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Modifier locus for exencephaly in *Cecr2* mutant mice is syntenic to the 10q25.3 region associated with neural tube defects in humans

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Davidson CE, Li Q, Churchill GA, Osborne LR, McDermid HE. Modifier locus for exencephaly in *Cecr2* mutant mice is syntenic to the 10q25.3 region associated with neural tube defects in humans. *Physiol Genomics* 31: 244–251, 2007. First published July 10, 2007; doi:10.1152/physiolgenomics.00062.2007.—Neural tube defects (NTDs), the second most common birth defect in humans, are multifactorial with complex genetic and environmental causes, although the genetic factors are almost completely unknown. In mice, >100 single gene mutations cause NTDs; however, the penetrance in many of these single gene mutant lines is highly dependent on the genetic background. We previously reported that a homozygous *Cecr2* mutation on a BALB/c background causes exencephaly at a frequency of 74% compared with 0% on an FVB/N background. We now report that a major genetic modifier on chromosome 19, mapped using whole genome linkage analysis, increases the relative risk of exencephaly by 3.74 times in homozygous BALB embryos vs. BALB/FVB heterozygotes. Scanning electron microscopy revealed that the modifier does not affect the location of neural tube closure site 2, a known murine susceptibility factor for exencephaly. Crossing the *Sp* (*Splotch*) mutation in the Pax3 gene onto the FVB/N background for two generations indicated that this resistant strain also decreases the penetrance of spina bifida. The chromosome 19 modifier region corresponds to a linkage region on human chromosome 10q25.3 mapped in a whole genome scan of human NTD families. Since the FVB/N genetic background affects susceptibility to both exencephaly and spina bifida, the human homolog of the chromosome 19 modifier locus may be a better candidate for human NTD susceptibility factors than genes that when mutated actually cause NTDs in mice.

mouse model; whole genome linkage analysis; Pax3

NEURAL TUBE DEFECTS OCCUR when the neural tube, the precursor to the brain and spinal cord, fails to close (12). The type of NTD depends on which region of the neural tube is affected. Anencephaly and the murine equivalent, exencephaly, occur when the cranial region of the neural tube fails to close, whereas spina bifida is caused by a lack of closure of the posterior neuropore. NTDs occur in ~1/1,000 live births, making them the second most common birth defect in humans (12). NTDs are generally multifactorial with both environmental influences, such as folate level in the diet, and complex genetics factors. The existence of genetic factors is illustrated by a 2–5% recurrence in siblings, which is up to a 50-fold increase over the occurrence risk in the general population (15). Efforts to identify genetic susceptibility factors for NTDs in humans have met with little success. Many homologs of genes that cause NTDs in mice have been sequenced in human cases of NTDs, including *NCAM1* (13), *MTHFR* (41), *Noggin* (5), and *TERC* (6). Human linkage analysis has also focused on regions of mouse NTD genes, including some genes in the retinoic acid pathway (14). Several candidate genes from the folate pathway have also been examined in human NTD cases, because periconceptional folate can prevent NTDs (1), but only methylenetetrahydrofolate reductase (MTHFR) has been found to be associated with human NTDs. A thermolabile isoform of MTHFR, a protein that reduces 5,10-methylenetetrahydrofolate to 5-methylenetetrahydrofolate, has been associated with NTDs in some cases (41) but has not been implicated in other studies (7). This MTHFR variant is associated at most with 11–19% of human cases (8). A recent whole genome screen for human NTDs (33) found several suggestive linkage regions, the most significant of which were at chromosomes 7p22 (associated specifically with one large family) and 10q25.3. The identification of the specific susceptibility genes at these loci would be a major step toward understanding the genetic causes for NTDs.

In contrast to the multifactorial inheritance of human NTDs, there are >100 single gene mutations in mice that lead to NTDs (21). Some of the processes affected in these mouse models include apoptosis, neural patterning, proliferation defects in adjacent tissues such as the mesenchyme and ventral tail bud, convergent extension, and actin organization. A few mutant mouse lines are multifactorial and depend on more than one gene to cause the NTD phenotype, including the SELH/Bc strain, which is predicted to have ~3 genes that contribute to the exencephaly phenotype (23), and the *curly tail* strain, which depends on the *ct* gene and at least one other strong modifier, the *mct1* gene, for its phenotype (27). These models may be useful in studying human NTDs, which also show a multifactorial inheritance pattern. In addition, many mouse mutants, including the *Sp* (29), *ct* (31), *Ski* (11), and *Cecr2* (4) mutant lines, are highly susceptible to genetic background effects, and strain-dependent incomplete penetrance is very common, but little is known about such modifier loci. The etiology of human NTDs makes the identification of low penetrance or minor effect genes in mice even more compelling, as human NTDs likely result from a summation of gene variants with minor effects and environmental factors. Small effect genes are difficult to map, and the use of highly penetrant mutations to uncover their effects may allow for their identification.

