GENETIC ANALYSIS OF CEREBELLAR FOLIAL PATTERN IN CROSSES OF A/J AND C57BL/6J INBRED MICE

by

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Abstract

Modern ideas of the development of functional localization in the brain, which include pattern formation, were developed in the nineteenth and twentieth centuries. Cerebral sulci and cerebellar fissures are associated with functional localization. Previous genetic analysis of the folial pattern of the cerebellum has led to the mapping of four genes in crosses of C57BL/6J with DBA/2J and BALB/cByJ mice. *Cfp1*, on the distal end of Chromosome 4, and *Cfp4*, on the proximal end of Chromosome 5, have pattern-specific effects on cerebellar folial pattern. *Cfp2* and *Cfp3*, which were provisionally mapped to Chromosomes 5 and 7, respectively, have general effects on cerebellar fissure number.

In the present study, five or more genes with a probability of linkage of at least 90% were found to influence cerebellar folial pattern in AXB and BXA RI strains derived from crosses of A/J and B6 mice. *Cfp1* on the distal end of Chromosome 4 was associated with the marker *D4Mit16*. The A/J allele of *Cfp1* was found to increase the frequencies of the uvular and nodular sulci. Three loci, *Cfp5*, *Cfp6* and *Cfp9*, on Chromosomes 8, X and 13, respectively, were found to have pattern-specific effects on cerebellar folial pattern. In addition, *Cfp7* and *Cfp8* on Chromosomes 3 and 7, respectively, had general effects on cerebellar fissure number.

Cloned genes in the vicinity of our mapped genes that are developmentally regulated in the central nervous system were considered candidates. *Pou3f1*, *Pax7* and *Lmyc* were identified as candidate genes for *Cfp1*. *En2* and *Zic3* were identified as candidate genes for *Cfp4* and *Cfp6*, respectively. In addition to the four previously mapped genes, there were five newly mapped genes that were discovered. Therefore, the data shows that change in cerebellar folial pattern is useful in identifying genes involved in brain development.

Abbreviations

CNS	Central Nervous System
LIS	Lingular Sulcus
PRCF	Precentral Fissure
ICF	Intraculminate Fissure
DES	Declival Sulcus
PSF	Posterior Superior Fissure
UVS	Uvular Sulcus
NOS	Nodular Sulcus
SDP	Strain Distribution Pattern
A	An inbred mouse strain
B6	An inbred mouse strain
D2	An inbred mouse strain
C	An inbred mouse strain
RI	Recombinant Inbred
AXB	A recombinant inbred mouse strain originally derived by crossing an A/J female with a C57BL/6J male and then brother-sister mated for a minimum of 20 generations.
BXA	A recombinant inbred mouse strain originally derived by crossing a C57BL/6J female with an A/J male and then brother-sister mated for a minimum of 20 generations.

BXD	A recombinant inbred mouse strain originally derived by crossing a C57BL/6J female with a DBA/2J male and then brother-sister mated for a minimum of 20 generations.
CXB	A recombinant inbred mouse strain originally derived by crossing a BALB/cByJ female with a C57BL/6J male and then brother-sister mated for a minimum of 20 generations.
B6AF1	F1 hybrid derived by crossing a C57BL/6J female with an A/J male
AJB6F1	F1 hybrid derived by crossing an A/J female with a C57BL/6J male
DNA	Deoxyribonucleic acid
F1 (or F_1)	First filial generation
F2 (or F_2)	Second filial generation
ANOVA	Analysis of Variance

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INTRODUCTION

Functional Localization

The brain is subdivided into topographically distinct areas that display functional localization. It is thought that cerebral localization arises by differentiation of neurons into functionally distinct neuronal types, regionalization of neurons and topographic connections. Functional localization may be defined as the distribution of sensory and motor functions to specific topographic areas of the brain. An example of functional localization in humans is the anatomical representation of the body for sensory and motor functions in the cerebral cortex. Theories about the development of functional localization include pattern formation, positional information, combinatorial gene interactions and plasticity. We have approached this problem by examining the patterning of the mouse cerebellum during development.

Nineteenth Century

Evidence in support of the theory of functional localization could be traced back to the late 1700s when the neuroanatomist Franz Gall, proposed that human characteristics such as hope and combativeness were localized to discrete regions of the brain (Young, 1970). Maps of the brain were constructed based on his hypothesis which became known as phrenology. An important feature of these early maps was that the brain was bilaterally symmetrical; that is, the human traits were for the most part, represented on both hemispheres.

In the early 1860's electrophysiological studies were initiated by Eduard Hitzig and his collaborator Theodor Fritsch in which electrical stimulation of the cerebral cortex in dogs resulted in muscle movements (Young, 1970). In essence, they had discovered the motor strip in the precentral gyrus.

In the late 1800s Pierre Broca and Carl Wernicke were able to localize language of cerebral cortical areas by examining patients with aphasias (Young, 1970). By studying patients who could comprehend language but could not speak and subsequent postmortem examination of their brains, Broca was able to localize lesions on the left cerebral hemisphere which is responsible for speech (Broca's area). Similarly, Wernicke studied patients with another type of aphasia; he examined patients who could not comprehend language but were otherwise able to speak. He was able to localize lesions that were responsible for speech comprehension near the junction of the temporal and parietal lobes (Wernicke's area).

Twentieth Century

In the early twentieth century neurobiologists, who were inspired by the work of Wernicke, generated a number of maps of the cerebral cortex in several mammals based on cytoarchitectural differences among various parts of the cortex. They suggested that each cytoarchitectural region was a separate entity or "organ" with a specific function. Based on cytoarchitectonics, Brodmann distinguished fifty-two areas in the human cerebral cortex (Haymaker and Schiller, 1970). He also suggested that each area was functionally distinct.

Modern neurobiologists of the mid-twentieth century used more sophisticated techniques to study functional localization. For example, they have taken the theory of functional localization a step further by studying and mapping the somatosensory and visual cortices in more detail by recording with microelectrodes.

With the aid of microelectrodes, Vernon Mountcastle in the 1950s was able to record from individual neurons of the somatic sensory cortex in cats as well as monkeys (Mountcastle, 1957; Powell and Mountcastle, 1959). By doing so they were able to make a major contribution of the mapping of the body upon the somatic sensory cortex.

Hubel and Wiesel in the 1950s and 60s also used microelectrodes to record from individual neurons of the visual cortex in the macaque monkey (Hubel and Wiesel, 1968). They studied the processing of retinal information in the lateral geniculate body and the visual cortex and were able to associate primary visual cortex with the visual field. In addition, their studies also increased our understanding of the organizational properties of the visual cortex.

In the 1950s during neurosurgery for epilepsy, Wilder Penfield used microelectrodes to stimulate the areas of the cerebral cortex that were involved with language to ensure that the those areas were not injured during surgery (Penfield and Roberts, 1959). Thus, with the use of modern techniques Penfield confirmed the areas that Broca and Wernicke had previously discovered.

Somatotopic Maps and Sulci

Because of the work of early neuroanatomists, it has been demonstrated that sensory and motor areas of the cerebral cortex are organized somatotopically. This is true for mice, monkeys, humans as well as all other mammals. In addition there seems to be a correlation between functional localization and sulcation. For example, the central sulcus in humans divides the motor cortex in the precentral gyrus from the somatosensory cortex in the postcentral gyrus. Another example is the calcarine sulcus in the occipital lobe; it demarcates the upper and lower visual fields.

Cerebral Convolutions

In humans, cerebral convolutions appear in the developing fetus in the fifth month and continue until the first postnatal year (Richman et al., 1975). The primary convolutions such as the central and calcarine fissures are the first to appear. The secondary convolutions such as the parieto-occipital sulcus not only appear after the primary ones, but they are relatively constant in their location. The tertiary convolutions appear in the third trimester and are not fully distinguishable until months after birth (Rakic and Sidman, 1970).

Relatively little is known about the mechanics of cerebral convolutions. Richman et al. (1975) proposed a mechanical model based on the differential growth between the inner and outer cortical layers of two human congenital cerebral malformations, microgyria (small convolutions) and lissencephaly (smooth-brain).

Cerebellum

Neurogeneticists have focused their attention on the mouse cerebellum because the mouse cerebrum is smooth-surfaced or lissencephalic . Unlike the cerebrum, the cerebellum has folia which makes the pattern easily discernible. Like the cerebral cortex, the cerebellum also has somatotopic maps (Fulton and Dow, 1937). The spinocerebellum has, in fact, two somatotopic maps. One of those maps is located in the anterior lobe while the other is located in the posterior lobe. The two are inverted with respect to one another.

Cerebellar Fissures

The cerebellar cortex has extensive foldings in the form of folia, which are separated by fissures. Welker (1990) argued that each individual folium is an independent processing module. He also stated that cerebellar fissures are not cortical entities and that they merely separate adjacent folia and are useful as landmarks.

The nomenclature used to describe cerebellar fissures (see Fig. 1 and Fig. 2) generally follows that of Larsell (1952). Garretson and Neumann (1993) constructed a table comparing the nomenclature that was used by other investigators (Larsell, 1952; Inouye and Oda, 1980; Wahlsten and Andison, 1991).

In a study of cerebellar folial pattern variation in mice, Garretson and Neumann (1993) reported that cerebellar fissures were found in sixteen locations. Six fissures are considered constant features: the intracentral (ICNF), preculminate (PCF), primary (FPR), prepyramidal (PPF), secondary (FSEC), and the posterior lateral fissure (PLF). One specimen was described that lacked the nodular sulcus (NOS) (Garretson and

Neumann 1993). The constant fissures are the deepest fissures which also appear earliest in development (Mares and Lodin, 1970).

