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DEVELOPMENT AND DISEASE

Genetic modifiers of otocephalic phenotypes in *Otx2* heterozygous mutant mice

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SUMMARY

Mice heterozygous for the *Otx2* mutation display a craniofacial malformation, known as otocephaly or agnathia-holoprosencephaly complex. The severity of the phenotype is dependent on the genetic background of a C57BL/6 (B6) strain; most of the offspring of *Otx2* knock-out chimeras, which are equivalent to the F_1 of CBA and B6 strains, backcrossed with B6 females display reduction or loss of mandible, whereas those backcrossed with CBA females do not show noticeable phenotype at birth. The availability of phenotypically disparate strains renders identification of *Otx2* modifier loci possible. In this study, a backcross of chimera with B6 was generated and genome-wide scans were conducted with polymorphic markers for non-mendelian distribution of alleles in *Otx2* heterozygous mutant mice displaying abnormalities in the lower jaw. We identified one significant locus, *Otmf18*, between D18Mit68 and D18Mit120 on chromosomes 18, linked to the mandibular phenotype (LOD score 3.33). A similar replication experiment using a second backcross (N3) mouse demonstrated the presence of another significant locus, *Otmf2* between D2Mit164 and D2Mit282 on chromosome 2, linked to the mandibular phenotype (LOD score 3.93). These two modifiers account for the distribution of the craniofacial malformations by the genetic effect between B6 and CBA strains. Moreover, *Otmf2* contain a candidate gene for several diseases in mice and humans. These genetic studies involving an otocephalic mouse model appear to provide new insights into mechanistic pathways of craniofacial development. Furthermore, these experiments offer a powerful approach with respect to identification and characterization of candidate genes that may contribute to human agnathia-holoprosencephaly complex diseases.

Key words: *Otx2*, Otocephaly, Agnathia-holoprosencephaly complex, Genetic modifier, Neural crest, Mandible, Mouse

INTRODUCTION

Gene targeting is an important technology for analysis of gene function during embryogenesis; moreover, it offers the means for the generation of animal models for human congenital disease. Hundreds of mutant mice have been developed; furthermore, thousands will be available in the near future, providing researchers with an immense new resource and information regarding the developmental biology field. Additionally, numerous targeted loci show disparate phenotypes that depend on the genetic background of mouse strains, thus affording an even broader understanding of gene function (Horan et al., 1995; Matsuo et al., 1995; Proetzel et al., 1995; Rozmahel et al., 1996; LeCouter et al., 1998; Wojnowski et al., 1998; Wawersik et al., 1999). One powerful approach leading to identification of genes that are involved in a specific phenotype is the mapping of loci that modify the severity of the phenotype employing naturally occurring variations in existing inbred strains (Lander and Kruglyak, 1995). Because the genetic contributions to these traits are often caused by a combination of effects at multiple loci, these traits are termed complex traits. However, the identification of

genetic loci that modify developmental malformations in knockout mutant mice as an experimental model has not been attempted.

Mouse *Otx2* is a *paired*-like type homeobox gene functioning as a transcriptional activator (Simeone et al., 1992; Simeone et al., 1993). It is sequentially expressed in the epiblast, anterior visceral endoderm, anterior definitive endoderm and anterior neuroectoderm prior to and during gastrulation; at the subsequent neurula stage, *Otx2* is expressed in the entire rostral brain region (Simeone et al., 1992; Ang et al., 1994; Acampora et al., 1998; Kimura et al., 2000). Indeed, several knockout and compound mutations of the *Otx2* gene suggest that it is involved in several steps for early AP patterning and rostral brain development in cooperation with other regulatory genes (Matsuo et al., 1995; Acampora et al., 1995; Acampora et al., 1997; Acampora et al., 1998; Ang et al., 1996; Suda et al., 1996; Suda et al., 1997; Suda et al., 2001; Kimura et al., 2000; Kimura et al., 2001; Tian et al., 2002).

In addition, *Otx2* is expressed in the cephalic mesenchyme, including the mesencephalic neural crest cells, which are distributed to the premandibular and distal regions of the mandibular regions (Kimura et al., 1997). Reflected by *Otx2* expression in the cephalic mesenchyme, *Otx2* also plays a crucial role in craniofacial development. *Otx2* single heterozygous mutant mice displayed craniofacial malformations that were strictly dependent on the genetic background of the murine strains (Matsuo et al., 1995). Previously, the *Otx2* knockout chimera has been generated in TT2 ES cells, which are derived from F_1 embryos obtained from crosses of inbred C57BL/6 (B6) and CBA strains (Yagi et al., 1993; Matsuo et al., 1995). Upon backcross of these chimeras with B6 females, the majority of heterozygous mutants are dead at birth, accompanied by severe craniofacial malformations, which are designated as otocephaly in many mammalian species and agnathia-holoprosencephaly complex in humans (Bixler et al., 1985; Juriloff et al., 1985; Winter, 1996; Wallis and Muenke, 2000). Notably, these mutants displayed reduction or absence of the lower jaw and/or eyes externally as well as holoprosencephaly by histological examination (Matsuo et al., 1995). However, when the chimeras were crossed with CBA females, most of the *Otx2* heterozygous mutant pups exhibited no noticeable phenotype. This evidence explicitly suggests the presence of several genetic modifier genes exerting strong effects on the expressivity of the *Otx2* heterozygous mutant phenotype.

On the basis of facilitated recognition and quantitation of small changes in *Otx2* activity through lower jaw development, in particular, the length of the mandible, this phenotype provides a simple and sensitive assay for allelic differences at secondary loci interacting with the *Otx2* gene product. Changes in the gene products of secondary loci that lie upstream, downstream or interact directly with the *Otx2* protein would all impact the expression of the *Otx2* mutant mandible phenotype. With the advent of simple sequence length polymorphism (SSLP) markers, which are distributed throughout the entire genome (Dietrich et al., 1992), it is now possible to rapidly map the loci that contribute to such complex genetic traits. This situation provides an ideal opportunity for defining the genes that control the severity of otocephaly. Moreover, genetic analysis of well-defined experimental models of otocephaly offers the potential to markedly accelerate the genetic analysis of human agnathia-holoprosencephaly complex. In this study, two different mouse strains, B6 and CBA, were employed in order to identify and map modifier loci acting upon the expression of mandible abnormalities of *Otx2* heterozygous mutant mice. The modifier loci thus identified regulate a genetic pathway of craniofacial development interacting with *Otx2*; furthermore, these loci may also be possible genetic causes of human agnathia-holoprosencephaly complex diseases.

