD7 (7,26). We did not find any genital abnormalities in male Whi/+ mice (data not shown). The majority of female Whi/+ mice had both clitoral hypoplasia and vulval abnormalities, something never observed in wild-type littermates (Fig. 4H–J and Table 2). We confirmed the occurrence of vulval and clitoral defects in mutants from the Crsl and Flo lines (data not shown).
All Whi/+ or wild-type animals used for this study were initially scored for circling, headshaking and hyperactivity. On the basis of the genotyping of all the mice used for this study, we analysed the penetrance of this behaviour. In total, we detected circling behaviour in 27 mice out of 28 mice with a confirmed Whi/+ genotype, giving a penetrance of 96% (Table 2). One Whi/+ animal without circling behaviour did show severe headshaking. This suggests that the inner ear phenotype is fully penetrant. However, we found that only 30% of the offspring from a mating between a wild-type and a Whi/+ individual displayed circling behaviour and had a Whi/+ genotype after weaning (Table 3). This was confirmed in Crsl and Flo mutant lines, where similar percentages of circling mice were observed after weaning (Table 3). We obtained similar percentages of Whi/+ animals at P2, but this difference was not significant due to the low number of animals analysed (n = 25) (data not shown). This suggests that ~50% of the heterozygous Chd7 mutant mice die before weaning.

The number of affected mice and the total number of mice are shown, with percentages given in brackets. χ²-tests showed significantly reduced numbers of mutants, with p < 0.005 for all three lines.

Some Whirligig mutant embryos have cardiovascular defects

To understand the early postnatal lethality of Whi/+ animals, we analysed the phenotype at prenatal stages. We initially focussed on E15.5–16.5, as at these stages most organs affected in CHARGE syndrome, including the eyes, choanae, palate and cardiovascular system, are well developed. At E15.5, in wild-type embryos, the toes of front and hind limbs were separated, pinnae were visible and the blood vessels were well developed (Fig. 5A). In some Whi/+ littermates, the toes of the hind limbs were not fully separated and the pinna primordium is smaller, indicating a small developmental delay (Fig. 5C). Forty-five percent of the Whi/+ embryos had either mild or severe oedema (Fig. 5B and C and Table 4), indicating cardiovascular insufficiency (27). In a small number of embryos, we found some mild or more severe haemorrhaging (Fig. 5C and Table 4). At E16.5, development of hair follicles and feet were used to stage wild-type and Whi/+ embryos. Whi/+ embryos had developed to the proper stage, but some showed abnormalities in the cardiovascular system similar to those described earlier (data not shown).
Some Whirligig embryos have cleft palate and choanal defects

Cleft palate and choanal atresia are two aspects of CHARGE syndrome that could correlate with reduced viability in mice. The palate develops from pharyngeal arch ectoderm and endoderm in close association with the underlying mesenchyme. In mice, the closure of the palate initiates from E12.5 and the palate is normally fully closed by E15.5 (29). Choanae are the spaces between the oral and nasal cavities and the development of the primary choanae is closely linked with palatal morphogenesis. Interactions between the mesenchyme and the epithelial component are essential for proper development of both the palate and choanae.

We analysed the development of the palate by scanning electron microscopy. At E15.5, the palate was fully closed in wild-type controls (Fig. 6A). In most wild-types, six to eight rugae are visible (Fig. 6A). The palate of the majority of Whirligig embryos was indistinguishable from wild-type palates (Fig. 6B and Table 4). However, 35% of Whirligig embryos had palatal defects, ranging from only partial closure to a complete cleft palate (Fig. 6C and D and Table 4). Interestingly, some Whirligig embryos with cleft palate develop some (but mostly fewer) rugae, suggesting that anterior–posterior patterning in the palate was not affected and that the development of the embryos was not severely delayed. The cleft palate might be due to a delay, rather than a complete block, in palatal morphogenesis. Therefore, we analysed the development of the palate at E16.5. In wild-type and normal Whirligig embryos, the closure point located at the midline of the palate had completely disappeared, and rugae are further developed than at E15.5 (Fig. 6E and F). In some Whirligig animals, the midline of the palate had not fused, although the palatal shelves were touching (Fig. 6G). In other Whirligig animals, the palate remains open and did not show any sign of closure (Fig. 6H).