The frequency of the "variable fissures" is correlated with the visual depth of these fissures so that the deepest are more frequent. The remaining fissures have been considered "extra": the lingular sulcus (LIS), precentral fissure (PRCF), intraculminate fissure (ICF), declival sulcus (DES), posterior superior fissure (PSF), uvular sulcus (UVS) and NOS. The PSF and NOS were the most common fissures in crosses of B6 and D2, and B6 and C mice. The LIS, ICF and UVS were intermediary with frequencies of 30 - 55%. The PRCF and DES were uncommon or rare in these populations.

Genetics of Cerebellar Folial Pattern

The first genetic study on the cerebellar folial pattern of mice was done by Inouye and Oda (1980). They examined the folial pattern of 13 inbred mouse strains and one closed colony (non-inbred). (An inbred mouse strain is the result of brother-sister mating for a minimum of 20 generations. The process of inbreeding eventually results in inbred mice that are genetically homogeneous and homozygous at all loci.) They found little variation in cerebellar folial pattern between inbred colonies and non-inbred colonies, and they concluded that the variation in cerebellar folial pattern was under genetic control.

Neumann et al. (1990) examined the variation of a single fissure, the ICF, in crosses of C57BL/6J (B6) and DBA/2J (D2) mice because Inouye and Oda had

examined two closely related strains, C57BL/6NCrj (B6) and DBA/2NCrj (D2), and they had reported that the two strains had a similar folial pattern. They found that B6 animals lacked the PRCF, ICF and DES while D2 animals had a deep ICF and to a lesser extent, the DES.

There are several advantages for using B6 and D2 inbred strains. These animals could easily be bred for "classical" genetic studies. For example, B6 and D2 animals can be crossed to produce F1 and F2 hybrids. F1 hybrids can be backcrossed to either D2 or B6 to produce B1 or B2 backcross animals, respectively. The principle advantage, however, is the availability of BXD recombinant inbred (RI) strains with several marker loci typed for that set. BXD RI strains were derived by initially crossing B6 and D2 inbred mice to produce F1 hybrids; F1 hybrids were subsequently brother-sister mated for a minimum of 20 generations to produce the inbred strains.

By examining the variation in the frequency of a single cerebellar fissure, the ICF, in crosses of B6 and D2 inbred mice and BXD RI strains, Neumann et al. reported that a locus on the distal end of Chromosome 4, which they named *Cfp1*, was involved in variation of cerebellar fissure number. Specifically, B6D2 and D2B6 F1 hybrids, B1 and B2 backcrosses and 21 BXD RI strains were utilized. Unlike Inouye and Oda, who reported that all B6 animals lacked an ICF, Neumann et al. found that the ICF was present in about 8% of the B6 population. The absence of the ICF was an incompletely penetrant trait. In addition, they found that all D2 animals and 60% of the F1 hybrids had the fissure. The data was consistent with a single locus mode of

inheritance (i.e., 1:2:1 ratio), and they concluded that the presence or absence of the ICF between the B6 and D2 strains was largely due to allelic differences at a single locus on the distal end of Chromosome 4, which they named *Cfp1*.

In a study of CXB RI strains, which are derived from crosses of BALB/cByJ (C) and B6 inbred mice, Wahlsten and Andison (1991) reported variation in the number of cerebellar fissures and brain size. They also found a positive correlation between cerebellar size, and frequency and depth of extra sulci. The declival sulcus (DES) was consistent with a single locus mode of inheritance, but the proposed locus, *dsc*, could not be mapped due to the existence of only seven RI strains in the CXB RI set.

Garretson and Neumann (1993) studied F2 classical crosses of BALB/cByJ (C) and B6 mice, and suggested that *dsc* is *Cfp1*. The purpose of their study was to determine whether allelic differences at the *Cfp1* locus, which is linked to the brown locus (*b*) on Chromosome 4, influences cerebellar fissure number. They found that the variation of one fissure, the DES, was associated with the *b* locus. The DES was also found to be positively correlated with the PRCF and ICF. Linkage analysis indicated that the recombination between *b* and *Cfp1* was not significantly different than the recombination between *b* and *dsc*, the locus proposed by Wahlsten and Andison. They concluded that *dsc* and *Cfp1* are the same locus.

Cooper et al. (1991) mated four inbred strains (C57BL/10J, DBA/2J, BALB/cJ, and SJL/J) of mice in a diallel cross and examined the cerebellum of the offspring for the presence or absence of the ICF. (A diallel cross is one approach to quantitative

genetics; it mostly addresses dominance issues.) The diallel cross was generated by crossing the four inbred strains in all possible ways to produce sixteen genetic groups. They reported that the DBA/2J strain expressed the ICF while the SJL/J strain was mostly absent for the ICF while the C57BL/10J and BALB/cJ strains expressed variability with regards to the ICF. They reported that analysis of the diallel cross showed significant additive (increase) and dominance effects for the presence of the ICF. They also reported evidence of environmental effects including a maternal effect in crosses involving the SJL/J strain and perhaps nutritional factors or parental behaviours accounting for the variance between and within 12 of the 16 groups.

We (Neumann et al., 1993) reported that at least three loci were involved in variation of cerebellar fissure number in crosses of B6 and D2. This study was significant because we were able to conclude that cerebellar folial pattern variation had a multifactorial (genetic and environmental) mode of inheritance. We were also able to conclude that in addition to the previously mapped locus, *Cfp1* on the distal end of Chromosome 4, there were at least two other loci, *Cfp2* and *Cfp3*, that were involved in folial pattern. *Cfp2* and *Cfp3* were provisionally mapped to Chromosomes 5 and 7, respectively; the probability of linkage in each of these cases was between 50 and 75%, respectively. We also reported that *Cfp2* may influence fissure number through genetic variation in thyroid hormone function.

Cerebellar fissure formation is determined by two classes of genes (Neumann et al., ms. in preparation). The first class of genes has a general effect on the number of fissures in the cerebellum, either increasing or decreasing overall fissure number.

In crosses of B6 and D2, variation in fissure number is due to two genes, *Cfp2* and *Cfp3* that act through thyroid hormone. $Cfp2^{b}$ and $Cfp3^{b}$, the B6 derived alleles, have a general effect on the shape of the cerebellum by increasing fissure number. The second class of genes has fissure or pattern-specific effects. *Cfp1* belongs to this class. In crosses of B6 and D2 mice, *Cfp1* had pattern-specific effects on cerebellar folial pattern by decreasing the LIS and increasing the frequencies of the PSF, UVS and NOS. In addition, in crosses of B6 and BALB/cByJ, $Cfp1^{c}$ increased the frequencies of the ICF, DES, and UVS. Further studies in crosses of B6 and D2 strains by Neumann et al. (ms. in preparation) showed that a locus on mouse Chromosome 5, which is tightly linked to *Mpmv13*, and we named *Cfp4*, had fissure-specific effects on the frequency of the UVS. Specifically, *Cfp4* acted as a decreaser of the UVS.

Homeotic Genes

Joyner et al. (1991) reported that a targeted deletion of the *En2* homeobox containing-gene ($En2^{hd}$) in transgenic mice resulted in normal animals except that cerebellar size was reduced along with a morphological alteration of the posterior lobe of the cerebellum. More specifically, observation of published photomicrographs reveals an altered folial pattern in the posterior cerebellar lobe effecting the PSF, UVS and perhaps the NOS. Millen et al. (1994) produced transgenic mice in which the first exon of *En2* was deleted ($En2^{ntd}$). They reported that mice in the second knockout experiment were phenotypically indistinguishable from the first knockout. We have

proposed that *En2* is a candidate locus for *Cfp4* because *En2* is located in the same region of Chromosome 5 as *Cfp4* and each gene affects the same region of the cerebellum (Neumann, et al., ms. in prep.). In addition, *Cfp4* decreased the frequency of the UVS, which is in the posterior lobe of the cerebellum and the primary site of *En2* knockout activity.

The homeobox was originally found with the aid of recombinant DNA technology in two homeotic gene complexes in Drosophila (McGinnis et al., 1984). The homeobox is a 180 base pair DNA sequence that encodes a 60 amino acid sequence called the homeodomain. The homeobox has been highly conserved on the evolutionary time scale; it is present in a host of species including mice and humans. Although the homeobox genes were initially discovered in homeotic gene clusters, they are also present apart from these clusters in other genes such as *En2*. Homeobox genes are part of a regulatory cascade of development. Their expression is regulated by other genes, and they, in turn, encode the homeodomain transcription factors that bind to DNA and act as regulators of other genes. In Drosophila the homeodomain proteins are important in specifying the identity of developing embryonic segments.

The homeotic genes are one class of genes where the homeobox is present. The *Hox* genes are homeotic genes, and they are expressed in the developing nervous system. In mice they are thought to play a role in the differentiation of the various regions of the developing anterior-posterior axis. Le Mouellic et al. (1992) used gene targeting to knockout the *Hoxc8* gene. They found that the anterior vertebra of the transgenic mice were transformed to resemble posterior vertebra. In another study by

Krumlauf (1993) in which mouse embryos were treated with exogenous retinoic acid, anterior regions of the hindbrain had been transformed to resemble posterior segments. The r2/3 rhombomeres, which normally do not express *Hoxb1*, expressed it when the embryos were treated with retinoic acid. Although the mechanism underlying the expression patterns of the *Hox* genes is not known, there is enough evidence that indicates that the *Hox* genes are involved in the patterning of the developing nervous system.

Paired-box Genes

Another group of transcription factors that effect the developing CNS is a group of genes known as the paired-box genes (Pax). The Pax genes were initially identified as a conserved sequence in Drosophila (Noll 1993). To date a total of nine Pax genes have been identified (Gruss and Walther, 1992).