MATERIALS AND METHODS

Mice and phenotypic analysis

Inbred B6 and CBA strains were purchased from Charles River. *Otx2* mutant chimeras were generated as described (Matsuo et al., 1995). Mice were housed in environmentally controlled rooms of the Center for Animal Resources and Development, Kumamoto University under the guidelines of Kumamoto University for animal and recombinant DNA experiments. Backcross embryos (N2 and N3) were obtained at 18.5 dpc. External views of each embryo were photographically recorded. Subsequently, tail samples were removed for genomic DNA analyses. Genotype of mutant and wild-type alleles was determined by PCR analysis as described (Matsuo et al., 1995). Cartilage and bones were stained with Alcian Blue and Alizarin Red based on the method of Kelly et al. (Kelly et al., 1983). Subsequently, the lengths of left and right mandibles of embryos were measured in millimeters and an average of both lengths was obtained (Figs 3, 4). According to the lengths of each mandible, the mutant embryos were qualitatively classified into three phenotypes, normal mandible (longer than 5.0 mm), small mandible (0.5 to 4.9 mm) or no mandible (0 mm).

Genotypic analysis

Genomic DNA was prepared by standard procedures (Sambrook et al., 1989). Genotypes were determined by PCR amplification of polymorphic DNA fragments containing simple sequence repeats (Dietrich et al., 1994). For initial genome-wide N2 and N3 linkage analysis, 92 and 51 markers, respectively, were used to genotype animals displaying three phenotypes, normal mandible, small mandible or no mandible. Markers were selected so as to provide a spacing interval of approximately 20 cM. For subsequent fine mapping, 35 and 15 additional SSLP markers were used to genotype these N2 and N3 mice, respectively (Figs 6, 7; data not shown). Sequences of the PCR primers are described at http://www.informatics.jax.org Primers for the majority of these markers were purchased from Research Genetics (Huntsville, AL). PCR products were separated on 3% agarose gels (generally clear resolution by greater than 5 bp difference was achieved with Agarose-1000, Gibco). Staining was effected with EtBr for genotype determination.

Linkage analysis

Statistical analyses were performed by composite interval mapping (Zeng, 1994; Jiang and Zeng, 1995) across the genome in 2 cM intervals by QTL cartographer (Basten et al., 2001). LRS scores were converted to logarithm of odds ratios (LOD scores) via division by 4.6. Suggestive and significant linkages were defined in accordance with the guidelines of Lander and Kruglyak (Lander and Kruglyak, 1995) as LOD score thresholds 1.9 and 3.3, respectively. Statistical evidence of suggestive and significant linkages would be expected to occur one time and 0.05 times at random in a genome scan, respectively (Lander and Kruglyak, 1995). A transmission distortion was not detected in the ratio of alleles at five modifier loci identified in this study for 100 random heterozygous N2 or N3 backcross mutant embryos (data not shown).

RESULTS

Variation and classification of *Otx2* heterozygous mutant phenotypes

In an effort to map loci responsible for modification of the severity of craniofacial defects in *Otx2* heterozygous mutant mice, two strains of mice, displaying disparate phenotypes of *Otx2* heterozygosity, were used (Figs 1, 2). We have generated *Otx2* mutant chimeras employing a TT2 ES cell line derived from F1 embryos of B6 females and CBA males (Yagi et al., 1993; Matsuo et al., 1995). Upon backcross of chimeric males with wild-type B6 females to generate heterozygous mutant mice, severe craniofacial malformations occurred in the majority of the *Otx2* heterozygous mutants at 18.5 dpc (Figs 1, 2). External abnormalities were mainly characterized as the reduction or loss of the lower jaw and eyes (Fig. 1D,E; Fig. 2). Additionally, the severity of the phenotype varied greatly from a normal condition to the appearance of acephaly (Fig. 1C-I). By contrast, when chimeric males were backcrossed with wild-type CBA females, craniofacial malformations were not observed (Fig. 1B).

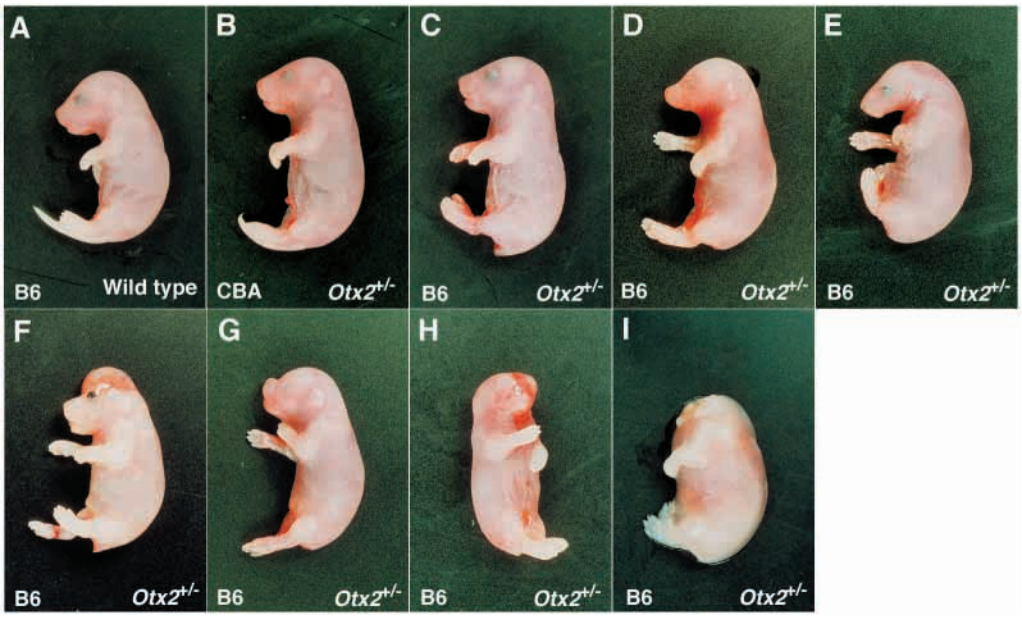

Fig. 1. Variation of external craniofacial morphology of *Otx2* heterozygous mutant embryos at 18.5 dpc. (A) Wild-type mouse backcrossed *Otx2* knockout chimeras with wild-type B6 females. *Otx2* heterozygous mutant mice (N2) backcrossed *Otx2* mutant chimeras with wild type CBA females (B) and with wild type B6 females (C-I). No noticeable malformations are evident in the mutant mouse on the CBA strain genetic background (B). The severity of the phenotype varies from normal to acephaly (C-I). No apparent external abnormalities are observed in the mutant mouse on the B6 strain genetic background (C). The mutant mouse displays reduction of the lower jaw (D). The mutant mouse lacks an entire lower jaw (E). The mutant mouse displays excencephaly (F). The distal region of the face is shortened in the mutant mouse (short nose) (G). The face is cleft in the mutant mouse (cleft face) (H). The entire head is lacking (acephaly) in the mutant mouse (I).