A known genetic susceptibility factor for the development of exencephaly in mice involves the location of a specific neural tube closure site. In mice the neural tube closes at three discrete points and then "zippers up" from these points (20). Defects in the fusion of the neural folds in the midbrain region, the area of closure site 2 formation, lead to exencephaly. The locations

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of closure points 1 and 3, at the hindbrain/cervical boundary and at the most rostral point in the forebrain respectively, are uniformly located in all strains examined to date whereas significant variation has been seen in closure 2 location (24). This inherent variability in closure 2 location has been suggested to affect penetrance in relation to environmental and genetic causes of exencephaly. The SVW/Bc strain, which has a more rostral closure 2 site within the forebrain, is more susceptible to exencephaly induced by hyperthermia (18) and exposure to valproic acid (17) than other strains, including the LM/Bc strain, which closes at the forebrain/midbrain boundary. The penetrance in the Sp^{2H} mutant line was reduced by ~39% when it was crossed from a rostrally closing strain to a caudally closing strain (19). Therefore, a more caudal closure 2 location is thought to result in resistance to exencephaly.

In this study we have used linkage analysis to map modifier loci for exencephaly penetrance in gene trap-induced *Cecr2* mutants. Human CECR2 is known to form a complex with SNF2L in HEK-293 cells (4). This CERF (CECR2-containing remodeling factor) complex has ATP-dependent chromatin remodeling activity. *Cecr2* mutant mice develop exencephaly in a female predominant manner with a penetrance highly dependent on the genetic background (4). Homozygous *Cecr2* mutants on a BALB/c background develop exencephaly at a penetrance of 74%, but exencephaly was not observed in *Cecr2* homozygous mutants on the FVB/N background. By using embryos resulting from intercrossing the FVB/N and BALB/c strains and backcrossing to BALB/c, we mapped a strong modifier on chromosome 19 with an odds ratio of 3.74. The genetic background was found not to affect closure 2 location between the two strains, as closure 2 does not vary between FVB/N and BALB/c embryos. The FVB/N strain was also found to modify NTD penetrance in the *Sp* mutant line, which develops exencephaly, spina bifida, and defects in neural crest cell-derived structures. The effects of the FVB/N strain on spina bifida suggest that the modifiers act on neurulation in general rather than specifically on cranial neurulation. This is particularly interesting as the chromosome 19 region mapped in this study is syntenic to the chromosome 10q25.3 region mapped in the whole genome screen for human NTDs (33), suggesting that the same gene may underlie the susceptibility to NTDs in both species.

MATERIALS AND METHODS

Mice. All mice used in this study were housed at the Health Sciences Laboratory Animal Services facility (University of Alberta). All animal protocols were reviewed and approved by the Health Sciences Animal Policy and Welfare Committee of the University of Alberta. The mice were maintained on a 14-h light/10-h dark cycle at 22 ± 2°C and supplied with Laboratory Rodent Diet 5001, with the exception of breeding females, which were fed Mouse Diet 9F 5020 (LabDiet). *Cecr2* mutant mice were produced as previously described (4) and backcrossed for ≥10 generations onto FVB/N and BALB/c backgrounds. Heterozygous *shroom* (*shrm*) mice on a C57BL/6J background were purchased from the Jackson Laboratory (Bar Harbor, ME), and heterozygous *Sp* mice on a C57BL/6J background were provided by Dr. Alan Underhill, University of Alberta. *Sp* and *shrm* heterozygotes were backcrossed onto the FVB/N background for two generations, and the resulting N2 mice were intercrossed to produce *Sp* and *shrm* homozygous mutant embryos. To obtain timed embryos, dams were euthanized at different time points following the detection of a vaginal plug [considered embryonic day (E) 0.5]. Embryos were dissected in PBS. All embryos were scored for phenotype (presence of an NTD) and extraembryonic membranes were collected for DNA extraction.

Cecr2, Sp, and shrm genotyping. Genomic DNA was isolated from tail biopsies or extraembryonic membranes. Genotyping for the *Cecr2* (4) and *Sp* (28) mutations was by done by PCR as described previously. Genotyping for the *shrm* mutation was done using a multiplex PCR reaction containing two *shrm* intron 3-specific primers (ShrmFor2: 5′-ggccccagactcaccataatc-3′ and ShrmRevLD: 5′-tggctatcctctggctacaca-3′) and one GTROSA53 gene trap-specific primer (ShrmR1: 5′-gagtttgtcctcaaccgcgagc-3′) (Jeff Hildebrand, personal communication).