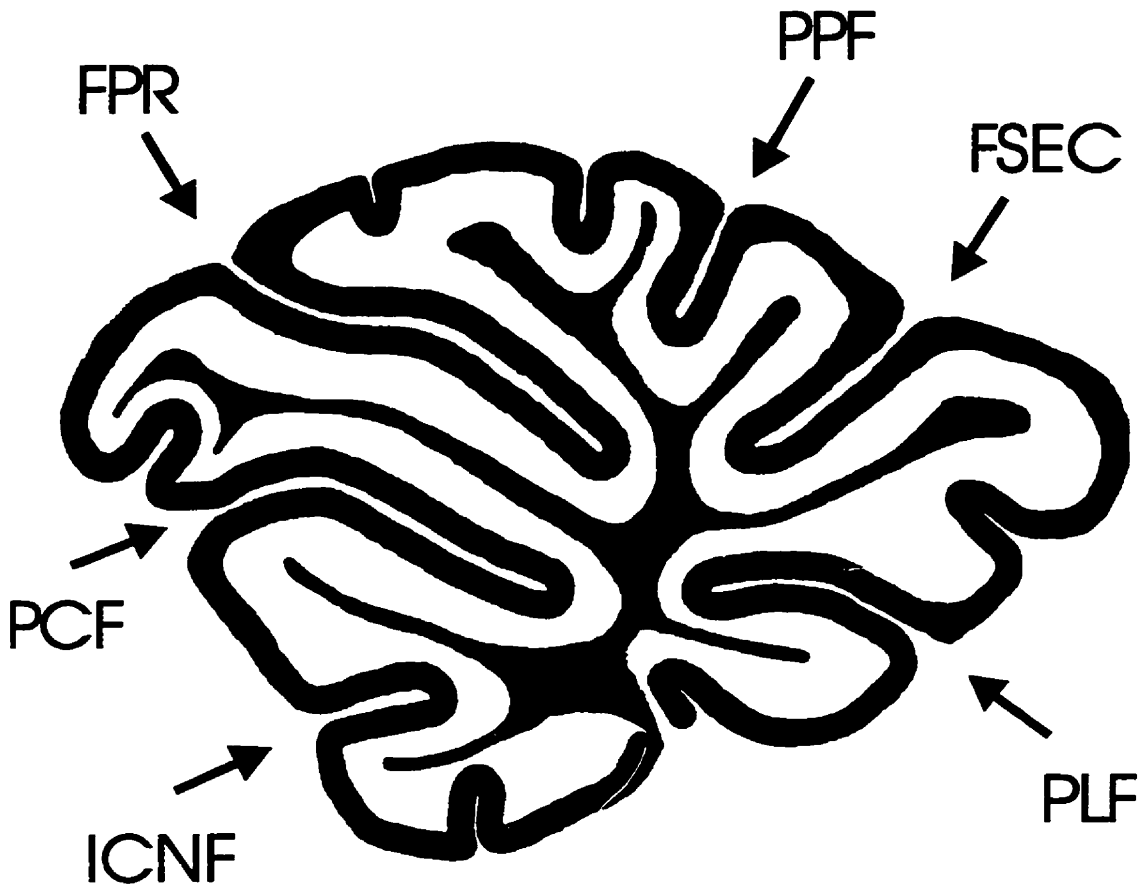
One of the Pax genes that effects the developing cerebellum is *Pax5*. By disrupting *Pax5* in embryonic stem cells, Urbanek et al., (1994) compared the phenotypic effects of the mutants to that of normal animals. They found that *Pax5* was not important in embryonic development and spermatogenesis; however, there was retarded growth, immune deficiencies, and an altered foliation of the anterior cerebellum.

Thus far, our studies regarding cerebellar folial patterning have yielded four mapped genes, though none of these genes have been cloned and characterized at the molecular level. *En2* and *Pax5* represent cloned sequences which have been shown to

act on the developing cerebellum. These genes may provide insight into the functions of some of the genes that we have mapped but not yet cloned.

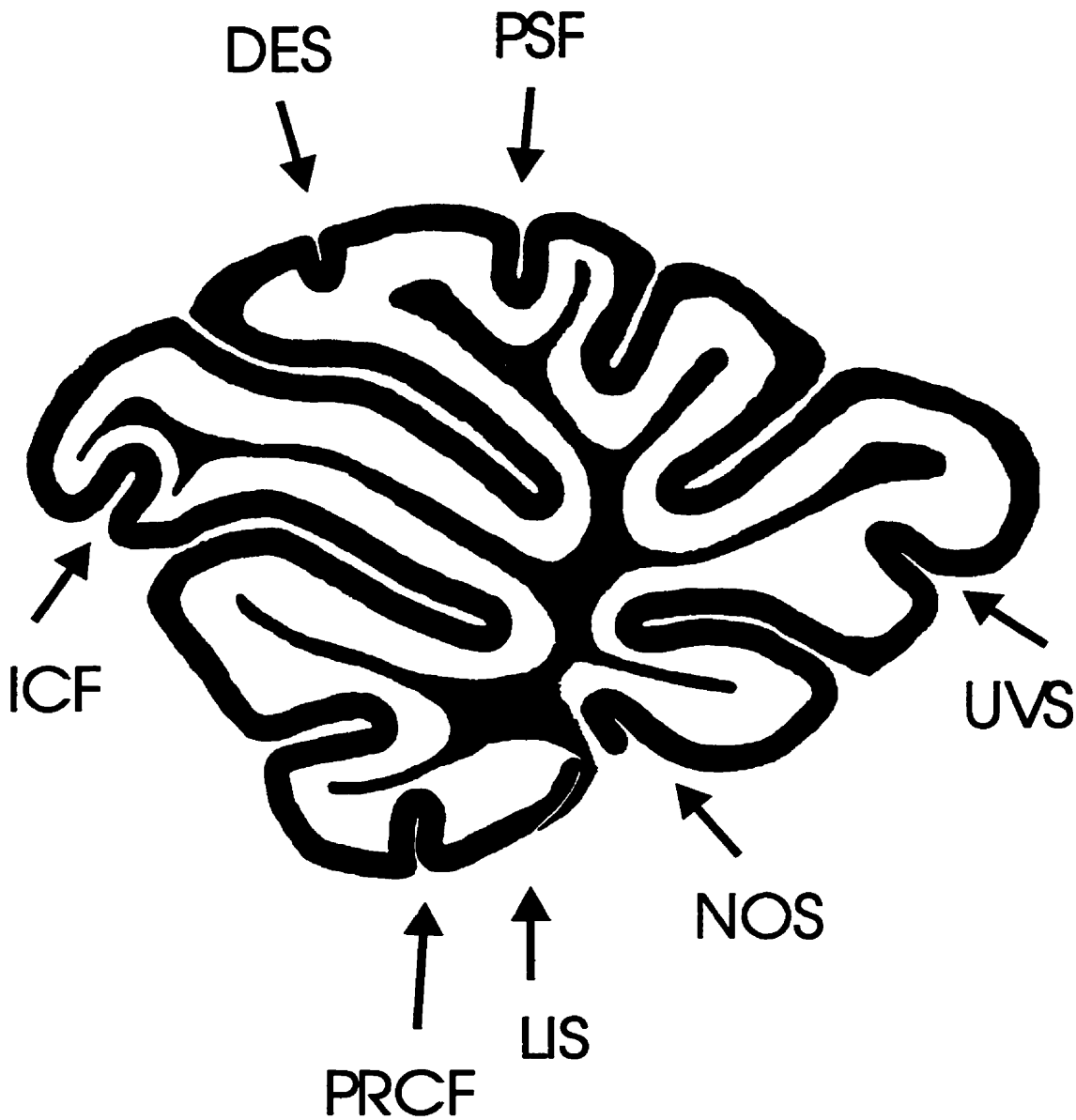
Here, we report a genetic analysis of fissure number and frequency of specific fissures in crosses of B6 and A/J inbred mice. The genetic analysis includes segregation analyses to determine the mode of inheritance, and linkage tests to map pertinent genes.

Figure 1. Schema Depicting Constant Fissures in Crosses of A/J and C57BL/6J Inbred Mice.



Note: ICNF = Intracentral fissure; PCF = Preculminate fissure; FPR = Fissura Prima; PPF = Prepyramidal fissure; FSEC = Fissura Secunda; PLF = Posterolateral fissure.

Figure 2. Schema Depicting Variable Fissures in Crosses of A/J and C57BL/6J Inbred Mice.



Note: LIS = Lingular sulcus; PRCF = Precentral fissure; ICF = Intraculminate fissure; DES = Declival sulcus; PSF = Posterior superior fissure; UVS = Uvular sulcus; NOS = Nodular sulcus.

MATERIALS AND METHODS

Animals

Mice used in this study were obtained from The Jackson Laboratory (Bar Harbor, ME). These included A/J and C57BL/6J (B6) inbred mice, AXB and BXA recombinant inbred (RI) mice and reciprocal F1 hybrids (AB6F1 and B6AF1) produced by crossing A/J and B6 animals. Adult mice were anesthetized with ether and perfused intracardially with 3.7% formaldehyde at The Jackson Laboratory. The heads were sent to Dalhousie University (Halifax, NS).