Initially, in order to investigate the variation of severity of craniofacial malformations, the chimeras were backcrossed with wild-type B6 females, resulting in N2 heterozygous offspring. Subsequently, the external abnormalities of these offspring were examined at 18.5 dpc (Fig. 1, Fig. 2A). Descriptions of eye and holoprosencephaly malformations were excluded in this investigation owing to the difficulty associated with judging defects from an external perspective; moreover, further histological analysis is required for the precise description of these abnormalities (Matsuo et al., 1995). Thirty-seven percent of heterozygous pups (N2) did not exhibit prominent abnormalities in jaw, nose or head externally (Fig. 1C, Fig. 2A). Nineteen and 21.5% of these offspring displayed reduction and absence of the lower jaw, respectively (Fig. 1D,E, Fig. 2A). A small percentage of mutants exhibited excencephaly (7.0%; Fig. 1F, Fig. 2A), short nose (3.0%; Fig. 1G, Fig. 2A), cleft face (2.0%; Fig. 1H, Fig. 2A) and acephaly, showing loss of the entire head (5.5%; Fig. 1I, Fig. 2A). The remaining small percentage of mutants revealed additional phenotypes, including ethmocephaly (5.0%; Fig. 2A; and data not shown). Consequently, the distribution of these craniofacial abnormalities is characteristic of a monogenic trait that is caused by modifier loci (Figs 1, 2) (Lander and Schork, 1994).

The most frequently observed phenotype was lower jaw abnormality; consequently, we focused on the jaw anomalies. In order to investigate the phenotype of lower jaws more precisely, the morphology of the mandibular skull following bone and cartilage staining by Alcian Blue and Alizarin Red was further examined (Fig. 3). We found that even in reduced lower jaws, mandible formation was affected to varying extents, ranging from simple fusion of the anterior tips of the incisors to involution of the entire mandible in a small single median bone (Fig. 3F,H,J). Furthermore, to determine the severity of the anomalous mandibles, the length of each was measured (Figs 3, 4). Normally, lengths of wild-type mandibles of B6 and CBA strains were consistently longer than 5.0 mm at 18.5 dpc (Fig. 3B, Fig. 4A; data not shown). Similarly, lengths of *Otx2* heterozygous mutant mandibles on CBA genetic background were also longer than 5.0 mm (data not shown). By contrast, mandibles of heterozygous mutants backcrossed to B6 females exhibited varying lengths (Fig. 3, Fig. 4B). Mutant mandibles demonstrated lengths in excess of 5.0 mm as well as in the range of 0.5 to 4.9 mm; additionally, the mandible was absent in several samples (Fig. 3C-K, Fig. 4B).

In order to define the genetics underlying this dramatic variation in mandible phenotype, a whole genome search for modifier loci involved in the modulation of mandible abnormalities was conducted. Thus, all mutant individuals exhibiting no apparent abnormalities, reduction of lower jaw and loss of lower jaw (Fig. 2), were genotyped; however,

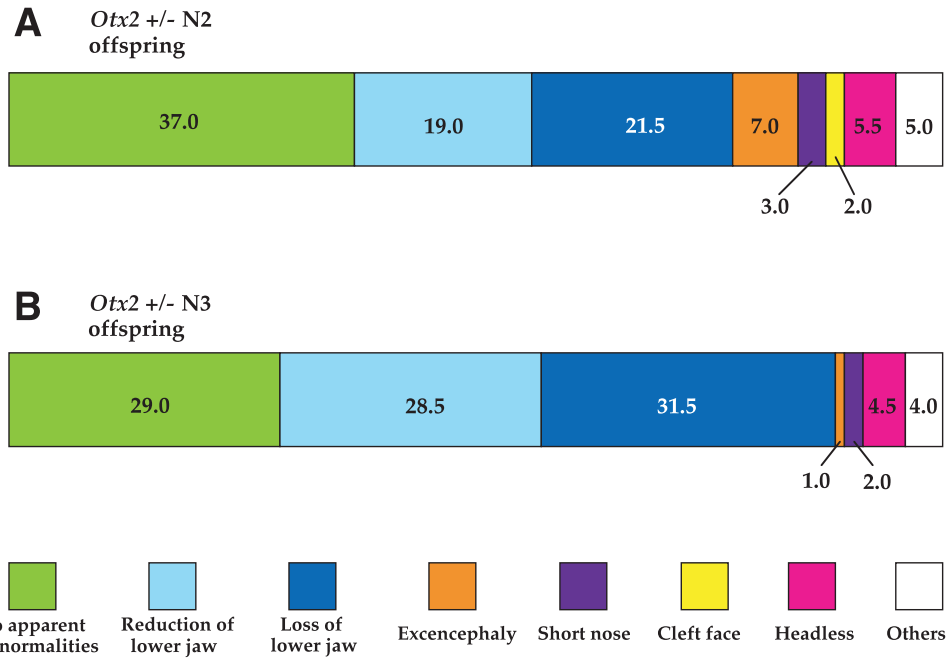

Fig. 2. Frequency distribution of external malformations in mutant mice with B6 background. (A) *Otx2* heterozygous mutant N2 embryos (n=200) at 18.5 dpc obtained by backcrossing chimeras with wild-type B6 females are phenotypically classified into eight groups according to their external morphology. (B) *Otx2* heterozygous mutant N3 embryos (n=200) at 18.5 dpc obtained by backcrossing the N2 male with wild-type B6 females are phenotypically classified into eight groups according to their external morphology.

mutant embryos displaying other external phenotypes, such as excencephaly, short nose, cleft face, acephaly, etc., were not investigated with respect to further genotyping experiments (Figs 1, 2).

Linkage analysis using N2 offspring

We hypothesized that the variable severity in the *Otx2* heterozygous mutant mandible of the B6 strain was due to the variation in genetic background, particularly involving modifier loci, the alleles of which differed between B6 and CBA. With the discovery of the highly polymorphic and simple genotyping protocols of simple sequence length polymorphisms (SSLPs) (Love et al., 1990), these markers are very applicable to the mapping of the location of genetic loci involved in genetic background-dependent phenotypic differences. However, the usefulness of CBA for genetic mapping studies has been limited by the lack of information regarding DNA variants alleles (Dietrich et al., 1992; Dietrich et al., 1994) (http://www.informatics.jax.org/). In order to map locations of modifiers of *Otx2* mutant mice, we first surveyed variant SSLP markers between CBA and B6 strains for the entire genome scan (Fig. 5). Of the 293 markers tested, 180 were variant based on agarose gel electrophoresis (data not shown). This rate of variant alleles is comparable with that observed in other inbred laboratory mouse strains (Dietrich et al., 1994). Given the high frequency of variant alleles and large litter size, the CBA strain could be useful in mapping studies of genetic modifiers in transgenic or knockout mice that are widely generated in the CBA and B6 genetic background.