Microsatellite and single nucleotide polymorphism genotyping. FVB/N homozygous *Cecr2* mutants were crossed to BALB/c *Cecr2* heterozygotes, and the resulting F1 *Cecr2* homozygous nonpenetrant animals were backcrossed to *Cecr2* heterozygous BALB/c mice. All crosses were done reciprocally. Embryos were collected from this cross and scored for exencephaly. All exencephalic embryos were used for linkage analysis as well as an equal number of randomly selected nonexencephalic control embryos. The exencephalic samples consisted of 61 female and 33 male embryos, and the unaffected control samples consisted of 45 female and 49 male embryos due to a female predominance of exencephaly. DNA was extracted from 94 exencephalic and 94 unaffected *Cecr2* homozygous mutant embryos, and the 94 exencephalic embryos were genotyped using 112 microsatellites spread at ~20 cM throughout the genome. The unaffected samples were genotyped using 15 markers that differed from the expected segregation pattern in the exencephalic samples: D2Mit92, D2Mit249, D2Mit101, D2Mit395, D14Mit60, D15MIT270, D15Mit209, D17Mit51, D19Mit31, D19MIT63, D19Mit88, DXMit140, DXMit46, DXMit79, and DXMit121. Following this initial analysis, 16 additional single nucleotide polymorphisms (SNPs) spaced ~3 Mb apart on chromosome 19 were used to genotype all exencephalic and unaffected embryos to confirm a potential linkage region.

DNA extraction and genotyping were performed at The Centre for Applied Genomics, Toronto. DNA was extracted from embryo tissue by standard procedures followed by PCR amplification of individual microsatellite markers using fluorescently tagged primers (IDT, Coralville, IA). Cycles were performed as follows: 94°C for 3 min; 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 30 s; and a final extension of 72°C for 5 min. The labeled products were then multiplexed and analyzed on a BaseStation automated sequencer (MJ Research, Waltham, MA) to determine, for any given marker, how many alleles from either FVB/N or BALB/c had been inherited. SNP genotyping was performed using single-strand specific nuclease digestion of heteroduplexed PCR products (38). PCR-amplified products spanning each SNP were amplified from the target DNA and heteroduplexed as follows: 95°C for 10 min; ramp to 85°C at 2°/s, ramp to 25°C at 1°/s. Single strand-specific nuclease was added at a concentration of 2 units/reaction, followed by incubation at 45°C for 20 min. Products were analyzed on agarose gel.

Statistical analysis. Initial analysis was done by comparing allele frequencies of the expected genotypes (BALB/BALB from the exencephalic group and FVB/BALB from the unaffected group) and the unexpected genotypes (FVB/BALB from the exencephalic group and BALB/BALB from the unaffected group) with expected random segregation frequencies (1 BALB/BALB: 1 FVB/BALB) using the χ^2-test of goodness of fit. This analysis method takes into account the mirror image effect expected between the two groups: that the expected genotypes should be increased in the region of a modifier locus, while the unexpected genotypes should be decreased. A P value of ≤0.001 was considered significant, because only 50 independent tests are possible due to the size of the mouse genome, and an effective P value of 0.05 requires an observed P value of 0.001 (0.5/50) (36).

Further linkage analysis was done using R/qtl (10). A main scan of the data for single quantitative trait locus (QTL) regions was done to

find regions of significance (95% threshold) and of suggestive linkage (37% threshold). Logarithm of the odds ratio (LOD) scores representing significance thresholds of 37% and 95% were determined by 1,000 permutation tests. The 37% significance threshold is the standard level of suggestive linkage reported for a whole genome QTL analysis that uses a permutation based method to determine the significance thresholds (26). X chromosome significance thresholds were computed as described by Broman et al. (9). Sex was included as a covariate in all genome scans. Pair-scan analysis was also used to analyze the data, but no evidence for interacting modifier loci was found. An odds ratio for the genotypic effect on the binary outcome, exencephaly, was computed at the marker locus nearest to the QTL peak. The odds ratio provides an estimate of the relative risk of disease that is independent of the sampling strategy (3). Direct estimation of QTL effect, such as percentage of variance explained, will be biased by the case-control sampling strategy employed in this study.

Scanning electron microscopy. Embryos on the BALB/c background were collected at 6:00 h on E9 and FVB/N embryos were collected at 18:00 h on E8 (±1 h). Embryos were dissected in PBS and were scored for somite number and degree of closure 2 progression. Embryos for which the closure 2 fusion location could be determined were fixed for 1–3 days in 2.5% glutaraldehyde in PBS. Embryos were dehydrated through an ethanol series and dried using hexamethyldisilizane. Dried embryos were sputter coated with gold using a Hummer sputtering system (Anatech) and viewed using a Philips/FEI LaB6 Environmental Scanning Electron Microscope.