Histology

Perfused heads were sent to Dalhousie University (Halifax, NS), where the cerebella were dissected. A total of 375 mice were used in this study. Each cerebellum was sagitally sectioned on a vibratome, and ten 100μm serial sections spanning the midline were collected and placed on a glass slide. The sections were allowed to air dry overnight before dehydrating in alcohol solutions. The specimens were immersed in increasing strengths of 70%, 95% and 100% ethanol solutions for five minutes and then placed in xylene for 15-20 minutes. The specimens were then rehydrated in decreasing strengths of 100%, 95% and 70% ethanol solutions for five minutes, respectively, before immersing in 0.1% cresyl violet (Chroma-Gesellschaft Schmid GmbH & Co., Stuttgart-Unterturkheim, Germany). After staining for 1 minute, the specimens were dehydrated in increasing concentrations of ethanol, as

previously described, and then placed in xylene. The specimens were cover slipped with PRO-TEXX™ mounting medium and examined for the presence or absence of specific cerebellar fissures. Five mice cerebella were excluded due to damage during preparation. All mice were evaluated for the absence or presence of seven variable cerebellar fissures (see introduction).

Experimental Design

The sample size for each of the populations was determined empirically from previous studies (Neumann et al., 1993; Garretson and Neumann, 1993; Neumann et al., in prep.). Based on a previous study (Neumann et al., 1993), a sample size of 10 animals (half from each sex) from each set was deemed sufficient to demonstrate differences in our trait of interest. These included the following sets of animals: 21 A/J and 35 B6 inbred mice, 11 AB6F1 and 13 B6AF1 reciprocal hybrids, and 10 animals from each of the available RI strains (17 AXB and 14 BXA RI strains), except for the BXA-9 RI strain of which there were only 5 animals available. One of these, AXB-25, was not included in segregation and linkage analyses because it was found to be genetically contaminated (Marshall et al., 1992). A total of 375 histological specimens was utilized in this study; this does not include five specimens that were discarded and replaced, nor does it include the AXB-25 RI strain set. Each animal was scored for fissure number and the presence of specific fissures. The results were compiled in tables containing descriptive statistics.

Segregation Analyses

The number of fissures (fissure number) was determined for each of the animals of the parental strains and the reciprocal F1 hybrids. A comparison was made of the distribution of fissure numbers for the parental strains. A/J mice were compared to B6 mice to determine whether there was a strain difference, and males were compared to females to determine whether there was a sex difference. The reciprocal F1 hybrids were similarly treated. The distribution of fissure numbers for AB6F1 and B6AJF1 mice were compared to each other to determine whether there was evidence of parental effects or sex-linkage, and the distribution of fissure numbers of the F1 males was compared to that of the F1 females to determine whether there was a sex difference. Hypothetical models were proposed that might possibly account for fissure number variation that was observed in the parentals and F1 hybrids. Hypothetical models were also proposed for each of the most variable fissures.

The segregation analyses of the RI strains consisted of descriptive statistics and analysis of variance (ANOVA). Initially, the distribution and range of mean fissure numbers for the 31 RI strains were examined. The range of mean fissure numbers of the RI strains was compared to that of the parentals. ANOVA was then used to compare the distribution of fissure number among the 295 RI mice and to determine which factor(s) accounted for most of the variation. In the first ANOVA, the fissure numbers were partitioned according to sex to determine the proportion of the variance that could be attributed to sex; this was testing the possibility of a sex-influenced trait. In the second ANOVA, the fissure numbers were partitioned according to RI set to

determine the percentage of the variance that could be attributed to set difference; this was testing parental effects for the original cross such as sex-linked genes, mitochondrial genes as well as environmental effects such as post-natal rearing or uterine environment. In the final ANOVA, the fissure numbers were partitioned according to RI strains to determine the percentage of the variance that could be attributed to RI strains.

The number of effective loci ($\hat{L}$) was estimated by a^2/V_G where a is the additive genetic component of the means (i.e., half the difference between the highest and lowest means of the RI strains) and V_G is the genetic component of the RI strain variance (Wright, 1952; Taylor, 1976). This calculation was a conservative estimate; therefore, the actual number of loci is probably greater than what this equation predicts.

Linkage Analyses

Linkage analyses consisted of tests of association between our traits of interest (fissure number and fissure-specific frequencies) and the strain distribution patterns (SDPs) of multiple marker loci contained in version 2.5 (March 1994) of the Map Manager database (Manly and Elliot, 1991). Student *t*-tests of the difference between the number of cerebellar fissures in AXB and BXA RI strains with the A/J derived allele, n^a, and that of those with the B6 derived allele, n^b, were performed for a set of 269 marker loci using published strain distribution patterns in an attempt to detect linkage. Significance in tests of association indicate lack of independence or, in

genetic terms, suggest linkage. Significance levels were interpreted using criteria previously established (Neumann, 1992). (See Appendix 2 for an example of the analysis described above.)

In addition to the exploratory analysis with all marker loci, there was also a confirmatory test of allelic substitution between the A/J derived allele, n^a, and the B6 derived allele, n^b, on the distal end of Chromosome 4 where *Cfp1* had been previously mapped (Neumann et. al., 1990). Allelic substitutions had an effect in previous crosses of C57BL/6J x DBA/2J and C57BL/6J x BALB/c (Neumann et al., 1993; Garretson and Neumann, 1993). The statistical tests were similar to the ones described above with the exception that a lower significance level was used (p=0.05) because *Cfp1* had been previously mapped to this chromosomal region (Neumann, 1992).

RESULTS

Descriptive Statistics

Four tables containing descriptive statistics are presented in Tables 1-4. Tables 1 and 2 contain the descriptive statistics pertaining to the A/J, B6 and reciprocal F1 hybrids. Tables 3 and 4 contain the descriptive statistics pertaining to the AXB and BXA RI strains. In addition, the absolute frequencies of the cerebellar fissures are presented in Tables 1 and 3, and the relative frequencies of the seven variable fissures are presented in Tables 2 and 4.

Thus the strain, sex, sample size, fissure number, mean fissure number and standard deviation of the A/J, B6 and the F1 hybrids are presented in Table 1. The strain, sex, sample size and relative frequencies of the seven variable fissures of the A/J, B6 the F1 hybrids are presented in Table 2. The RI strain, sample size, fissure number and mean fissure number for each of the RI strains are presented in Table 3. The RI strain, sample size and relative frequencies of the seven variable fissures are presented in Table 4.

Segregation Analyses

Analysis of Parental Strains and F1 Hybrids

The mean fissure numbers for the variable fissures in B6 and A/J inbred mice were 8.7 (s.d.=0.75) and 8.2 (s.d.=0.40), respectively; this difference was statistically

significant (χ^2=15.04, 3 d.f., p=0.002). The mean cerebellar fissure number of B6 animals raised at The Jackson Laboratory was not significantly different from that which we reported in B6 animals raised at The Children's Hospital (Boston) (Neumann et al., 1993).

The mean number of fissures for the reciprocal F1 hybrids was 9.4 (s.d.=0.65). Since the F1 mean was greater than both parental means, this was suggestive of overdominance. Male B6AF1 hybrids had a greater mean fissure number than the other three F1 hybrid populations suggesting sex-linkage; however, this difference was not statistically significant (χ^2=16.45, 6 d.f., p=.01).

Thirteen cerebellar fissures were present at various frequencies in the mice examined. Six fissures were present in all mice included in this study: the ICNF, PCF, FPR, PPF, FSEC and PLF (see Fig. 1). The frequencies of the seven variable fissures in the parentals and F1 hybrids (see Fig. 2) are presented in Table 2. Among the "extra fissures", three of these were uncommon and provide little material for the study of heritable variation: the precentral fissure (PRCF), the declival sulcus (DES), the intraculminate fissure (ICF). The posterior superior fissure (PSF) was excluded because it was present in 95% of the animals; thus, it also provides little material for genetic analysis.

Three of the inconstant fissures and fissure number present more promising variation for genetic analysis. As previously stated, since the mean fissure number of the F1 hybrids was greater than either of the parental means, this suggested overdominance. Each of the inconstant fissures was incompletely penetrant. The LIS

was present in 69% of the B6 animals but absent in A/J mice and was present in only 8% of the F1 hybrids; therefore, the absence of the LIS in A/J mice was partially dominant. The UVS was present in all A/J and F1 hybrids but absent in 89% of B6 mice; therefore, the presence of the UVS was a dominant trait. The NOS was present in 89% of B6 mice and 19% of A/J mice but was present in all of the F1 hybrids; therefore, the presence of the NOS was a dominant trait.

The simplest explanation for the observed frequencies of fissure numbers in the parentals and F1 hybrids would be a single autosomal gene with two alleles that are overdominant in the heterozygous F1 hybrids. Allelic differences at such a locus on an autosomal chromosome could explain the observed frequencies. Because B6 mice had a greater fissure number than A/J mice, the B6 allele would act as an increaser of fissure number relative to the A/J allele. When both alleles are present together in the heterozygous F1 hybrids, the increased fissure number in the F1 hybrids relative to the parentals could be attributed to overdominance. Hence, because segregating populations (F2s or backcross animals) were not available for this study, the proposed model consisting of a single, autosomal gene with two alleles cannot be ruled out nor can a multifactorial model consisting of multiple genes.

Because sex-linkage was previously suspected, an alternative model to explain the frequency of fissure numbers in the parentals and F1 hybrids would be a single X-linked gene. Allelic differences at a hypothetical locus on the X Chromosome between the A/J allele and the B6 allele could explain the increased fissure number in the B6 mice relative to the A/J mice where the B6 derived allele acts as an increaser

of fissure number relative to the A/J derived allele. This was observed in the parental strains when B6 mice were compared to A/J mice. However, if this model were correct, then one would predict a phenotypic value of approximately 8.9 in the B6AF1 males and a phenotypic value of approximately 8.3 in the AB6F1 males because B6AF1 males have an X Chromosome derived from B6 background while AB6F1 males have an X Chromosome derived from the A/J background. The observed phenotypic values were 10.1 and 9.2 for the B6AF1 and AB6F1 males, respectively. This difference was statistically significant (χ^2=32.08, 9 d.f., p=0.00019), and this model is improbable. However, a multifactorial model consisting of an X-linked gene that affects fissure number is certainly possible. Such a model would predict that the BXA RI strains would have a greater fissure number relative to the AXB RI strains.