In order to map the regions of the genome containing modifying loci, 199 mutant pups displaying no apparent abnormalities, reduction of lower jaw and loss of lower jaw (Figs 1, 2) were initially selected from the first generation of B6 backcrossed animals (N2). These were subjected to further skull staining and the lengths of each mandible were measured. Then, the severity of the lower jaws phenotypes was designated as normal mandible (the mandible length is longer than 5.0 mm), small mandible (the mandible length corresponds to 0.5 to 4.9 mm) and no mandible (the length is 0 mm). Subsequently, 92 SSLP markers were chosen, covering approximately 20 cM intervals throughout the entire genome with the exception of two chromosomes: chromosome 14, on which the *Otx2* gene is located, and chromosome X, which is derived solely from the B6 strain. These 199 offspring were then genotyped using 92 PCR markers for the initial genome scan (Fig. 5). Markers (e.g. chromosomes 2, 10 and 18) showing trends for the potential linkage (P<0.05) were subjected to extended genotyping so as to include a total of 439 mutant embryos displaying the phenotype of normal mandible, small mandible or no mandible together with 35 additional microsatellite DNA markers surrounding potential loci (Fig. 6).

Thus, linkage analysis was conducted with the composite interval mapping of QTL-cartographer program (Fig. 6) (Basten et al., 2001); in addition, to investigate whether the genetic loci can modify the phenotype for small mandible (the mandible length corresponds to 0.5 to 4.9 mm) or no mandible (0 mm) qualitatively, genetic analysis was also performed with mutant individuals displaying normal mandible (the mandible length is longer than 5.0 mm) and no mandible, or those displaying normal mandible and small mandible, respectively (Fig. 6). Consequently, one significant linkage on chromosome 18, which was defined as *Otx2* modifier (*Otmf*) *18*, was obtained exhibiting a peak LOD score of 3.33 at 11.1 cM (Fig. 6C, Table 1). One suggestive linkage was found on chromosome 10, with a peak LOD score of 2.56 at 38.1 cM (Fig. 6B, Table 1). These two loci exert effects on both the no mandible and small mandible phenotypes (Fig. 6B,C, Table 1). Unexpectedly, *Otmf18* was derived from the CBA strain (Table 1), suggesting epistatic interactions between modifiers. Additionally, two weak linkages were also detected on chromosome 2; these linkages exhibited peak LOD scores of 1.59 at 17 cM and 1.8 at 66.9 cM, respectively (Fig. 6A). Thus, these findings acquired via the survey of N2 offspring indicate that at least one modifier locus *Otmf18* is significantly involved in the severity of mandible phenotypes in *Otx2* mutant embryos.

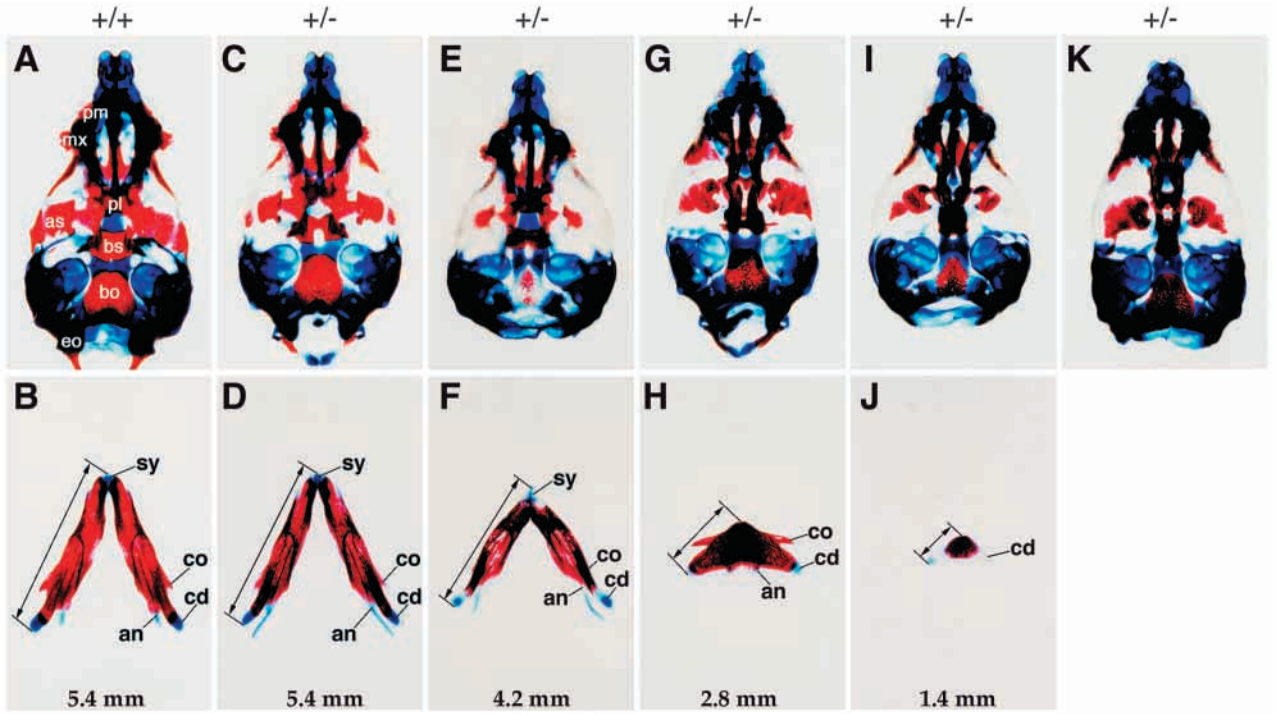

Fig. 3. Variation of mandible length in *Otx2* heterozygous mutant mice. Whole-mount views of skull morphology of wild type (A,B) and heterozygous mutant embryos (C-K) at 18.5 dpc following cartilage and bone staining. Mandibles have been separated in cases in which they were present (A-J). (K) An embryo lacking a mandible. The length of each mandible from proximal to distal is 5.4 mm (A), 5.4 mm (C), 4.2 mm (E), 2.8 mm (G) and 1.4 mm (I). an, angular process; as, alisphenoid; bs, basisphenoid; bo, basioccipital; cd, condyloid process; co, coronoid process; eo, exoccipital; mx, maxillar; pl, palatine; pm, premaxillar; sy, symphysis.

Linkage analysis using N3 offspring

As described previously, small numbers of N2 backcross heterozygous mutant mice survived to weaning, followed by fertility that afforded further progeny (Figs 1, 2) (Matsuo et al., 1995). In order to refine modifier location, a single N2 male was selected; subsequently, allele distribution between B6 and CBA was genotyped employing the 92 polymorphic markers from the N2 initial genome scan (Fig. 5). Chromosomes 3, 6, 7, 12 and 17 were found to be homozygous for B6 in this male mouse; chromosomes 2, 5, 9, 11, 13 and 16 were heterozygous for CBA, whereas chromosomes 1, 4, 8, 10, 15, 18 and 19 contained both B6 homozygous and B6 and CBA heterozygous regions (Fig. 5; marked in gray or black). Consequently, among the chromosomes on which the modifier candidates by N2 linkage analysis were located, chromosome 2 was heterozygous for B6 and CBA and chromosomes 10 and 18 were homozygous for B6. Thus, the latter two chromosomes were excluded from further N3 analysis.