RESULTS

Collection of embryos for microsatellite and SNP genotyping. The penetrance of exencephaly in *Cecr2* homozygous mutant embryos was previously found to be 74% (35/47) on the BALB/c background and 0% (0/42) on the FVB/N background (4). The exencephaly penetrance from the FVB/BALB F1 cross was only 2.9% (1/35), similar to the FVB/N strain penetrance (Table 1). The FVB/N modifier(s) are therefore able to produce an almost complete resistance to exencephaly in a heterozygous state and can be considered dominant or semidominant. Embryos were collected from reciprocal crosses of *Cecr2* homozygous mutant FVB/BALB F1 mice to heterozygous *Cecr2* mutants on a BALB/c background. The penetrance in the backcross embryos was 28.1% (101/360). Of the exencephalic embryos, 66% are female and 33% are male due to the female predominance of exencephaly in *Cecr2* mutants. Since a single dominant modifier would be expected to give ~37% penetrance, these data suggest that a small number of major modifiers may be segregating independently.

Linkage analysis. Both the BALB/c and FVB/N *Cecr2* mutant lines used in this experiment were congenic at generation F10 or greater, with at least 99.9% of background alleles derived from the BALB/c and FVB/N genomes respectively. Embryos for whole genome linkage analysis were collected from a BALB/c × FVB/N cross followed by a backcross to BALB/c, with all parentals containing at least one copy of the *Cecr2* mutation. Using 112 microsatellites spread at ~20 cM throughout the genome, we genotyped 94 exencephalic embryo samples to correlate the exencephaly phenotype to region(s) of the genome that were homozygously derived from the susceptible BALB/c strain significantly more frequently than expected through random segregation. A further 94 nonexencephalic embryos were genotyped for the 15 microsatellites that differed from the expected segregation pattern in the exencephalic samples. These 15 markers were analyzed by combining the "expected genotypes" and the "unexpected genotypes" from both groups, which allows the mirror image effect between the two groups to be analyzed and corrects for any segregation distortion. The only significant region found, based on a level of significance of $P \leq 0.001$, was on chromosome 19 in the region of D19MIT63 and D19Mit88. The region on chromosome 2, with a peak at D2Mit249, had a P value of 0.00558 that may be suggestive of a weaker modifier. This region, however, showed an opposite effect to that expected, with the heterozygous genotype, rather than homozyogous BALB/c genotype, seen more frequently than expected in the exencephalic embryos. The exencephalic and nonexencephalic embryos were all tested for 16 additional SNPs on chromosome 19, which allowed an examination of markers spaced ~3 Mb apart along the entire chromosome. The linkage peak was found to be located at ~40.1 Mb, which corresponds to the rs3677115 marker (Fig. 1).

A further analysis of the microsatellite and SNP data was done using Rqtl 1.05–2 software (R version 2.4.0). This analysis confirmed the chromosome 19 linkage and the possible linkage on chromosome 2 that was found using the χ^2 statistical analysis described. The rs3677115 marker at ~40.1 Mb on chromosome 19 shows the strongest linkage and has an LOD score of 4.35. This result is above the 95% significance level, which is represented by an LOD score of 2.73 (Fig. 2). The relative risk for exencephaly in B/B vs. F/B individuals was estimated by an odds ratio to be 3.74. The risk of exencephaly is 3.74 times greater in individuals with the homozygous BALB/c genotype. The region located on chromosome 2 at ~48 cM or 103 Mb has an LOD score of 1.49, which is slightly above the suggestive threshold (Fig. 2). This analysis also located a suggestive linkage region on the X

Table 1. *Penetrance of exencephaly in Cecr2 mutant embryos on various genetic backgrounds*

Strain	BALB/c*			FVB/N*			FVB/BALB F1			FVB/BALB × BALB/c	
Genotype	m/m	m/+†	+/+	m/m	m/+	+/+	m/m	m/+	+/+	m/m	m/+
Exencephaly	35	0	0	0	0	0	1	1	0	101	1
Normal	12	110	70	42	99	59	34	38	3	259	338
Total, *n*	47	110	70	42	99	59	35	39	3	360	339
Penetrance, %	74.5	0	0	0	0	0	2.9	2.6	0	28.1	0.3

*Data previously reported (18). †m represents the $Cecr2^{Gt45Bic}$ allele; + represents the wild-type *Cecr2* allele. Note: Previously reported BALB/c and FVB/N data were collected after 5–6 generations of backcrosses of a mixed 129/BALB/FVB strain to the 2 pure strains (18). FVB/BALB F1 data were collected by intercrossing FVB/N and BALB/c *Cecr2* mutant mice that were at generation N7 or N8. FVB/BALB F1 *Cecr2* homozygous mutant mice were backcrossed to BALB/c *Cecr2* heterozygous mutant mice using mice that had been backcrossed to the pure strains for at least 10 generations. BALB/c heterozygotes were crossed to FVB/N homozygous Cecr2 mutant mice to collect FVB/BALB F1 data with the exception of a single heterozygous cross.