Similarly, single autosomal gene models cannot be ruled out for the presence or absence of each of the following fissures in the parental strains and F1 hybrids: LIS, UVS and NOS. The presence of each of the fissures was an incompletely penetrant trait. For each of these inconstant fissures, allelic differences between the A/J derived alleles and the B6 derived alleles at an autosomal locus for each of the traits may explain the observed results.

Since the data for each of the three inconstant fissures and fissure number is consistent with a single locus model, the question is raised as to whether a single locus could be responsible for the variation of the three inconstant fissures and fissure number. A single autosomal gene model cannot be ruled out, and it would have to have a pattern-specific effect similar to *Cfp1*.

Analysis of RI Strains

The mean number of cerebellar fissures in the RI strains was presented in ascending order in Table 3. AXB-4 had the lowest mean fissure number (7.1) while BXA-11 had the highest mean fissure number (11.1). Hence, the range of mean fissure numbers for the 31 RI strains was 7.1 to 11.1. This was eight times the parental difference (0.5), and in genetic terms, this phenomenon where the offspring are more extreme than either parent is called transgressive inheritance.

In this study 295 RI mice were scored for the presence or absence of cerebellar fissures. The segregation analyses consisted of tests of ANOVA. In the first analysis, the RI strains were partitioned into two groups according to sex (see Table 5). One group consisted of 148 females while the other group consisted of 147 males. By partitioning the data according to sex, no significant difference ($p=0.54$) was observed, and the ANOVA test would have explained less than 1% of the variance.

In the second analysis the RI mice were partitioned into two groups according to set. The AXB RI mice ($n=160$) were grouped into one set while the BXA RI mice ($n=135$) were grouped into a second set (see Table 6). By partitioning the data according to set, a significant difference ($p=1.2 \times 10^{-06}$) between the reciprocal RI sets was found that explained approximately 14% of the variance.

In the final analysis, the RI mice were partitioned into thirty groups based on the thirty RI strains that were scored (see Table 7). Each group consisted of 10 mice except BXA-9 of which there were only 5 mice available. By partitioning the data according to RI strains, a significant difference ($p=9.9 \times 10^{-55}$) among the thirty RI

strains was found that explained approximately 69% of the variance.

A conservative estimate of the number of effective loci ($\hat{L}$) was made with the following expression a^2/V_G , where *a* is the additive genetic component of the means. However, in this study the extreme means in the RI set were used to calculate *a*. Given the observed means for AXB-4 and BXA-11 *a* was estimated to be 4, and the genetic component of the variance (V_G) was estimated to be 1.09 (see Table 7). The estimate of the number of effective loci was 3.67. Therefore, this equation predicts that there are a minimum of four genes involved in cerebellar folial pattern in crosses of A/J and B6 mice.

Linkage Analysis

Linkage Analysis with Marker Loci

The association of marker loci, *Cph1* and *Pmv6*, may represent linkage with *Cfp5* and *Cfp6*, respectively (Table 8). *Cph1* on Chromosome 8, was associated with differences in fissure number (p=0.0003) while *Pmv6* on the X Chromosome, was associated with differences in the LIS (p=0.0001). The estimate of the probability of linkage with p=0.0003 and p=0.0001 is 95% and 98%, respectively (Neumann, 1992). The A/J derived allele $Cph1^a$ was associated with a decrease in fissure number. The A/J derived allele $Pmv6^a$ was associated with an increased presence of the LIS. The results suggest that *Cfp5* and *Cfp6* have pattern-specific effects on cerebellar folial patterns.

The association of marker loci, *D3Mit7*, *Iapls3-49* and *Xmv13*, may represent linkage with *Cfp7*, *Cfp8* and *Cfp9*, respectively. *D3Mit7* on Chromosome 3 and *Iapls3-49* on Chromosome 7 were associated with differences in fissure number (p=0.0004) while *Xmv13* on Chromosome 13 was associated with differences in the NOS (p=0.0004). The estimate of the probability of linkage with p=0.0004 is 90% (Neumann, 1992) (see Table 9). *D3Mit7* on Chromosome 3 was associated with a general effect on fissure number (p=0.0004); the A/J derived allele *D3Mit7*^{a} was associated with a decrease of fissure number. *Iapls3-49* on Chromosome 7 was also associated with a general effect on fissure number; however, the A/J derived allele *Iapls3-49*^{a} acted as an increaser of fissure number. *Xmv13* on Chromosome 13 was associated with the NOS and had a pattern-specific effect on the cerebellar foliation; the A/J derived allele *Xmv13*^{a} acted as a decreaser of the NOS and fissure number.

Twenty loci from 14 chromosomes were associated with either fissure number or specific fissures. Significant associations were found (p=0.007; Table 10); estimates of the probability of linkage were 50% (Neumann, 1992). In some cases a locus that was listed in Table 10 was selected from a group of tightly linked loci that were clustered together in a specific chromosomal region. Only one locus was selected from this cluster. The criteria used to select a particular locus was based on the following: the number of strains that had been genotyped for the loci, phenotypic effects and the p-value of the *t*-tests.

Tests of Confirmation of the Affects of Cfp1 on Distal End of Chromosome 4

Cfp1 was previously mapped to mouse Chromosome 4 in the BXD RI crosses

(Neumann et. al., 1993). In addition, it had been determined that *Cfp1* was localized in a region flanked by the markers *Pmv47* and *Gpd1*. It had also been reported that *Cfp1* was associated with the marker *Mpmv19*, which was on the proximal end of that chromosomal region (i.e. closer to *Pmv47* than *Gpd1*) (Neumann et al., ms. in prep.). In order to confirm linkage in the present cross in the same chromosomal vicinity, a p-value of 0.05 was needed because linkage had been previously detected. Among Chromosome 4 marker loci in the AXB and BXA RI strains, another marker, *D4Mit16*, displayed the greatest association with fissure number and thus, *Cfp1* (see Table 11). *D4Mit16* is located approximately midway between the two flanking markers, *Pmv47* and *Gpd*.

Cfp1 Effects on Folial Pattern

In previous crosses of CXB RI strains, the allele from the C background increased the frequency of the DES, ICF, and UVS relative to the B6 background. In crosses of BXD RI strains the allele from the D2 background increased the frequency of the PRCF, ICF and UVS, but decreased the frequency of the LIS relative to the B6 background. In the present cross the allele from the A/J background increased the frequency of the UVS and NOS relative to the allele from the B6 background (Table 11).

Table 1. Absolute Frequencies of Cerebellar Fissure Number in C57BL/6J and A/J Inbred and Reciprocal F1 Hybrid Mice.

Generation	*Sex*	*n*	*Fissures*					*Mean*	*S.D.*
			7	8	9	10	11		
A/J	Female	11	-	10	1	-	-	8.09	0.30
	Male	10	-	7	3	-	-	8.30	0.48
C57BL/6J	Female	19	2	7	8	2	-	8.53	0.84
	Male	16	-	3	11	2	-	8.94	0.57
AB6F1	Female	6	-	-	6	-	-	9.00	0.00
	Male	5	-	-	4	1	-	9.20	0.45
B6AF1	Female	6	-	-	6	-	-	9.00	0.00
	Male	7	-	-	1	4	2	10.14	0.69

n is the number of mice examined in each population. "-" indicates the relative frequency was 0.

Table 2. Relative Frequencies of the Seven Variable Fissures in C57BL/6J and A/J Inbred and Reciprocal F1 Hybrid Mice.

Generation	*Sex*	*n*	LIS	PRCF	ICF	DES	PSF	UVS	NOS
A/J	F	11	-	-	-	-	1.0	1.0	0.09
	M	10	-	-	-	-	1.0	1.0	0.30
C57BL/6J	F	19	0.63	-	-	-	0.79	0.16	0.95
	M	16	0.75	0.06	0.06	-	1.0	0.06	1.0
AB6F1	F	6	-	-	-	-	1.0	1.0	1.0
	M	5	0.20	-	-	-	1.0	1.0	1.0
B6AF1	F	6	-	-	-	-	1.0	1.0	1.0
	M	7	0.14	0.14	0.57	0.29	1.0	1.0	1.0

"-" indicates the relative frequency is 0. *n* is the number of mice examined in each population.

Table 3. Absolute frequencies of cerebellar fissure number in the AXB and BXA RI strains.