Next, this N2 male was backcrossed with wild type B6 females, resulting in heterozygous N3 animals. External phenotypes were classified as described above (Fig. 1). The frequency of external phenotype in these N3 mutant embryos is summarized in Fig. 2B. Twenty-nine percent of heterozygous pups did not display prominent abnormalities in jaw, nose or head (Fig. 2B). Mutant progeny exhibited reduction (28.5%) of and loss (31.5%) of the lower jaw (Fig. 2B). All mutant animals exhibiting no apparent abnormalities, reduction of lower jaw and loss of lower jaw (Fig. 2) were subjected to skeletal staining and the lengths of each mandible were measured (Fig. 4C). Then, the severity of the mandibular phenotype was designated as normal mandible (the mandible length is longer than 5.0 mm), small mandible (the mandible length corresponds to 0.5 to 4.9 mm) and no mandible (the length is 0 mm). For the modifier mapping, these 202 mutant N3 pups were genotyped initially with 51 microsatellite markers that were not homozygous for B6 strain allele in this male (Fig. 5, marked in gray). Thus, genetic analysis was conducted with the composite interval mapping of QTL-cartographer program as described (Fig. 7) (Basten et al., 2001). For markers (e.g. chromosome 2) with potential linkage ($P<0.05$), extended genotyping was performed along with 16 additional SSLP markers (Fig. 7). Furthermore, to investigate whether the loci can modify the phenotype for small mandible or no mandible qualitatively, genetic analysis was also performed with mutant individuals displaying normal mandible and no mandible or those displaying normal mandible and small mandible, respectively (Fig. 7). Consequently, we found that one significant locus was mapped on chromosome 2. *Otmf2*, which was also linked weakly in the N2 linkage analysis (Fig. 6A), regulates the phenotype displaying no mandible with a peak LOD score of 3.93 at 77 cM (Fig. 7). Furthermore, one suggestive locus, which was characterized by a peak LOD score of 3.13 at 96 cM on chromosome 2, regulates the phenotype of the small mandible (Fig. 7). The above results, in conjunction with N2 linkage data, indicate that at least two distinct modifier loci, *Otmf2* and *Otmf18*, regulate the severity of the otocephalic phenotypes in *Otx2* heterozygous mutant mice.

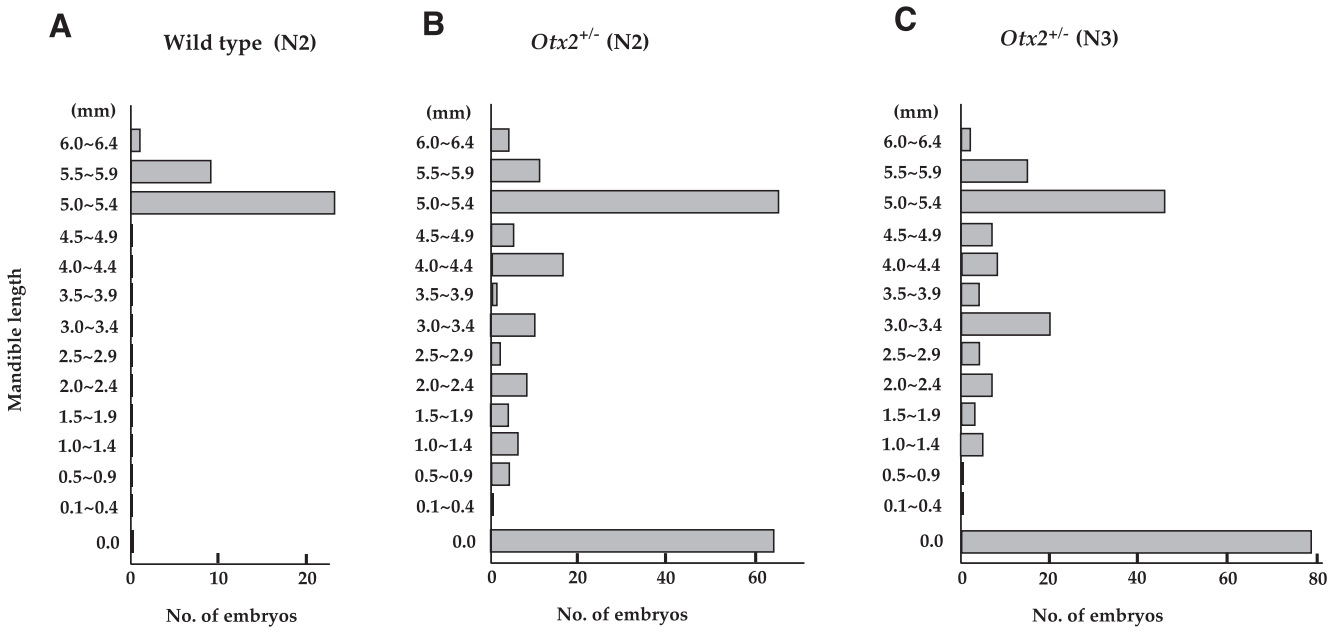

Fig. 4. Frequency distribution of mandible length in 18.5 dpc embryos. Distribution of mandible length in N2 wild-type embryos (*n*=30) chimeras crossed with wild type B6 females (A), heterozygous N2 mutants (*n*=200) crossed with wild-type B6 females (B) and heterozygous N3 mutants (*n*=200) backcrossed twice with wild-type B6 females (C).

DISCUSSION

In the present investigation, genetic linkage analysis (Lander and Botstein, 1989; Lander and Kruglyak, 1995; Darvasi, 1998) was employed to identify genetic loci modifying the otocephalic phenotype in *Otx2* heterozygous mutant mice. A genome-wide screen comparing the pattern of strain means to the severity with SSLP markers detected two significant modifier loci, *Otmf2* and *Otmf18* (Figs 6, 7; Table 1). This data offers the first evidence that these genetic loci regulate the severity of the otocephalic phenotype. Furthermore, the findings indicate that these loci are genetically associated with *Otx2* locus. In addition, these modifier may interact with other unidentified modifier loci epistatically. One locus, *Otmf18*, was mapped on the CBA allele (Table 1). As the otocephalic phenotype is not evident on the CBA genetic background (Fig. 1), the *Otmf18* locus on the CBA strain alone appears to be insufficient to induce mandible abnormalities. Thus, a second undetermined modifier, probably located on the B6 strain, may be required for expression of mandible abnormalities.