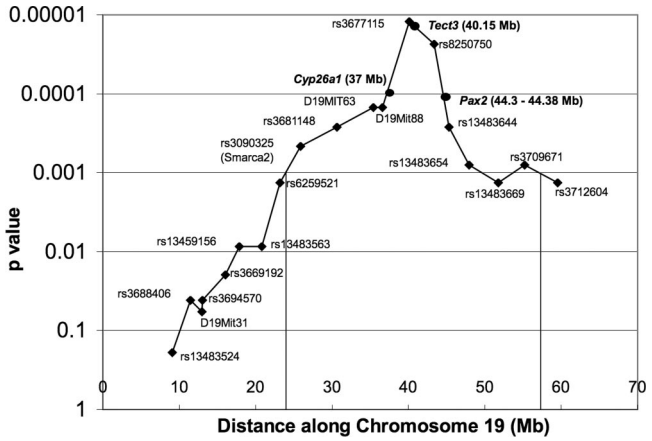

Fig. 1. Graphical representation of the *P* values of all the microsatellite and single nucleotide polymorphism markers on chromosome 19. The *P* values, which we determined by comparing the expected to the unexpected genotypes, were plotted against their location along the chromosome in Mb. The chromosomal region between the 2 lines defines the modifier region, in which the *P* value is >0.001. The location of candidate genes *Cyp26a1*, *Pax2,* and *Tect3* are shown.

chromosome at ~55 cM nearest to the DXMit79 marker with an LOD score of 3.04 (Fig. 2). Analysis of the X chromosome QTL requires that we subdivide the sample into groups by sex and direction of the backcross. In this case there were too few animals in some classes to draw a statistically supported conclusion regarding the nature of the QTL, though it appears to be male specific with a protective FVB allele.

Effect of FVB/N strain background on Sp and shrm. To explore whether the modifier(s) in the FVB/N strain that reduce the penetrance of exencephaly in the *Cecr2*^{Gt45Bic} mutant line can also reduce penetrance in other NTD mutant lines, two other mutations were bred onto predominantly FVB/N backgrounds. Mutants for the *Pax3* and *shrm* genes were chosen because these genes and their causative roles in neurulation are fairly well established and because they result in multiple types of NTDs. The *Sp* mutation in the *Pax3* gene arose spontaneously on a C57BL/6J background. The *Sp* homozygous mutant embryos develop two types of NTDs, exencephaly and spina bifida. The penetrance of spina bifida on a background that is ~75% FVB/N dropped significantly, from 100% (35/35) on C57BL/6 J to 73.1% (19/26) (χ^2 goodness-of-fit test, P = 0.001) (Table 2). Exencephaly also dropped from 25.7% (9/35) on C57BL/6 J to 11.5% (3/26) on ~75% FVB/N (χ^2 goodness-of-fit test, P = 0.168). The decrease in the exencephaly phenotype, although suggestive, is not statistically significant due to the small number of embryos that develop exencephaly on either background.

The *shroom*^{GtROSA53Sor} mutation is a gene-trap insertion that was originally characterized on a C57BL/6J background (22). The homozygous mutants develop four defects: exencephaly at 100% penetrance (93/93), facial clefting at 87% penetrance (68/78), spina bifida at 23% penetrance (21/93), and ventral closure defects at 12% penetrance (7/59) (Table 2). In our analysis of the C57BL/6J background, while exencephaly was seen at 100% penetrance, 87.5% (7/8) of homozygous mutants developed spina bifida, as opposed to the 23% observed by Hildebrand and Soriano (22). The exencephaly phenotype was also completely penetrant on the predominantly FVB/N background (23/23), as on the original C57BL/6J background. However, the penetrance of spina bifida on the predominantly FVB/N background was only 8.7% (2/23), which was significantly lower than the 87% penetrance on the C57BL/6J background seen in our analysis (χ^2 goodness-of-fit test, P = 0.000023). The variation between the spina bifida penetrance on the FVB/C57 mixed background (8.7%) and the C57BL/6J background as observed by Hildebrand and Soriano (23%), however, did not differ significantly (χ^2 goodness-of-fit test, P = 0.13) (22).

Closure 2 location. The location of closure 2 is known to have an effect on the susceptibility to exencephaly in mice. However, we found that closure 2 in both BALB/c and FVB/N strains occurs at the boundary between the forebrain and the midbrain, which is the most common closure 2 site in mice (Fig. 3). A difference was seen in absolute timing: BALB/c embryos undergo closure 2 at about embryonic day (E) 9.25, whereas FVB/N embryos undergo closure 2 earlier, at ~E8.75. However, in both BALB/c and FVB/N embryos, closure 2 occurred most frequently at the 9-somite stage but varied between 8 and 10 somites for both strains. Thus, developmental stage and the location of fusion at closure point 2 did not vary between BALB/c and FVB/N strains, and cannot account for the differences in NTD penetrance seen between these strains.