RI Strain	*n*	*Fissures*								*Mean*
		6	7	8	9	10	11	12	13	
AXB-4	10	1	7	2	-	-	-	-	-	7.1
AXB-13	10	1	5	4	-	-	-	-	-	7.3
AXB-18	10	1	3	5	1	-	-	-	-	7.6
AXB-8	10	1	2	6	1	-	-	-	-	7.7
BXA-12	10	-	4	5	1	-	-	-	-	7.7
AXB-15	10	-	5	3	1	1	-	-	-	7.8
AXB-14	10	-	1	9	-	-	-	-	-	7.9
AXB-20	10	-	2	6	2	-	-	-	-	8.0
AXB-19	10	-	1	7	2	-	-	-	-	8.1
A/J										8.2
AXB-11	10	-	2	4	3	1	-	-	-	8.3
BXA-26	10	-	-	6	4	-	-	-	-	8.4
AXB-25	10	1	1	3	2	3	-	-	-	8.5
C57BL/6J										8.7
BXA-1	10	-	-	2	7	1	-	-	-	8.9
BXA-8	10	-	-	3	4	3	-	-	-	9.0
BXA-13	10	-	-	-	10	-	-	-	-	9.0
BXA-17	10	-	1	1	5	3	-	-	-	9.0
AXB-12	10	-	-	-	9	1	-	-	-	9.1
BXA-14	10	-	2	-	4	3	1	-	-	9.1
AXB-23	10	-	-	2	5	2	1	-	-	9.2
BXA-25	10	-	-	1	6	3	-	-	-	9.2
AXB-10	10	-	-	-	6	4	-	-	-	9.4
BXA-2	10	-	-	-	7	2	1	-	-	9.4
BXA-7	10	-	-	-	2	8	-	-	-	9.8
AXB-5	10	-	-	-	1	9	-	-	-	9.9
AXB-1	10	-	-	-	-	10	-	-	-	10.0
BXA-24	10	-	-	1	-	7	2	-	-	10.0
AXB-6	10	-	-	-	1	6	3	-	-	10.2
BXA-9	5	-	-	-	1	2	2	-	-	10.2
BXA-4	10	-	-	-	1	4	5	-	-	10.4
AXB-2	10	-	-	-	-	3	7	-	-	10.7
BXA-11	10	-	-	-	1	1	5	2	1	11.1

n is the number of mice examined in each population. "-" indicates that the relative frequency is 0.

Table 4. Relative frequencies of specific cerebellar fissures in the AXB and BXA RI strains.

RI Strain	*n*	LIS	PRCF	ICF	DES	PSF	UVS	NOS
AXB-1	10	1.0	-	-	-	1.0	1.0	1.0
AXB-2	10	-	-	1.0	0.7	1.0	1.0	1.0
AXB-4	10	-	-	-	-	1.0	-	0.4
AXB-5	10	0.9	-	-	-	1.0	1.0	1.0
AXB-6	10	1.0	0.1	0.2	-	1.0	0.9	1.0
AXB-8	10	0.1	0.1	-	-	0.8	0.1	0.6
AXB-10	10	0.9	-	-	-	1.0	0.4	1.0
AXB-11	10	-	-	0.3	-	1.0	0.5	0.5
AXB-12	10	-	-	0.1	-	1.0	1.0	1.0
AXB-13	10	-	-	-	-	0.9	-	0.4
AXB-14	10	-	-	-	-	1.0	-	0.9
AXB-15	10	0.6	0.2	-	-	1.0	0.1	-
AXB-18	10	0.5	0.3	-	-	0.8	-	-
AXB-19	10	0.7	0.6	-	-	0.8	-	-
AXB-20	10	0.6	0.5	-	-	1.0	-	-
AXB-23	10	1.0	-	0.1	-	0.8	0.3	1.0
AXB-25	10	0.7	-	-	-	0.8	0.7	0.3
BXA-1	10	-	0.2	-	-	0.8	0.9	1.0
BXA-2	10	-	-	0.3	0.1	1.0	1.0	1.0
BXA-4	10	-	0.6	-	0.8	1.0	1.0	1.0
BXA-7	10	-	-	0.8	-	1.0	1.0	1.0
BXA-8	10	-	0.5	-	-	1.0	0.8	0.7
BXA-9	5	0.2	0.8	-	-	1.0	1.0	1.0
BXA-11	10	0.6	-	0.6	0.7	1.0	1.0	1.0
BXA-12	10	-	0.6	-	-	1.0	-	-
BXA-13	10	-	-	-	-	1.0	1.0	1.0
BXA-14	10	0.3	0.1	0.3	-	1.0	0.6	0.8
BXA-17	10	0.1	0.6	-	-	1.0	0.4	0.9
BXA-24	10	0.1	-	0.9	0.1	1.0	0.9	1.0
BXA-25	10	0.3	0.1	-	-	1.0	0.8	1.0
BXA-26	10	0.2	-	-	-	1.0	0.2	1.0

n is the number of mice examined in each population. "-" indicates that the relative frequency is 0.

Table 5. Analysis of Variance of Mean Fissure Number in the AXB and BXA RI Strains.

Grouping by Sex.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	*F* Statistic	P-value
Between Groups	1	0.58	0.58	0.37	0.54
Within Groups	293	458.01	1.56		
Total	294	458.59			

Total Variance (s^2_T)	1.56
Within Group Variance (s^2_E)	1.56
Between Group Variance (s^2_A)	-0.01
Variance Explained	<1%

Table 6. Analysis of Variance of Mean Fissure Number in the AXB and BXA RI Strains.

Grouping by Set.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	*F* Statistic	P-value
Between Groups	1	35.57	35.57	24.64	1.2×10^{-06}
Within Groups	293	423.02	1.44		
Total	294	458.59			

Total Variance (s^2_T)	1.56
Within Group Variance (s^2_E)	1.44
Between Group Variance (s^2_A)	0.23
Variance Explained	13.8%

Table 7. Analysis of Variance of Mean Fissure Number in the AXB and BXA RI Strains.

C. Grouping by Strain.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	*F* Statistic	P-value
Between Groups	29	324.09	11.18	22.02	9.9×10^{-55}
Within Groups	265	134.50	0.51		
Total	294	458.59			

Total Variance (s^2_T)	1.56
Within Group Variance (s^2_E)	0.51
Between Group Variance (s^2_A)	1.09
Variance Explained	68.1%

Table 8. Marker Loci with Greater than 95% Probability of Linkage to Loci Influencing Cerebellar Foliation.

Locus	Chr.	*n*	Fissure No.	LIS	PRCF	ICF	DES	PSF	UVS	NOS
*Cph1**	8	28	**-1.41**[a]	-0.22	0.27	-0.33	-0.12	-0.01	-0.54	-0.45
Pmv6	X	30	-0.27	**0.43**[b]	-0.11	-0.18	-0.11	-0.03	-0.21	-0.10

Association at the 0.0003 level is indicated with the superscript a.
Association at the 0.0001 level is indicated with the superscript b.

For each marker locus on this table, the difference between the means of RI strains containing the A/J allele and those of RI strains containing the B6 allele for cerebellar fissure number and relative frequencies of each of the variable fissures is presented. The probability of linkage with a p-value of 0.0003 is 95%. *Cfp5* was associated with marker locus *Cph1*. *Cfp6* was associated with marker locus *Pmv6*.
* - *Cph1* was previously designated *Cpe*.

Table 9. Marker Loci with Greater than 90% Probability of Linkage to Loci Influencing Cerebellar Foliation.

Locus	Chr.	*n*	Fissure No.	LIS	PRCF	ICF	DES	PSF	UVS	NOS
D3Mit7	3	30	**-1.28**[a]	-0.07	0.02	-0.21	-0.14	-0.03	-0.36	**-0.43**
Iapls3-49	7	30	**1.32**[a]	-0.06	-0.06	0.33	0.22	0.03	**0.49**	**0.33**
Xmv13	13	30	-1.00	-0.01	**0.28**	-0.26	-0.05	-0.02	**-0.43**	**-0.47**[a]

Associations that are significant at the 0.007 level are printed in bold typeface. Associations that are significant at the 0.0004 level are indicated with the superscript a.

For each marker locus on this table, the difference between the means of RI strains containing the A/J allele and those of RI strains containing the B6 allele in cerebellar fissure number and relative frequencies of each of the variable fissures is presented. The probability of linkage with p-values of 0.007 and 0.0004 is 50% and 90%, respectively. *Cfp7* was associated with marker locus *D3Mit7*. *Cfp8* was associated with marker locus *Iapls3-49*. *Cfp9* was associated with marker locus *Xmv13*.

Table 10. Marker Loci with Greater than 50% Probability of Linkage to Loci Influencing Cerebellar Foliation.

Locus	Chr	*n*	Fissure No.	LIS	PRCF	ICF	DES	PSF	UVS	NOS
Vil	1	28	0.17	**-0.39**	0.03	0.03	0.11	0.03	0.11	0.21
At3	1	24	0.69	**-0.33**	0.02	0.08	0.12	0.06	**0.46**	0.28
D2Mit6	2	30	-0.99	0.02	0.09	-0.13	-0.16	-0.02	**-0.43**	-0.36
D3Mit6	3	30	**-1.19**	-0.16	0.04	-0.09	-0.06	-0.04	**-0.40**	**-0.43**
Mpmv13	5	30	-0.34	**-0.41**	-0.07	0.16	0.06	0.02	-0.09	-0.02
Iapls1-50	5	30	-0.19	0.07	**0.21**	-0.17	-0.12	-0.02	-0.12	-0.07
D6J1	6	29	**1.06**	0.10	0.02	0.02	0.06	0.05	**0.39**	**0.36**
Iapls2-25	6	30	0.87	0.01	0.01	-0.03	-0.05	0.00	**0.45**	**0.46**
Pmv4	7	30	**-1.08**	-0.10	0.16	-0.22	**-0.23**	0.01	-0.31	**-0.34**
c	7	29	0.89	-0.29	-0.13	**0.31**	0.19	0.06	**0.39**	0.32
Es1	8	30	**-0.99**	-0.14	0.13	-0.06	-0.02	0.00	-0.52	-0.40
Fps14	10	11	0.86	-0.38	-0.26	0.49	0.02	0.07	**0.55**	0.40
Pmv37	12	29	-0.42	-0.07	**0.27**	-0.08	0.01	-0.03	-0.15	**-0.38**
Odc	12	30	-0.75	-0.08	**0.24**	-0.13	0.01	-0.01	**-0.42**	**-0.36**
D13Mit3	13	30	-0.65	-0.04	**0.27**	-0.23	-0.08	0.00	-0.26	**-0.32**
D13Mit13	13	30	-0.89	0.03	**0.26**	**-0.31**	-0.09	0.02	**-0.45**	**-0.33**
*D15Ag1**	15	17	0.32	**0.48**	-0.01	-0.13	-0.20	-0.01	0.17	0.03
Iapls1-22	15	30	0.12	**0.37**	0.08	-0.04	-0.03	-0.02	-0.03	-0.17
D16Mit4	16	30	**1.12**	0.01	-0.03	0.18	**0.24**	0.05	0.35	0.28
D18Mit17	18	30	**-1.09**	-0.15	0.17	-0.23	-0.05	-0.02	**-0.45**	-0.31
Iapls3-44	19	30	**1.15**	-0.21	-0.08	0.30	0.21	0.03	**0.43**	**0.40**
D19Mit19	19	30	**1.12**	0.28	-0.12	0.09	0.11	0.03	**0.45**	0.26

* This locus was previously designated *D15Citb1*.
Associations that are significant at the 0.007 level are printed in bold typeface.