Therefore, these findings, in conjunction, suggest that the genetic mechanism of the otocephalic phenotype is substantially more complex than originally expected. Nevertheless, the modifier loci account for the genetic effect between B6 and CBA strains and can, in part, explain the distribution of craniofacial malformations brought about by haploinsufficiency of the *Otx2* gene. Indeed, identification and characterization of these genetic loci provide new insights into mechanistic pathways of mandible development derived from mesencephalic neural crest. Furthermore, the otocephalic mouse model may afford a powerful approach with respect to identification and characterization of candidate genes that may contribute to human agnathia-holoprosencephaly complex diseases.

Otx2 modifier loci may control several distinct steps for the formation of neural crest cells

The modifier loci identified in this study are considered to regulate the developmental processes of mandible, which originates from mesencephalic neural crest. Fate-mapping experiments in chicks have suggest that skull bones of the premandibular and the distal regions of the mandibular regions originate from cephalic neural crest mainly at the level of mesencephalon (Couly et al., 1993; Koentges and Lumsden, 1996). Similarly in mouse, mesencephalic neural crest cells contribute to the mesenchyme of premandibular and mandibular regions (Osumi-Yamashita et al., 1994; Imai et al., 1996). Notably, endogenous *Otx2* is expressed in neural plate, neural crest and neural crest cells at the level of mesencephalon; moreover, distal elements of mandibular arch skeletons are lacking or severely affected in *Otx2* heterozygous mutants (Matsuo et al., 1995; Kimura et al., 1997) (this study). Thus, the *Otx2* heterozygous mutant defects relate primarily to *Otx2* function in the formation of mesencephalic neural crest (Kimura et al., 1997).

As the genetic modifier loci were crucial for development of neural crest-derived structures, it is likely that they play an important role in the induction, guidance, migration or differentiation of mesencephalic neural crest in the identical genetic pathway of the *Otx2* gene. Neural crest is induced at the dorsolateral edge of the neural plate; from that point, neural crest cells delaminate and migrate along specific routes to many destinations in the vertebrate embryo (Le Douarin, 1982). Grafting experiments in the chick have shown that interactions between embryonic non-neural ectoderm (presumptive epidermis) and neural plate induce the formation of neural crest cells at their interface, and that each of these tissues contributes to the neural crest (Selleck and Bronner-Fraser, 1995; Liem et al., 1995). After induction, neural crest

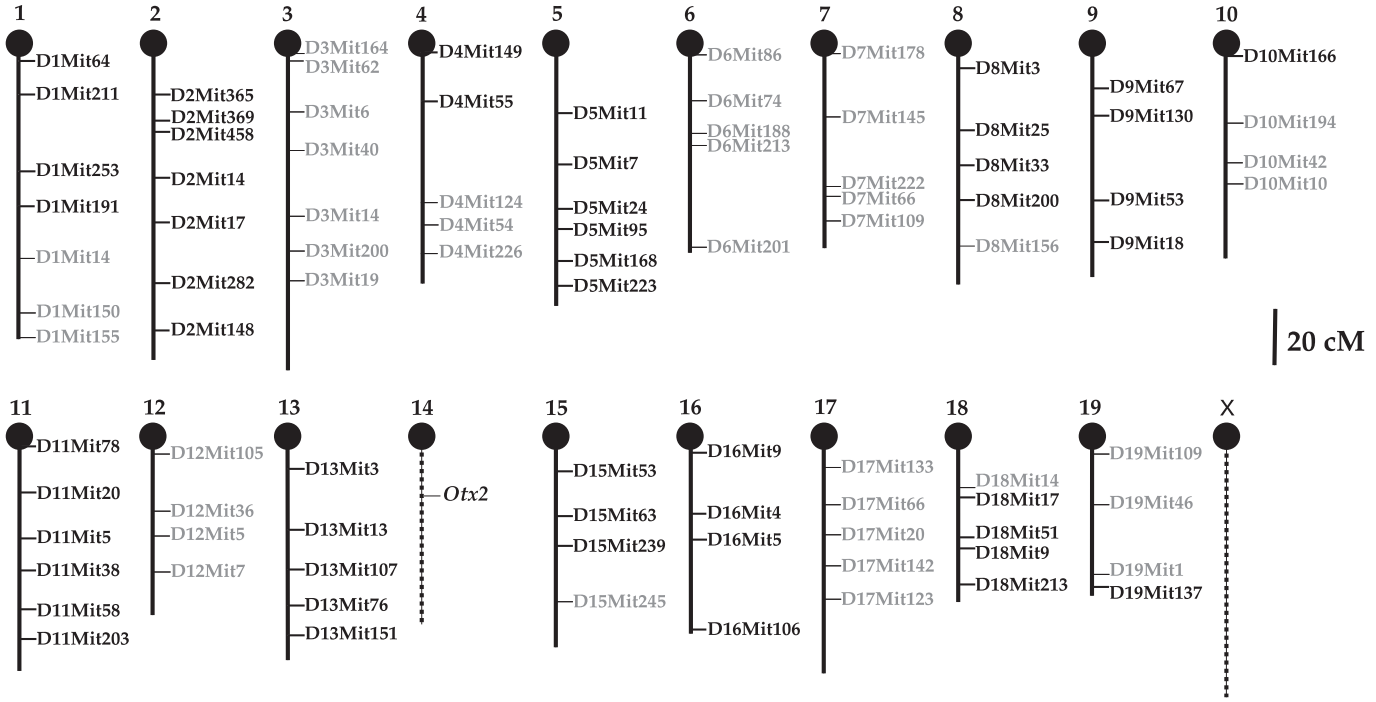

Fig. 5. Chromosomal location of microsatellite markers selected for analysis of phenotypic pools in the first level screen. Marker positions were obtained from the microsatellite map distributed by the MIT mouse genome database. All SSLP markers colored in both black and gray are employed in the N2 analysis. Black markers represent a heterozygous (B6/CBA) and gray markers represent a homozygous (B6/B6) allele in the N2 male. Black SSLP markers representing heterozygous (B6/CBA) allele are used in the N3 mapping analysis.

delaminates from neural tube; that is, neural crest undergoes an epithelial to mesenchymal conversion and begins to migrate along specific pathways, differentiating into several structures. An important link exists between the guidance and differentiation of neural crest cells. In some cases, specified cells are targeted to the correct destinations, whereas in other instances, cells migrate to sites where they encounter inductive signals. These crest cells finally differentiate into a wide variety of cell types, including neurons and glial cells of the peripheral nervous system, melanocytes and smooth muscle cells, and cartilaginous and skeletal elements in the head (Le Douarin, 1982).