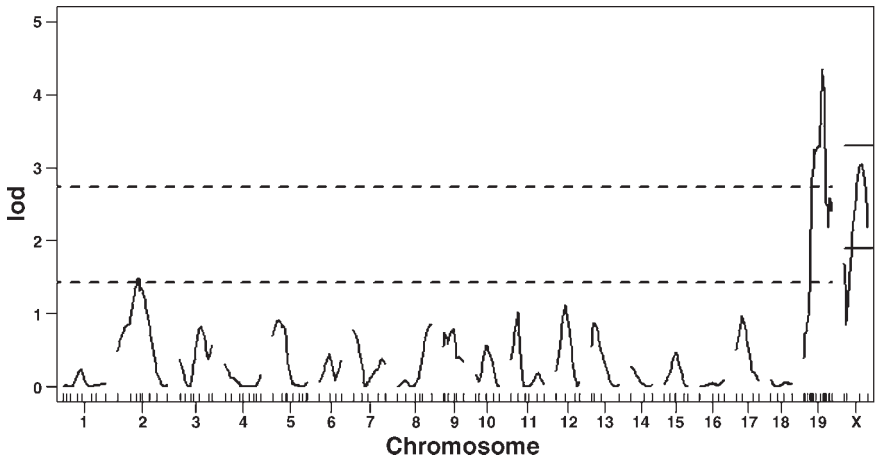

Fig. 2. Linkage analysis of excencephaly frequency. The genome scan plot shows the logarithm of the odds ratio (LOD) score for excencephaly frequency (*y*-axis) across genomic location (*x*-axis). The lower dotted line represents the suggestive threshold of 37% (LOD = 1.89), and the upper dotted line represents the 95% significance threshold (LOD = 2.73) for autosomes. Thresholds for the X chromosome were computed separately, as described by Broman et al. (9), and are shown as solid lines.

Table 2. *Penetrance of Splotch and shroom mutant lines on the original C57BL/6J backgrounds and an FVB/N N2 mixed background*

	Splotch						*shrm*						
Strain	FVB/N F2*			C57BL/6J			FVB/N F2			C57BL/6J			
Genotype	Sp/Sp	Sp/+†	+/+	Sp/Sp	Sp/+	+/+	−/−	−/+‡	+/+	−/−	−/+	+/+	−/−§
Exencephaly	3	0	0	9	0	0	23	0	0	8	1	0	100%
	11.5%	0%	0%	25.7%	0%	0%	100%	0%	0%	100%	8%	0%	
Spina bifida	19	1	0	35	1	0	2	0	0	7	0	0	23%
	73.1%	1.4%	0%	100%	1.6%	0%	8.7%	0%	0%	87.5%	0%	0%	
Normal	5	72	44	0	62	43	0	56	24	0	11	11	0%
	19.2%	98.6%	100%	0%	98.4%	100%	0%	100%	100%	0%	92%	100%	
Total	26	73	44	35	63	43	23	56	24	8	12	11	

*C57BL/6J heterozygous mutants were crossed to wild-type FVB/N mice and the heterozygous F1 progeny were backcrossed to FVB/N. Heterozygous progeny from this cross were intercrossed to generate the FVB F2 data, for which the genetic background is approximately 75% FVB/N. †Sp represents the *Sp* allele of *Pax3*; + represents the wild-type *Pax3* allele. ‡represents the $shrm^{GtRosa53}$ mutation and + represents the wild-type *shroom* allele. §Penetrance data reported for the shrm mutant line by Hildebrand and Soriano (22).

DISCUSSION

The difference in exencephaly penetrance caused by the *Cecr2* mutation on BALB/c and FVB/N genetic backgrounds suggests the presence of one or more modifier loci affecting susceptibility. By crossing the two mutant lines, we have shown that the resistance to exencephaly seen in FVB/N is a dominant effect. Using linkage analysis we have located a genomic region on mouse chromosome 19, at ~40.1 Mb, that contains one or more major modifiers of the *Cecr2* mutant phenotype. The remainder of the penetrance variation likely results from multiple minor effect gene variants, and linkage analysis did suggest the presence of other potential modifier loci. These minor modifiers are important, as together they are responsible for the majority of the penetrance variation, but the mapping of minor modifiers is difficult because they require much larger sample sizes to achieve significance. It is likely