The severe cleft palate gave us the opportunity to analyse the morphology of the primary choanae in some Whirligig embryos. In Fig. 6D and H, arrows point to the position of the primary choanae. Analysis of the choanae of three Whirligig embryos indicated that there was a defect in some but not all Whirligig animals. In one Whirligig embryo (E15.5), the surface ectoderm had clearly invaginated and the nasal– buccal membrane has resolved, leading to the formation of the choanae (Fig. 6I). In two other Whirligig embryos (E16.5), the choanae were clearly less well developed, and the nasal–buccal membrane appeared to persist (Fig. 6J and K).

From this analysis, we conclude that a significant number of Whirligig animals have palatal and choanal defects. Both cleft palate and choanal atresia can result in an inability to feed and breathing problems. These two aspects, together with the earlier described cardiovascular defects, could explain the early postnatal lethality of Chd7 mutant mice. The range of defects found in Whirligig mice combined with mutation in Chd7 indicates that these mutants represent a good model for CHARGE syndrome.

Table 4. Penetrance of the phenotypes identified in Whirligig embryos

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>+/+</th>
<th>Whirligig</th>
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<tbody>
<tr>
<td>Palatal defect (%)</td>
<td>5% (1/17)</td>
<td>35% (7/20)</td>
</tr>
<tr>
<td>Cleft palate</td>
<td>5% (1/17)</td>
<td>25% (5/20)</td>
</tr>
<tr>
<td>Mild palate defects</td>
<td>0% (0/17)</td>
<td>10% (2/20)</td>
</tr>
<tr>
<td>Oedema</td>
<td>0% (0/25)</td>
<td>45% (14/31)</td>
</tr>
<tr>
<td>Haemorrhages</td>
<td>0% (0/25)</td>
<td>10% (3/31)</td>
</tr>
</tbody>
</table>

Percentages are given for the numbers in brackets.
Is the Chd7 gene highly mutable?

The large proportion of de novo mutations in CHD7 in CHARGE syndrome patients (7) and the numerous mutations of mouse Chd7 described here suggests that there may be an increased susceptibility of Chd7 to mutation compared with other genes (2). Our finding here of nine different mutations spread over a wide stretch of the mouse Chd7 gene rules out a single mutation hotspot as an explanation for the frequency of mutation at this locus. Also, our recent finding of three new alleles (Crsl, Flo and Whi) derived from ENU mutagenesis of C3HeB/FeJ mice, and the report of a spontaneous mutation mapping to this locus and leading to the same phenotype (Wheels-like, Whll) indicates that the apparently high frequency of mutations at this locus is not due to any particular sensitivity of the BALBc strain to ENU mutagenesis as was originally hypothesized (2–4,30). Therefore, we examined the sequence of the human, mouse and zebrafish Chd7 genes to ask whether the usage of codons might lead to a greater chance that a random single base change would generate a stop codon. We analysed 30 randomly selected genes (Supplementary Material, Table S1) with single orthologues in all three species and compared codon usage with that of the Chd7 gene (Supplementary Material, Table S2). We looked at the effects of unweighted base changes and also base changes weighted for the type of mutation (changes due to ENU for mouse and zebrafish based on known increased likelihood of A or T mutations, and the spontaneous changes weighted for CpG dinucleotides in humans as these are known to show increased likelihood of spontaneous mutation). In general, the Chd7 gene showed a slightly greater risk of a non-silent mutation than the mean of the 30 random genes, but the difference was mostly not statistically significant. Human CHD7 showed a significantly greater risk of a nonsense mutation. Zebrafish chd7 showed a very small but significantly elevated risk of a missense mutation when weighted for the known ENU mutagenic profile. All other comparisons between the Chd7 and 30 random genes were not significant (Supplementary Material, Table S2).

Although there was little evidence in zebrafish and no evidence in mouse for an increased chance of a non-silent mutation due to codon usage in the Chd7 gene, we asked whether this was supported by empirical data. We used a large-scale reverse genetic screening approach in zebrafish called TILLING (Targeting Induced Local Lesions IN Genomes) to determine the ENU-induced mutation rate of the chd7 coding sequence (31,32). Zebrafish chd7 exons were chosen on the basis of homology to mouse and human CHD7 sequence and where mutations in orthologous human or mouse CHD7/Chd7 exons have been reported in CHARGE patients (7) or the mouse mutants reported here. To compare the chd7 mutability with average gene mutability,
we resequenced part of the coding region and splice sites of 17 control genes, chosen across the zebrafish genome from a variety of protein classes (Supplementary Material, Table S3). The zebrafish library used was a set of 3072 F1 fish derived from ENU-mutagenized males generated on a mixed genetic background. ENU-induced base pair changes were distinguished from single nucleotide polymorphisms by the fact that they occurred in only one individual fish from the library.