Table 11. Association of Chromosome 4 Marker Loci in the Vicinity of *Cfp1* with Differences in Cerebellar Fissure Number and Relative Frequencies of Variable Fissures in AXB and BXA RI Strains.

Locus	*n*	Fissure No.	LIS	PRCF	ICF	DES	PSF	UVS	NOS
Pmv47	30	-0.48	0.11	-0.05	-0.23	-0.14	-0.03	-0.19	0.04
D4Nds2	29	0.79	0.19	0.00	0.08	0.00	0.00	0.24	0.25
D4Mit12	29	0.79	0.19	0.00	0.08	0.00	0.00	0.24	0.25
D4Mit16	29	0.66	-0.03	-0.13	0.14	-0.03	0.02	**0.35**	**0.33**
Akp2	20	0.75	-0.05	0.22	0.00	0.21	0.05	0.24	0.06
Gpd1	30	0.20	0.22	0.08	-0.17	-0.09	0.05	0.04	0.04

Associations that are significant at the .05 level are printed in bold typeface.

DISCUSSION

Segregation and linkage analyses of crosses of A/J and C57BL/6J inbred mice indicate that at least five loci, which we have named *Cfp5*, *Cfp6*, *Cfp7*, *Cfp8* and *Cfp9*, were involved in variation of cerebellar fissure number. *Cfp5* and *Cfp6* were localized to Chromosome 8 and the X Chromosome, respectively. The probability of linkage for each of the two loci was at least 95%. *Cfp7*, *Cfp8* and *Cfp9* were localized to Chromosomes 3, 7 and 13, respectively. The probability of linkage for each of the three loci was at least 90%. *Cfp1* on the distal end of Chromosome 4 was associated with the marker *D4Mit16*. The A/J allele of *Cfp1* was found to increase the frequencies of the uvular and nodular sulci. Twenty-two additional loci were also mapped; however, the probability of linkage was approximately 50%.

Quantitative Genetics

Variation in most organisms is usually quantitative. A quantitative trait has a range of phenotypes differing by degree rather than a qualitative difference. Unlike Mendelian traits, quantitative traits have a genetic component as well as an environmental component. A good example of a Mendelian trait is sickle cell anemia in humans where a point mutation in a single gene results in abnormal erythrocyte production. More importantly, the defective gene accounts for 100% of the variance. Examples of quantitative traits include variation of bristle number in Drosophila or height in the human population. As compared to Mendelian genetics, quantitative

traits are much more difficult to analyze. Usually, geneticists have to use statistics to properly analyze their data pertaining to a quantitative trait locus.

Quantitative genetics is important to our studies because variation in cerebellar folial pattern is also a quantitative trait where the genetic variation is due to several genes (Garretson and Neumann, 1993; Neumann et al., 1993; Neumann et al., ms. in preparation) . The foundation of quantitative genetics was laid down in the early to mid-twentieth century. There was an incentive at that time to study corn genetics (Jones, 1917). By breeding different strains of corn, scientists were ultimately hoping to increase the yield of corn per acre. They contributed to quantitative genetics by developing mathematical tools to analyze their data. For example, they developed formulas to estimate the number of genes that effected a trait, and they also proposed models to explain segregation analyses (Wright, 1952). However, their efforts were limited because the tools required to map a gene (or genes) to a particular chromosome were not available.

Molecular Genetics

With the advent of recombinant DNA technology, the focus of modern genetics has been on the molecular structure of genes. This is a big departure from classical genetics in which a phenotype was studied and then a gene(s) associated with the trait was mapped. Currently, simply mapping a gene is not sufficient; one must characterize (i.e. sequence, expression studies) the molecular structure of a gene and that is usually done by mapping and cloning it.

The overall impact of recombinant DNA technology in biology and genetics in particular has been enormous. For example, in the AXB, BXA RI set there are hundreds of marker loci that have been mapped with the use of molecular techniques. In fact, there are so many markers added to the mouse genome, that "chromosome committees" have been established to review new markers that are added to ensure that they are accurately positioned on the relevant chromosomes. The genetic maps that are generated by the chromosome committees are maintained by The Jackson Laboratory, and they are made available to the scientific community via the internet (http://www.jax.org).

Nowhere has the impact of molecular biology been more evident than the studies of the fruit fly, Drosophila. Because of its simplicity as compared to mammals, Drosophila has been studied extensively in the past few decades. It was in Drosophila that genes involved in transcription were first isolated. The molecular information obtained was used as a molecular probe to screen for structurally related genes in the same and/or different species. The related genes that are sought are said to belong to a particular gene family. This strategy has been so successful with Drosophila geneticists that they often use it as a basis for grant applications for the isolation of mammalian genes.

Gene Families

During the course of evolution, novel genes arose by the duplication and divergence of older ancestral genes (Dickerson and Geis, 1983). Because of this,

many genes in mammalian genomes belong to a family of interrelated genes. Geneticists have been able to exploit gene families. The most difficult and laborious part is cloning the initial member of a family. When that is accomplished, the identification of subsequent members is relatively easy. Some of the gene families that were initially identified in Drosophila and then discovered in mice are the homeobox, paired-box and POU gene families (Scott et al., 1989; Bopp et al., 1986; Deutsch et al., 1988). Members of a gene family are likely to have related functions, but that is not always the case. We are interested in gene families because they may give us insight as to the type of genes that affect patterning of the cerebellum.

Transcription Factors

Although several gene families have been discovered, we are only interested in a select few. Specifically, we are interested in gene families that encode transcription factors because we believe that some of the genes that are involved in the patterning of the cerebellum may belong to these gene families. Transcription factors are proteins that bind to DNA and are involved in gene expression. Many of these transcription factors contain helix-turn-helix domains (Harrison, 1991). This type of domain was the first DNA binding motif to be discovered (Harrison and Aggarwal, 1990). The homeobox, which encodes a 60 amino acid homeodomain, also contains a helix-turn-helix motif (Scott et al., 1989).

Thyroxine (T_4) Levels and Fissure Number

In addition to gene families we are also interested in thyroid hormone related genes. Briefly, in the early 1970s Lauder et al. (1974) made newborn rats hypo- and hyperthyroid by using pharmacological agents. It was determined that hypothyroid rats had a significantly greater number of fissures. These findings were of interest to us because we wanted to know whether variation in fissure number in our studies was due to thyroid hormone. In our crosses DBA/2J mice were hyperthyroid relative to C57BL/6J (Neumann et al., 1993). Thyroxine levels and fissure number for seven BXD RI strains were determined, and it was found that there was a strong negative correlation between thyroid hormone serum levels and total fissure number.

The *Cfp1*^{d} allele did not seem to influence thyroid levels, but two other marker loci displayed associations with total T_4 serum concentration in the direction predicted by the inverse correlation. The two marker loci displayed potential associations with *Cfp2* and *Cfp3*. However, the probability of linkage in each case was only 50-75%. In addition only one of these loci (*Cfp2*) was associated with both fissure number and total T_4 serum concentration.

The AXB, BXA RI Set

There were advantages to using the AXB, BXA RI set. Previous studies utilized the BXD and CXB RI strains (Neumann et al., 1993; Garretson and Neumann 1993) . The biggest advantage that this set has to offer is that it is a reciprocal set. This means that two-thirds of the X Chromosomes in the AXB set are derived from the A/J background while two-thirds of the X Chromosomes in the BXA set are

derived from the B6 background. The AXB, BXA RI set was important to us because its features allowed us to investigate whether cerebellar folial pattern is influenced by maternal effects as reported by Cooper et al. (1991).

As compared to a previous study (Neumann et al. 1993), more genes were mapped in the present study. We previously found four genes with at least a 50% probability of linkage in the BXD RI set while in the present study we found twenty-seven genes with a probability of linkage of at least 50% in the AXB, BXA study.

The best explanation for this outcome may be the number of different alleles between the two RI sets. The B6 and D2 inbred strains, which were initially crossed to generate the BXD RI set, may have less allelic differences than the AXB, BXA RI set, which was generated by crossing the A/J and B6 inbred strains. In fact, Marshall et al. (1992) suggested just that in a genetic survey based on 31 AXB, BXA RI strains and DNA samples from eight extinct strains. The authors go on to say that because the A/J and B6 parental sets are less related than the B6 and D2 parental sets, it may be more useful for linkage analysis. This may be the best explanation as to why we mapped more genes in this study as compared to our previous studies.

Candidate Genes

Because hundreds of genes have been mapped (and many have been cloned as well), in the mouse genome, we are interested in determining whether the genes we mapped are equivalent to previously mapped genes. Thus, we evaluated candidate genes in the vicinity of *Cfp1* through *Cfp9*. It is important to note that the genes that

we mapped were mapped with a low level of resolution. In other words, the actual map positions of the genes may lie an estimated 20 cM from the marker locus that it was associated with. To reduce the number of candidate genes, we would like to position all of the genes on a high resolution genetic map. This would put us in a better position to clone the gene and to evaluate candidate genes.