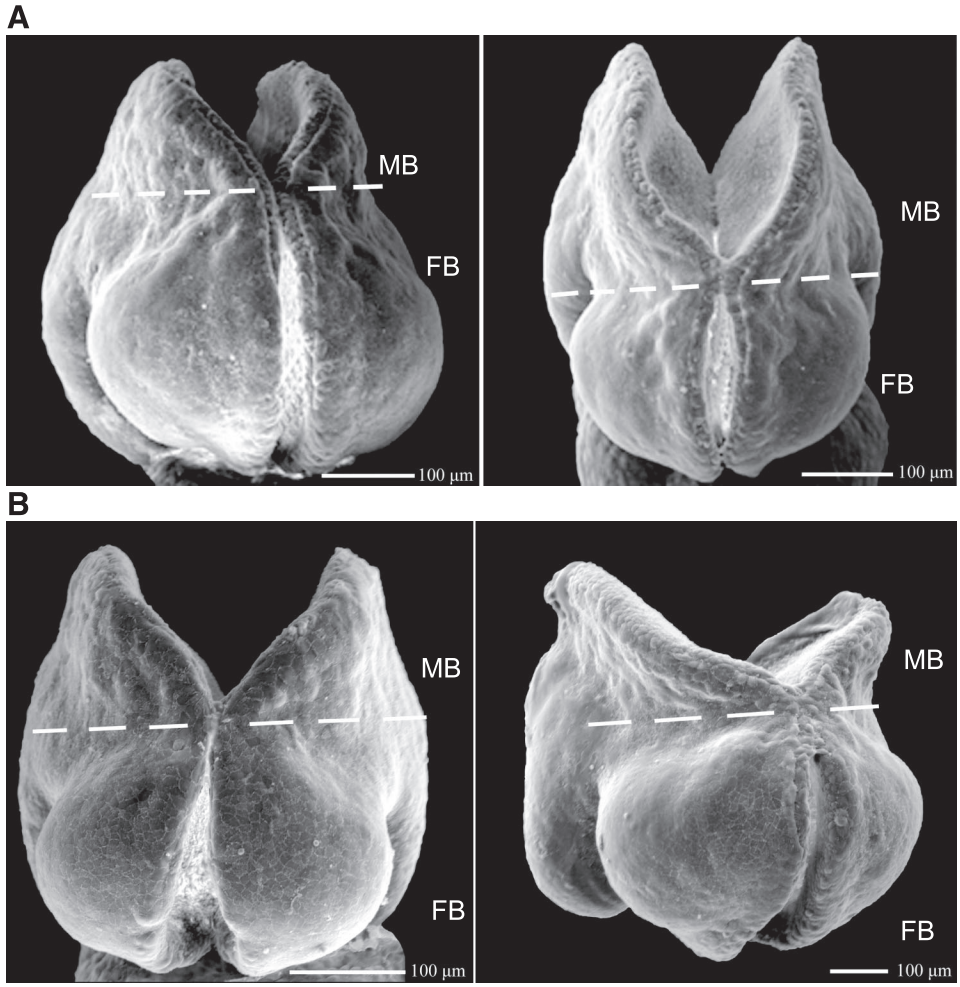

Fig. 3. Normal elevation and fusion of cranial neural folds at closure point 2 in BALB/c and FVB/N mouse embryos. Rostral scanning electron microscopic photographs of two embryonic day (E) 9.25 BALB/c mouse embryos at the 9-somite stage (*A*) and two E8.75 FVB/N mouse embryos at the 9 somite stage (*B*). The dotted line shows the boundary between the forebrain and the midbrain, at which closure 2 initiated in all of the BALB/c and FVB/N embryos collected. FB, forebrain region; MB, midbrain region.

that many differences between the two strains contribute to the penetrance variation, and there may be alleles in both strains that confer susceptibility or resistance. The suggestive QTL peaks found in our study were on chromosome 2, although in this genomic region BALB/c appears to be the resistant genotype since the heterozygous genotype is enriched in the exencephalic embryos, and on the X chromosome. These two suggestive linkage regions require further examination to be confirmed as modifier regions.

By moving other NTD-causing mutations onto a predominantly FVB/N background, we demonstrated that the FVB/N strain has a more general resistance effect on neurulation than just affecting *Cecr2* mutants. The FVB/N strain also affects both cranial and caudal NTDs, which would not be apparent in the Cecr2 mutant, which develops only exencephaly. The penetrance of spina bifida was clearly reduced in the *Sp* mutant embryos, from 100 to 73.1%, and the data suggest that the frequency of exencephaly is also reduced. Preliminary analysis of *shrm* mutant embryos on a predominantly FVB/N background also showed a decrease in the penetrance of spina bifida, although exencephaly did not appear to be affected. The reduction in penetrance of spina bifida and possibly exencephaly that occurs when the *Sp* and *shrm* mutations are crossed onto a partially FVB/N genetic background from a C57BL/6J background has not been shown to be caused by the chromosome 19 variation and may be due to genetic variants anywhere in the genome. It would be interesting to test both the FVB/N background, and the chromosome 19 region specifically, on other genetic and chemically induced NTDs. Neurulation defects in mice can be caused by a wide variety of genes, which likely affect different aspects of neural tube closure such as proliferation or apoptosis levels in the neuroepithelium or underlying mesenchyme, the bending of the neural tube at the hinge points or the fusion of the neural folds. The interactions and pathways of these genes are poorly understood, but locating a modifier that can act in a general manner to modify NTDs may be the most relevant to a large number of human NTDs.