In the 17 control genes, we found 58 mutations in a total sequence of 14.9 Mb, giving an average mutation rate of one mutation in 0.26 Mb (95% confidence intervals, CI: 0.20–0.34 Mb). The mutation rate of our control group is in agreement with an estimate of the library mutation rate as one mutation in ~0.27 Mb (F. van Eeden, personal communication). We screened approximately one-third (~2344 bp) of the coding sequence of zebrafish chd7 in 3072 zebrafish, or a total of 4.2 Mb and identified 12 mutations. This gave an average mutation rate of one mutation in 0.35 Mb (95% CI: 0.2–0.68 Mb) for chd7. The mutation rate for chd7 appears to be ~1.4-fold less than the average mutation rate in this library, but this is not a significant difference (z = 0.9763, P = 0.3289). This indicates that chd7 does not have a higher susceptibility to mutation.

We analysed the percentages of the different types of ENU-induced mutations in the zebrafish control and chd7 genes and compared this with published data (33). In contrast to the published data and the control genes reported here, we did not find any nonsense or splice site mutations in the chd7 gene (Supplementary Material, Fig. S2). However, this was not statistically significant, presumably due to the small sample size.

**DISCUSSION**

We have described in previous reports 12 mouse mutant lines with similar behavioural defects, all mapping to proximal chromosome 4 (1–4). We report here the identification of mutations in the mouse orthologue of the human CHD7 gene in nine of these mutant lines. CHD7 was previously shown to be mutated in patients with CHARGE syndrome (7–10). We demonstrate that these mouse mutants have similar abnormalities as described in CHARGE syndrome, as we observed inner ear, choanal, heart, eye, genital and palate defects in Whirligig mice (Table 5). Furthermore, abnormalities are found in human and mouse heterozygous carriers of CHD7/Chd7 mutations and no homozygotes have been reported to survive past birth.

CHARGE syndrome is diagnosed on the basis of several major and intermediate criteria but other associated malformations have also been reported (22,34). Inner ear abnormalities, especially anomalies of the semicircular canals and cochlea, are one of the most frequent findings in CHARGE syndrome and are used as a major diagnostic criterion (16,35,36). The Chd7 mutant mice were originally identified from large-scale ENU mutagenesis programs designed in part to detect new dominant hearing and/or balance-impaired mouse mutants (37,38). Detailed morphological analysis showed that the circling and headshaking behaviours of these mice are associated with truncations of the lateral and posterior semicircular canals (2). These malformations are very similar to those reported in CHARGE syndrome patients (12,16,18). CHARGE patients have been reported to have vestibular problems and hyperactivity (16,17), and this may be due to inner ear defects or might be related to a circadian rhythm defect as reported for the Wheels mutants (6). In addition, it was shown that at least one of these mutants, Edy, has mildly raised thresholds for cochlear responses (2). CHARGE patients have very characteristic short and wide pinae and osicle abnormalities, including a hypoplastic incus, have been reported (18). We did not find abnormalities in the external ears (pinae) of Chd7 mutant mice, but an increased incidence of minor ossicular anomalies was found (2).

Abnormalities of the external genitalia, the urinary tract and kidneys have been reported to be associated with CHARGE syndrome (26). We have not found any abnormalities in the urinary tract or kidneys of male and female Chd7 mutant mice. The external genitalia of male Whi/+ mice were normal, but most female Whi/+ mice had an abnormal vulva and a hypoplastic clitoris.