We have found two genetic loci that significantly modify the severity of mandible phenotypes of *Otx2* heterozygous mutants. Notably, the *Otmf18* locus appeared to be linked to phenotypes of no mandible and small mandible (Fig. 6C; Table 1). This finding suggests that *Otmf18* may direct the formation of mesencephalic neural crest cells fated to the entire mandible. The *Otmf2* locus was linked solely with the phenotype of no mandible (Fig. 7; Table 1), indicating that this locus may regulate earlier processes in neural crest formation, i.e. induction or delamination of neural crest. By contrast, one suggestive locus at 96.0 cM on chromosomes 2, was linked with the small mandible phenotype but not with the no mandible phenotype (Fig. 7), suggesting that this locus may regulate later processes, such as the migration or differentiation of mesencephalic neural crest cells, which exclusively contribute to the most distal region of the mandible. Thus, *Otx2* may regulate several distinct steps of neural crest formation at that stage, interacting with distinct modifier genes. Further precise mechanisms of mandible development by modifiers await the identification of modifier genes.

Candidate genes and mechanism of interaction with *Otx2*

We have identified two modifiers; however, mapping resolution is not sufficiently fine to determine the single gene that is responsible for modification of the mandible phenotype. Nevertheless, from this survey, many genes that are believed to interact with *Otx2*, such as the *Emx1*, *Emx2*, *Otx1*, *Cripto* and *Lim1* genes (Matsuo et al., 1995; Suda et al., 1996; Suda et al., 1997; Suda et al., 2001; Acampora et al., 1997; Acampora et al., 1998; Kimura et al., 2001; Zoltewicz et al., 1999), were excluded as a genetic modifier of *Otx2* in craniofacial development. A potential *Otmf2* candidate is *Alx4*. The modifier, *Otmf2*, identified on proximal chromosome 2, was located near the *Alx4* gene, which is located at 65.0 cM of chromosome 2 (Table 1) (Qu et al., 1998). *Alx4* is a closely related member of the family of paired-related homeobox genes named as Prx family (Qu et al., 1998). The Prx family consists of *Prx1* (previously referred to as *Mhox*), *Prx2*, *Cart1*, *Alx3* and *Alx4*. All of these genes are expressed in the cranial mesenchyme of the mandibular arch (Zhao et al., 1994; Zhao et al., 1996; Qu et al., 1997; Berge et al., 1998a; Berge et al., 1998b; Lu et al., 1999). Indeed, *Alx4*$^{-/-}$ mutation in mouse and haploinsufficiency of human *ALX4* cause ossification defects of the skull (Qu et al., 1997; Wu et al., 2000; Wuyts et al., 2000; Mavrogiannis et al., 2001). Furthermore, the *Alx4* heterozygous mutant phenotype is subject to strain-specific genetic modifying loci in mouse (Forsthoefel, 1962; Forsthoefel, 1968; Qu et al., 1999). Moreover, in *Alx4*$^{-/-}$;

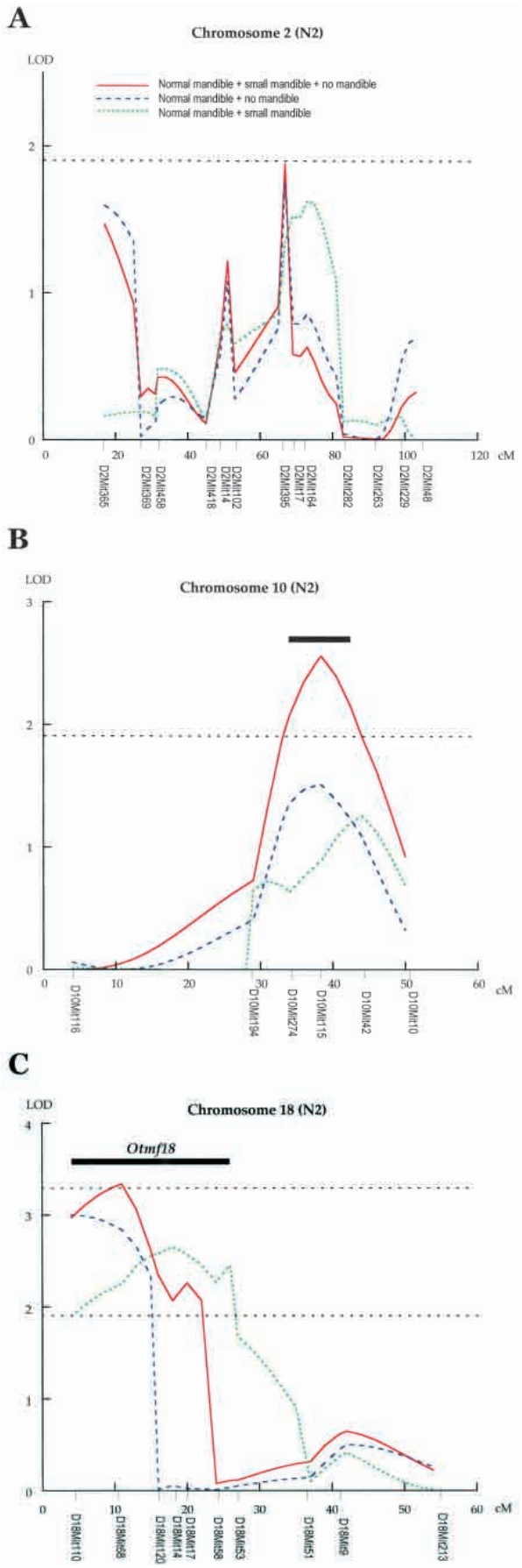

Fig. 6. Interval mapping of the modifier locus in the N2 analysis. Chromosome 2 (A), chromosome 10 (B) and chromosome 18 (C). The vertical and horizontal axes display LOD scores and the relative positions of the markers along the chromosomes from centromere (left) to telomere (right) in cM, respectively (determined by QTL cartographer). Two broken horizontal lines depict the LOD scores (1.9 and 3.3), which represent suggestive and significant linkages (Lander and Kruglyak, 1995), respectively. Red lines represent values obtained from mutant embryos displaying phenotypes of no mandible, small mandible and normal mandible. The most likely position for each locus, determined by its two LOD support intervals, is indicated by the black bar above the plot (B,C).

Cart1$^{-/-}$ double mutant mice, the distal region of the mandible was severely truncated (Qu et al., 1999). Indeed, based on our N2 analysis, no mandible and small mandible phenotypes were suggestively associated in chromosome 10, on which *Cart1* is located (Fig. 6B) (Zhao et al., 1994). Furthermore, expression of Prx family and *Otx2* genes was consistently co-localized in the mesenchyme of the mandibular arch (data not shown). These results support our hypothesis that *Alx4* may genetically interact with *Otx2* in skull development.