Although most genes associated with NTDs in mice have not shown an association with human NTDs, the linkage peak on chromosome 19 corresponds to one of the two major linkage peaks mapped in a whole genome scan for association with human NTDs (33). The phenotypes in this human linkage study included various NTDs: mainly lumbosacral level myelomeningocele (spina bifida), but also including anencephaly and craniorachischisis (33). The fact the *Sp* and *shrm* mutations on an FVB/N background showed effects on penetrance of spina bifida rather than just exencephaly adds further evidence that the murine chromosome 19 modifier locus may be the same as the human 10q25.3 susceptibility locus.

We have shown that the variation in exencephaly penetrance between the BALB/c and FVB/N strains is not due to a variation in closure 2 location, a known susceptibility factor for the development of exencephaly. This indicates that the modifiers are acting on some other aspect of neurulation. The existence of closure 2 in humans is controversial. Some human studies have observed a closure point around the forebrain/midbrain boundary, and other groups have postulated its existence based on the types of NTDs that exist in the human population (30, 40). Others, however, have reported an absence of closure point 2 (32, 37). The only difference found between BALB/c and FVB/N was in the timing of closure 2 in relation to gestational age, as FVB/N initiates closure 2 fusion at ~E8.75 compared with ~E9.25 in BALB/c. However, since there was no difference in developmental stage (somite number) this may not be a meaningful difference.

The chromosome 19 modifier region contains two genes, *Cyp26a1* and *Pax2,* which are known to cause exencephaly when knocked out in mice, and therefore variants of these genes may predispose the BALB/c strain to exencephaly (Fig. 1). *Cyp26a1* is located at 37 Mb and encodes a retinoic acid-metabolizing enzyme. Two independent *Cyp26a1* knockout mice have been produced, which developed a number of defects, including exencephaly, spina bifida, caudal agenesis, and vertebral homeotic transformations, and die at midgestation (2, 35). *Pax2*, which is located at ~44 Mb, is a member of the paired box family of transcription factors. *Pax2* mutant mice develop exencephaly and/or midbrain/cerebellum patterning defects depending on the genetic background (16, 39).

The most promising candidate gene in this region, however, could be *Tect3*, which is located at 40.15 Mb (Fig. 1). *Tect3* is located directly under the peak of both the mouse and human linkage regions. An analysis of the Ensembl SNP database, which allows the examination of polymorphisms between many mouse strains, revealed a nonsynonymous SNP in the *Tect3* gene that differs between BALB/c and FVB/N. In the BALB/c strain, the *Tect3* gene encodes a threonine at position 186, which is a polar, hydrophilic, neutral amino acid, whereas the FVB/N strain encodes a methionine, which is a hydrophobic, neutral amino acid (NM_001039153). The threonine at this site is conserved in rat (XM_001053561), cow (XM_588288), crab-eating macaque (AB168156), dog (XM_845574), and human (BC040113) *Tect3* homologs. Another family member, *Tectonic* or *Tect1*, has recently been found to be involved in patterning of the neural tube via the Sonic hedgehog signaling pathway, which determines cell fate in the developing neural tube (34). *Tect3* is 58% similar to *Tectonic*, which is a secreted protein; however, *Tect3* is predicted to have carboxy-terminal transmembrane domains and is likely an integral membrane protein. The function of *Tect3* is currently unknown; however, if its function is similar to that of *Tectonic,* then a variant *Tect3* could affect susceptibility to NTDs.

The modifier gene candidate region under the chromosome 19 peak currently contains ~540 identified and predicted genes. We will therefore narrow the candidate region by moving overlapping segments of the FVB/N resistance region onto the susceptible BALB/c background. The resulting subinterval congenic lines will be tested for a drop in exencephaly penetrance in the homozygotes, indicating the presence of the FVB/N copy of the modifier locus in that fragment of chromosome 19. Once the region is narrowed we will be able to examine candidate genes for sequence or expression differences between the two strains. Identification of the chromosome 19 modifier gene will then allow analysis of the effects of this gene in the human NTD population. Thus this mouse model system may provide a means to locate and test human NTD candidate gene(s). Very few genes have been linked to human NTD susceptibility to date, including MTHFR (40) and VANGL1 (25), and mapping additional human susceptibility factors would be a significant step toward understanding human NTDs.

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