We found that a significant number of Whi, Flo and Crst mutant mice die before weaning, as reported earlier for Wheels mutants (5,6). As we found normal Mendelian ratios at fetal stages up to E16.5 but fewer Whi/+ animals at P2, we hypothesized that these animals die perinatally. CHARGE syndrome is a very heterogeneous disease and survival is poor when patients have either cyanotic cardiac lesions, bilateral choanal atresia or oesophageal fistula/ataresia or combination of these three (39). Heart defects described in CHARGE syndrome patients include ventricular and atrial septal defects and conotruncal defects (40,41). We report here that some Whi/+ fetuses have signs of severe cardiovascular insufficiency (oedema) associated with a failure of interventricular septum closure. In this study, we have not investigated the development of the oesophagus, so the occurrence of oesophageal atresia or fistula in Chd7 mutant mice is currently unknown. However, analysis of the primary choanae in Whi/+ embryos indicated a persistence of the nasal–buccal membrane, suggesting a form of choanal atresia. In addition, we found a high incidence of cleft palate in Whi/+ embryos. Cleft palate is a minor associated finding in CHARGE, but in both humans and mice, the presence of cleft palate can affect mortality rates (42,43). The high penetrance of palatal defects in Whi/+ mice could be due to the presence of modifiers on the genetic background used (C3HeB/FeJ). We propose that cardiovascular insufficiency combined with choanal and palatal defects might account for the high perinatal mortality in Chd7 mutant mice.

One major criterion for CHARGE syndrome is ocular coloboma. This can affect the iris, ciliary body and/or the choroid due to a failure of the optic fissure to close (44). We inspected the eyes of E15.5, P2 and adult Whi/+ mice and did not find evidence for the occurrence of iris coloboma. At this stage, we cannot rule out the occurrence of retinal coloboma in Whi mice. However, Wheels mutants occasionally show small eyes (5), we have previously reported signs of cataracts in some of these mutants (2) and in the present study, we found that Chd7 mutants develop symptoms closely resembling the human dry eye condition, keratoconjunctivitis.
An abnormal tear film can lead to ocular inflammation and result in epithelial disease. Dry eye can result from dysfunction of the ocular surface-secretory glandular functional unit. This can be due to a dysfunction of the autonomic efferent or afferent neurons innervating the tear gland unit or the tear gland itself. Previously, it was shown that Neurturin-deficient mice develop keratoconjunctivitis due to defects in the autonomic neural network (45). Several ganglionic defects have been described in CHARGE patients, including defects in olfactory (I), visual and the facial–vestibulo-acoustic (VIII) ganglia associated with arhinencephaly, sensory-neuronal deafness, facial paralysis and feeding difficulties (46,47). Because CHARGE patients have defects in olfactory epithelia, eye and inner ear, it is not clear whether the defects in these cranial ganglia are primary or secondary defects due to abnormalities in the organs they innervate (15,48,49).

Expression of Chd7 was detected in the ganglionic eminence and facial, nasal, vestibulo-acoustic and dorsal root ganglia. In addition, we detected strong expression in the developing Meissner’s and Auerbach’s plexi that later form the enteric nervous system. As we find strong expression of Chd7 in the developing ganglia in mouse, the cranial nerve defects seen in CHARGE patients may be a direct result of CHD7 mutation.

It has been reported that CHARGE syndrome patients have a normal birth weight, but have retarded postnatal growth and remain small even after puberty (23). We report here that Chd7 mutant mice have a lower body weight from weaning onwards. Some reports on CHARGE syndrome suggested that lower growth hormone levels due to pituitary abnormalities are the underlying cause of the growth delay although other studies have not confirmed this (24). The Chd7 mice could be a valuable tool in studying the causes of the growth delay in CHARGE syndrome.

Earlier reports hypothesized that a disturbance in epithelial–mesenchymal interactions might underlie aspects of the phenotype of CHARGE syndrome or the semicircular canal truncation in the mutant mice (2,22,50). We show here that Chd7 is expressed in many organs affected in CHARGE syndrome including the olfactory epithelium, ganglia and several areas of the developing brain (22). However, Chd7 expression is widespread, including both epithelial and mesenchymal cells, so it may have a role in both cell layers.

Whereas CHARGE syndrome is mostly caused by de novo mutations in humans, the Chd7 mouse mutants appear to be hereditary models for CHARGE syndrome. Severe abnormalities, including problems with onset of puberty, lead to a small chance of reproduction of the mutation in humans (51). In the mouse, only a small fraction of the mutants will reproduce, as approximately half of the mutants die before weaning and heterozygous females tend to be poor mothers due to their circling behaviour. We did not find any signs of a delay or problems in onset of puberty in mice.