One possible interaction between *Otx2* and *Alx4* involves direct transactivation by these transcriptional factors of *Otx2* expression in cephalic mesenchyme. Consistent with this hypothesis, we previously found that DNA sequences, termed motif B (TAATTA), were highly conserved in *cis*-regulatory elements between mouse and pufferfish *Otx2*; additionally, these sequences were essential for *Otx2* expression in cephalic mesenchyme (Kimura et al., 1997). Motif B is a suitable candidate for the Prx family homeodomain binding sites (Cserjesi et al., 1992; Kimura et al., 1997; Cai, 1998; Qu et al., 1999). These Prx family proteins exhibit similar DNA-binding activity; moreover, these proteins also form heterodimers and activate transcription in a similar fashion (Qu et al., 1999). Furthermore, they are dose-sensitive genes and function in a partially redundant manner in mandible development (see above). These lines of evidences supports our hypothesis that Prx family transcription factors directly transactivate the level of *Otx2* expression in cephalic mesenchyme.

Differences in the amino acid sequences of these candidate genes between B6 and CBA could underlie subtle changes in the function of these proteins, affecting the mechanisms by which interaction occurs with downstream target genes or transcription factor complex. Alternatively, slight differences may exist between the B6 and CBA alleles in the temporal or spatial patterns and level of expression of these genes. Therefore, assessment of the aforementioned candidates as modifiers of *Otx2* will require high-resolution mapping studies employing congenic strains to obtain a more precise localization of these loci. Moreover, sequence comparisons and analysis of relative timing and expression levels in the B6 and CBA alleles are necessary.

Human agnathia-holoprosencephaly complex

Otocephaly, also referred to as agnathia-holoprosencephaly, is a lethal developmental field complex that is characterized by extreme hypoplasia or absence of the mandible, microstomia, aglossia and synotia (Bixler et al., 1985). Significant advances in the study of this disease have revealed the genetic and gene-environment bases of numerous common and rare craniofacial

Table 1. Summary of the genetic linkages

Locus	Chr	Position	LOD	Phenotypes	Origin	Candidates
Otmf2	2	77.0	3.93(N3)*	No mandible	B6 (recessive)	*Alx4*
Otmf18	18	11.1	3.33(N2)	Small mandible and no mandible	CBA (dominant)	

Listed are the provisional nomenclature of loci that significantly modify the otocephalic phenotypes of *Otx2* heterozygous mutnat mice (Locus), the chromosomes are on which they are located (Chr), the distance from the centromere in cM (Position), the maximum LOD scores (LOD), phenotypes that controlled by the modifier locus (Phenotypes), origin of regulatory alleles (Origin) and candidates genes (Candidates).
*A LOD score was generated by mutant mice exhibiting normal mandible and no mandible.

disorders (Winter, 1996; Wallis and Muenke, 2000). In humans, this condition can occur alone or in association with various other anomalies, including cyclopia, holoprosencephaly, cerebellar hypoplasia and other visceral anomalies (Opitz, 1980; Pauli et al., 1983). Moreover, the otocephalic phenotype has been observed in many animal species, including mouse (Juriloff et al., 1985), sheep (Willson, 1966; Smith, 1968), guinea pig (Wright and Wagner, 1934) and rabbit (Faller and Rossier, 1969). In mouse, the otocephaly (*oto*) mutation was identified in a screen for lethal mutations on chromosome 1 (Juriloff et al., 1985). This locus has been mapped between D1Mit79 and D1Mit134 in a region of synteny with human 2q35-36 (Zoltewicz et al., 1999). Strong linkage with the *oto* locus for mandible phenotypes of *Otx2* heterozygous mutants was not detected in the current investigation; however, further consomic or congenic analysis is required in order to finally determine whether the *oto* locus is associated with the *Otx2* mutant phenotype.

In addition to mandible abnormalities, most *Otx2*$^{+/-}$ mutant mice also displayed holoprosencephaly (Matsuo et al., 1995). In humans, holoprosencephaly is the most common developmental defect of the forebrain (Wallis and Muenke, 1999). It exhibits an incidence as high as 1 in 250 during early embryogenesis (Matsunaga and Shiota, 1977). The phenotype of holoprosencephaly is quite variable and proceeds in a continuous spectrum from severe manifestations with major brain and face anomalies to clinically normal individuals (Wallis and Muenke, 1999). Several distinct human genes for holoprosencephaly have been identified recently, including *SHH*, *ZIC2*, *SIX3*, *TGIF* and *HESX1* (Roessler et al., 1996; Brown et al., 1998; Wallis et al., 1999; Gripp et al., 2000; Dattani et al., 1998). Intrafamilial variability of clinical findings exists in kindreds carrying specific mutations in either *SHH* or *SIX3* (Nanni et al., 1999; Brown et al., 1998). Indeed, heterozygous carriers for mutations in either *SHH* or *SIX3* can appear phenotypically normal; by contrast, other heterozygous mutation carriers within the same family may be severely affected. This observation suggests the possibility of the occurrence of an undetermined second mutation in the same gene. Alternatively, other gene products or environmental factors may act in these pathways and alterations in the identical or additional genes or factors could be required for severe holoprosencephaly manifestations (Nanni et al., 1999; Brown et al., 1998). It is not known as to whether *OTX2* is involved in human holoprosencephaly. The modifier loci identified in this study might be suitable candidates for genetic causes of human craniofacial congenital diseases. Identification of human mutations of *OTX2* modifier genes and evaluation of interaction between these genes and environmental causes awaits molecular identification of these modifier genes.

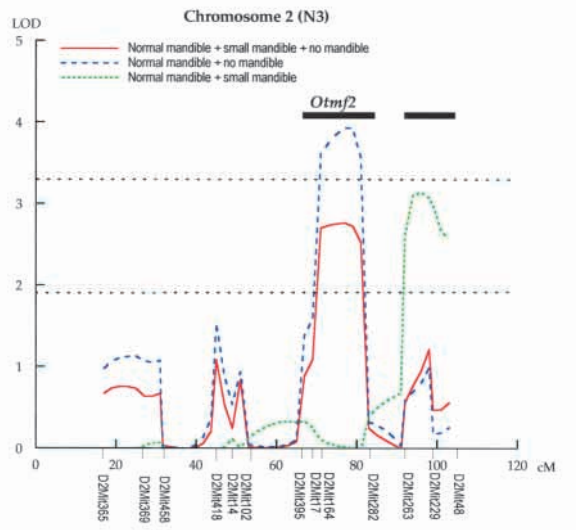

Fig. 7. Interval mapping of the modifier locus in the N3 analysis on chromosome 2. The vertical and horizontal axes show LOD scores and the relative positions of the markers along the chromosomes from centromere (left) to telomere (right) in cM, respectively (determined by QTL cartographer). Two broken horizontal lines indicate the LOD scores (1.9 and 3.3), which represent suggestive and significant linkages (Lander and Kruglyak, 1995), respectively. The most likely position for each locus, determined by its two LOD support intervals, are indicated by the black bars above the plot.

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