Previously, we hypothesized that the locus on mouse chromosome 4 may be highly mutable (2). Here, we showed that at least nine out of 12 reported mutations mapping to proximal chromosome 4 have a mutation in Chd7. Similar to the range of mutations found in the human CHD7 gene (9), we found a relatively high number of nonsense mutations and no missense mutations. CHARGE syndrome is generally considered as a sporadic disease, although familial cases with dominant inheritance have been described. The incidence of CHARGE syndrome is around 1:8500 to 1:10 000 (52,53). Why so many mutations have been identified in mouse and human CHD7 is currently unknown. We did not find any particular feature of the Chd7 gene, such as codon usage, that might explain the apparently high level of mutation in the mouse, although the human gene did show codon usage that should give a higher risk of a nonsense mutation than in our control set of genes. TILLING of a library of zebrafish derived from ENU mutagenized fathers did not
reveal a significantly higher mutation rate in the *chd7* gene compared with control genes. The fact that no nonsense or splice site mutations were identified in the *chd7* gene could indicate that zebrafish carrying such mutation have a dominant lethal phenotype or alternatively could be due to the small number of animals screened.

An alternative explanation for the finding of large number of mouse mutants with *Chd7* mutations arising from ENU mutagenesis programmes could be the combination of large gene size and an easily detectable phenotype. The mouse *Chd7* coding sequence is 8961 bp long, presenting a relatively longer and more conserved coding sequence, expression in a narrower range of tissues, the absence of closely related paralogues and a lower evolutionary selection pressure are likely to contribute to a high incidence of harmful mutations. We know *Chd7* is a large gene, but it is widely expressed. If a gene is under very high evolutionary pressure, a mutation is more likely to cause embryonic lethality. Mutations in other genes could occur as often or even more often than in *Chd7* but either a higher lethality early in pregnancy or more subtle phenotype would mean that such mutations are not identified in large-scale mutagenesis programmes or in human patients. The combination of long coding sequence and easily distinguishable phenotype could contribute to the identification of many mutations in humans and mice.

**CHARGE syndrome** is a very heterogeneous disease consisting of an association of several clinical features (56). The difference in expression of the phenotype between different individuals could be explained by the presence of genetic modifiers, the type of mutation in *CHD7* (nonsense, missense, splice site or microdeletion affecting several genes in the region) and interaction with the environment including exposure to teratogens. There is evidence that there is linkage of the CHARGE phenotype to other chromosomes, indicating that other genes could be involved in the same pathways as *CHD7* (12,28,57,58). In addition, the wide range of malformations found in CHARGE syndrome are found in other syndromes including Abruzzo-Erickson and Kabuki syndrome (59–61). Therefore, a careful diagnosis of such complex heterogeneous diseases is essential. In the *Chd7* mutant mouse, we found that the penetrance of different aspects of the phenotype varies. Ear abnormalities and eye defects of the female external genitalia are frequently found in *Whi/+* mice (>90%), whereas cardiovascular, palate and eye defects have a reduced penetrance (<50%) even on an inbred genetic background. The underlying causes of such reduced penetrance in *Chd7* mutant mice are currently unknown, although genetic background has been reported to have a significant influence on penetrance of the circling phenotype in *Wheels* mutants (5). On the basis of the overlap in clinical features between *Chd7* mutant mice and CHARGE syndrome, we believe that these mice will be a valuable tool in further analysis of the pathology underlying the abnormalities in CHARGE syndrome.

**MATERIALS AND METHODS**

**Mice, sequencing and genotyping**

Animal husbandry and experiments were carried out in accordance with UK Home Office regulations. *Cyclone* (Cyn, previously Cyn), *Dizzy* (D2), *Eddy* (Edy), *Flounder* (Flo), *Leda* (Lda), *Metis* (Mt), *Orbiter* (Obt), *Tornado* (Todo) and *Whirligig* (Whi) mice were described previously (2–4). Genomic DNA from mouse tails and ear clips was purified as described (33,62). Yolk sacs were lysed overnight at 55°C in 50 mM KCl, 10 mM Tris–HCl, pH 8.3, 2 mM MgCl2, 0.45% NP-40, 0.45% Tween-20 and 0.4 mg/ml proteinase K. Proteinase K was inactivated at 95°C and 1 µl was used for genotyping. For exon resequencing, primers were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) or in-house using ExoSeq (http://www.sanger.ac.uk/genetics/exon/). PCR was performed using ReddyMix (Abgene) according to manufacturer’s instructions with 1 µm primer and 3 pg/µl DNA. *Whi* mice were genotyped by a PCR (Fw: 5'-CTCAGGAAATAC CAATGGGAG-3'; Rv: 5'-CAAAGAAAAAGTCCACAG AAAC-3') followed by an MfeI restriction digest. An MfeI recognition site is present in the wild-type sequence but not in the *Whi* mutant sequence. The forward primer has a single base pair mismatch which allows for the introduction of a recognition site for MfeI within the primer itself, providing an internal control for digestion. Digesting PCR products with MfeI generates a single band of 204 bp for wild-type, two bands of 204 and 233 bp for *Whi/+* and one band of 233 bp for *Whi/Whi* animals.

**In situ hybridization**

Specific PCR primers for mouse *Chd7*, *Chd7-1* (Fw: 5'-GAGCCAGAGGCTAGCACTAACCT-3'; Rv: 5'-TAGCT GTCTCCTGGGAGGGCGT-3'), *Chd7-2* (Fw: 5'-GC TGCTGCTGTCGCCTCTACTT-3'; Rv: 5'-GAGGGGAGGTCCAGCCTTCTAC-3') and *Chd7-3* (Fw: 5'-CT ATGACCCCTACACGCCCCAGGG-3'; Rv: 5'-GGGACA CATCAGTGTTCCCTCGGC-3') were designed using in-house software. Forward and reverse primers were coupled to T7 and T3 promoter sequences, respectively. The DNA templates for *in vitro* transcription were amplified from mouse testis cDNA. Digoxygenin-labelled RNA probes were generated by *in vitro* transcription using T3 and T7 RNA polymerases. As the *Chd7* gene was annotated as four separate genes (Ensembl Gene IDs ENSMUSG00000045383, ENSMUSG00000059686, ENSMUSG00000050506 and ENSMUSG00000041235) in Ensembl version 31 (Mouse Genome Assembly NCBI m33), we wanted to confirm that these encode one transcript and thus have an identical expression pattern during embryonic development. We performed whole mount *in situ* hybridization using three probes recognizing these Ensembl predicted transcripts (*Chd7-1*, *Chd7-2* and *Chd7-3*) at three stages of embryonic development (Supplementary Material, Fig. S3). C57Bl/6J embryos from timed mating were dissected in ice-cold PBS at E7.5–16.5, with E0.5 at noon the day the plug was found. For whole mount *in situ* hybridization, embryos were fixed overnight at 4°C in 4% paraformaldehyde in PBS and processed as...
described (63). For in situ hybridization on sections, samples were fixed for 2 days at 4°C in 10% neutral-buffered formalin, embedded in paraffin and cut into 8 μm sections. The automated Ventana Discovery system and Ventana reagents (EZprep, cat. no. 950-100, LCS, cat. no. 50-010, Reaction buffer, cat. no. 950-300, Ribowash, cat. no. 760-105, Blue-map kit, cat. no. 760-120), Protease 3 (cat. no. 760-2020), Ribomap (cat. no. 760-102) and ISH Red counterstain (cat. no. 780-2186) were used according to manufacturer’s instructions. We optimized protease treatment, probe concentration and hybridization and wash temperatures until the three Chd7 probes gave a similar staining intensity. Optimal temperature for hybridization and post-hybridizing washes were at 68 and 90°C, respectively. After counterstaining, slides were washed vigorously with soapy tap water, rinsed well in tap water and briefly in distilled water, covered with Crystal/Mount (Abcam, cat. no. Ab8213), dried for 30 min at 50°C and covered with DEPEX and a cover slip.

Phenotype analysis

Whi mice were weighed at weekly intervals from P24–73. Adult mice at P73–264 were sacrificed by rising CO2 concentration and examined for gross abnormalities. Tail and pinnae were taken for genotyping, and the carcass was processed for skeleton staining (64). Whi mice at P1–2 were weighed, sacrificed by an overdose of pentobarbital sodium BP (Lethobarb) and examined for gross abnormalities. The skin was taken for genotyping. The carcass was prepared for bone and cartilage staining (65). For histological analysis, embryos were harvested at E15.5 in ice-cold PBS and yolk sacs were taken for genotyping. Embryos were fixed in 10% neutral-buffered formalin and embedded in paraffin. Sections of 7 μm were cut, dewaxed and stained for haematoxylin/eosin. For scanning electron microscopy, embryos from E15.5–15.6 were harvested in PBS and decapitated. After removal of the lower jaw, heads were fixed overnight in 2.5% glutaraldehyde in PBS, washed in PBS and processed for SEM by the OTOTO method (66). Samples were dehydrated in graded ethanol series, dried in a Bal-Tec critical point dryer according to manufacturer’s instructions and analysed in a Hitachi S-4800 scanning electron microscope at 5 kV.

Coding sequence and protein analysis

A random number generator was used to generate a list of 30 human genes from the Ensembl database with single orthologues in both mouse and zebrafish (Supplementary Material, Table S1). The coding sequence of each gene was analysed using the Coddle program (http://www.proweb.org/coddle/). The program was used to calculate the percentage of potential mutations that cause nonsense and missense changes. CODDLE allows different mutation spectra to be used to weight the likelihood of potential nonsense and missense mutations in a coding sequence depending on the species being looked at. For humans, a weighting appropriate to spontaneous mutations depending on CpG content was used. Weighting for the known mutational spectrum of ENU was used for both mouse and zebrafish. An unweighted analysis (all changes, equal weight) was also carried out. Statistical comparison of the percentage chance of nonsense and missense mutations in chd7 and the average of the 30 control genes was carried out using the non-parametric Wilcoxon signed rank test and the parametric one-sample t-test. The Shapiro–Wilks test was used to analyse normal distribution of the data sets. InterProScan (http://www.ebi.ac.uk/InterProScan/), ScanProsite (http://us.expasy.org/psort/) and NeedleP (http://www.sanger.ac.uk/rrs/) were used to search for protein families, functional domains and to calculate similarity/identity.

TILLING

Genomic DNA samples from a library of the F1 offspring of mutagenized zebrafish were kindly provided by Dr E. Cuppen and Dr F. van Eeden (33). Screening for ENU-induced mutations in chd7 was carried out by resequencing. Nested primers to amplify the Danio rerio chd7 exons were designed using LIMSTILL (http://limstill.niob.knaw.nl). The nested primers were coupled to M13 sequences at 5’ ends (M13F 5’-TGTAATACGACGGTTATCAGT, M13R 5’-AGGA AACAGCATGATGACCAT). The first round PCR samples contained 5 μl of a 1:50 dilution of genomic DNA from fin clips, 0.2 μM primer and 400 μM of each dNTP in PCR mix (10 mM Tris–HCl, 2 mM MgCl2, 50 mM KCl, 0.01% Tween-20 and 0.2 U Taq). First round PCR conditions were 92°C, 180 s; 15 cycles of 92°C for 20 s (65–0.5°C/cycle), 30 s and 72°C, 60 s; 25 cycles of 92°C, 20 s and optimal annealing temperature, 30 s and 72°C, 30 s; 72°C, 10 min. The second PCR contained 400 nl of the first round PCR in PCR mix as described earlier with 0.1 μM primer and 100 μM dNTPs. Second round PCR conditions were 92°C, 180 s; 30 cycles of 92°C, 20 s and optimal annealing temperature, 30 s and 72°C, 60 s; 72°C, 10 min. A 1 μl sample was run on a 2% agarose gel and the yield was estimated. Ten nanogram of second round PCR product was used for sequencing using M13 primers. In addition, we screened a sample of exons from 17 other genes to estimate the average mutation rate for the same library. These control genes were chosen with the aim of obtaining zebrafish knockouts for other studies. The screening conditions were identical for chd7 and the control coding sequence. Sequence data were analysed using an in-house program written by D. Stemple, which compares sequences to a Genbank-formatted file containing exon information, scans for heterozygous base positions and predicts amino acid changes. Nonsense mutations are automatically recorded, and all potential mutations are examined manually. Statistical comparison of chd7 and control mutation rates and power analysis were carried out in STATLETS version 2.1 software (StatPoint LLC). 95% CI was derived using a Poisson assumption for the distribution of mutation events.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.
ACKNOWLEDGEMENTS

We thank Steve Brown, Martin Hrabé de Angelis and Helmut Fuchs for providing the mouse mutants, Pat Nolan and Kelvin Hawker for supplying material, Liz Sheridan and Laurens Wilming for annotation, Edwin Cuppen and Freek van Eeden for access to the zebrafish library, Robert Andrews and Anja Kolb-Kokocinski for advice about scanning electron microscopy and animal house staff for excellent animal care. This work was supported by the Wellcome Trust, MRC, Defeating Deafness and the EC CT-97-2715 and QLG2-CT-1999-00988.

Conflict of Interest statement. None declared.

REFERENCES