Candidate genes were searched in an electronic database that is maintained by the Jackson Laboratory (http://www.jax.org). The database is a compilation of chromosome committee reports that are revised and updated annually. Loci within a 20cM area of the positions of the *Cfp* genes were potential candidate genes. Any genes that were involved in the central nervous system (especially the cerebellum) or development or contained a homeobox were considered good candidate genes. A summary of all candidate genes influencing cerebellar folial pattern is presented in Table 12.

Candidate Genes for Cfp1

There were three genes identified as candidates for *Cfp1*(see figure 3). All three candidate genes mapped to the distal end of Chromosome 4, and they all encode a DNA-binding motif. Two of these genes were homeobox-containing genes.

Pou3f1 is a candidate for *Cfp1* (see Figure 3). *Pou3f1* maps approximately 10cM proximal to *D4Mit16*, the marker that was associated with *Cfp1* (Avraham et al., 1993). The POU family of mouse genes encode transcription factors. In addition, they are also homeodomain-containing genes. The *Pou3f* genes are expressed in the

nervous system and have an overlapping pattern of restricted expression in the brain (Dominov and Miller, 1996). *Pou3f1* is up regulated during brain development, and it is primarily expressed in the telencephalon, mesencephalon and brain stem.

Pax7 is also a candidate for *Cfp1* (see Figure 3). *Pax7* is a candidate because it also maps approximately 10cM proximal to *D4Mit16*, and it is expressed in the midbrain and hindbrain during mouse development (Asano and Gruss, 1992). The *Pax* gene family encodes a paired-domain which contains gene products that are related to transcription factors (Gruss and Walther, 1992). In addition to a paired-box, many *Pax* genes also contain a homeobox.

Lmyc was also a candidate for *Cfp1* (see Figure 3). *Lmyc* maps approximately 10cM distal to *D4Mit16* (Ingvarsson et al., 1988). *Lmyc* encode a transcription factor with a helix-loop-helix motif. It is expressed in the developing mouse brain from mid-gestation through the perinatal period. A targeted mutation of another gene in the family, *Nmyc*, affected pattern formation.

Candidate Genes for Cfp6

One candidate gene, *Zic3*, was identified for *Cfp6* (see Figure 4). *Zic3* is a zinc finger protein that is expressed in the developing cerebellum (Yokota et al., 1996). Zinc fingers are found in proteins that contain DNA-binding motifs. *Zic* is homologous to a Drosophila pair-rule gene, and human *Zic* cDNA is 85% homologous to mouse *Zic* cDNA. By using immunohistochemistry, the investigators were able to detect protein in the nuclei of cerebellar granule cells.

Cfp1, Cfp2, Cfp3 and Cfp4

We were interested in seeing whether *Cfp1-Cfp4* had any effects on cerebellar folial pattern in this study. Previously, we mapped *Cfp1-Cfp4* in crosses of B6 and D2 animals. In the present study we were able to confirm an effect of *Cfp1* in the AXB, BXA RI strains but not for *Cfp2*, *Cfp3* or *Cfp4*.

The most likely explanation for this is that there were no allelic differences between A/J and B6 at the *Cfp2*, *Cfp3* and *Cfp4* loci. At any of the three loci, for example, it is likely that they are homozygous for either the A/J or B6 allele.

Another possibility is that there may be allelic differences at *Cfp2-Cfp4*, but those effects are compensated by the effects of *Cfp5-Cfp9*. In other words, *Cfp5-Cfp9* may have such strong effects on the patterning of the cerebellum that the effects of *Cfp2-Cfp4* cannot be detected. If that is the case, one possible way to detect the effects of *Cfp2-Cfp4* in the crosses of A/J and B6 would be to increase the sample size. However, that would not necessarily ensure detection of the effects on the patterning of the cerebellum.

Future Direction

As previously stated, none of the genes that we have mapped involving cerebellar folial pattern have been characterized at the molecular level. The next obvious step is to clone the genes that we have mapped. The problem that we are confronted with is that patterning of the cerebellum is not a Mendelian trait where all of the variance is due to one gene. We have previously shown (Neumann et al., 1993;

Garretson and Neumann 1993), and presently confirmed, that cerebellar pattern formation is a multifactorial trait where the variance is not only due to genetic mechanisms but also to environmental effects as well. This means that in our effort to clone any of the multiple mapped loci that constitute the genetic component, the environmental component will have a compounding effect. In addition since it is expensive and time intensive to clone all the genes, we must focus on a select few. Therefore, the best genes to select for cloning would be the ones that account for the largest percentage of the variance. Based on previous work (Neumann et al., 1993), *Cfp1* would fit this criteria in crosses of B6 and D2 animals, and based on the present work, perhaps *Cfp6* on the X Chromosome in crosses of A/J and B6 animals.

The next step in this project is to fine map *Cfp1* on Chromosome 4. We have mapped *Cfp1* on the distal end of Chromosome 4, and we do have three candidate genes. By fine mapping the interval of interest, we accomplish two major objectives. First, it will allow us to narrow the list of candidates. Of the three candidate genes, some or all of the candidates will be eliminated while some may be strengthened or new ones found. Second, it will prepare us for positionally cloning of our gene. Currently, *Cfp1* has been mapped within a 20-30cM interval. To put this in perspective, 1cM corresponds to two million base pairs in mouse which may contain as many as twenty genes. Therefore, a 20-30cM interval may contain as many as 600 genes! By fine mapping the interval, we would like to narrow the interval as much as possible. If we narrow the region to less than two cM, then we will be able to insert fragments of this area into cloning vector such as a yeast artificial chromosome

(YAC). A cloning vector such as a YAC merely acts as a vehicle for us to further manipulate our interval of interest containing *Cfp1*. Ultimately, we want to narrow the interval to the point where we only have *Cfp1*, in which case the gene could be cloned and sequenced.

The cloning process is long, laborious and expensive. It is in our interests, therefore, to continue to look for alternative routes to clone our genes. As previously discussed, one method is the candidate gene approach. Another method is to take advantage of transgenic mice that have had specific genes knocked out. It is interesting to note that while we are trying to clone genes by taking a phenotype, mapping the genes that effect the phenotype and cloning the genes of interest, other investigators are taking the opposite route, the so called "reverse genetics" (Kessel and Gruss, 1990). As previously discussed, investigators are using recombinant DNA technology to identify gene families and then knock out (and generating transgenic mice in the process) as many genes in a family as they can. Often, the transgenic mice that are generated have no known phenotype. It would be of interest if some of the transgenic animals that have been generated may contain a gene knockout involved in the cerebellar folial pattern complex. Hence, transgenic animals that are generated by others may be very useful to us.

We have shown that there are multiple genes involved in the folial pattern of the cerebellum. However, it is not the fissures that are necessarily important. The presence or absence of fissures does not make much of a difference to an animal. The important aspect is that we were able to map five additional genes involved in

cerebellar development. Hence, the number of genes involved in the development of the nervous system must not only be immense but also complex.

Figure 3. A Map of the Distal End of Chromosome 4 Depicting Candidate Genes for *Cfp1*.

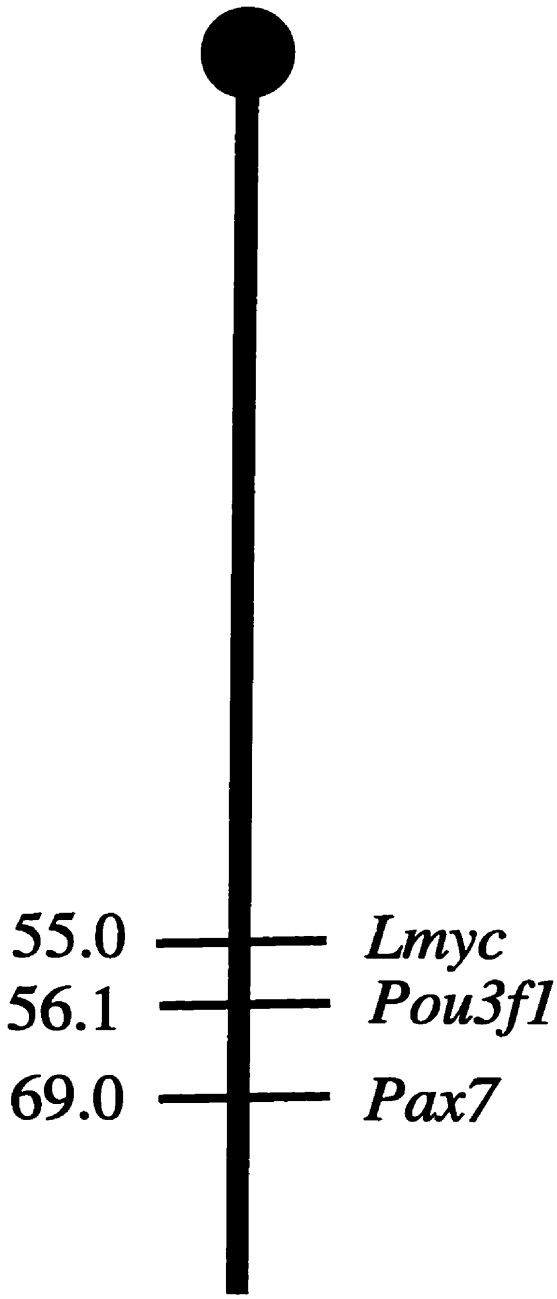


Figure 4. A Map of the X Chromosome Depicting a Candidate Gene for *Cfp6*.

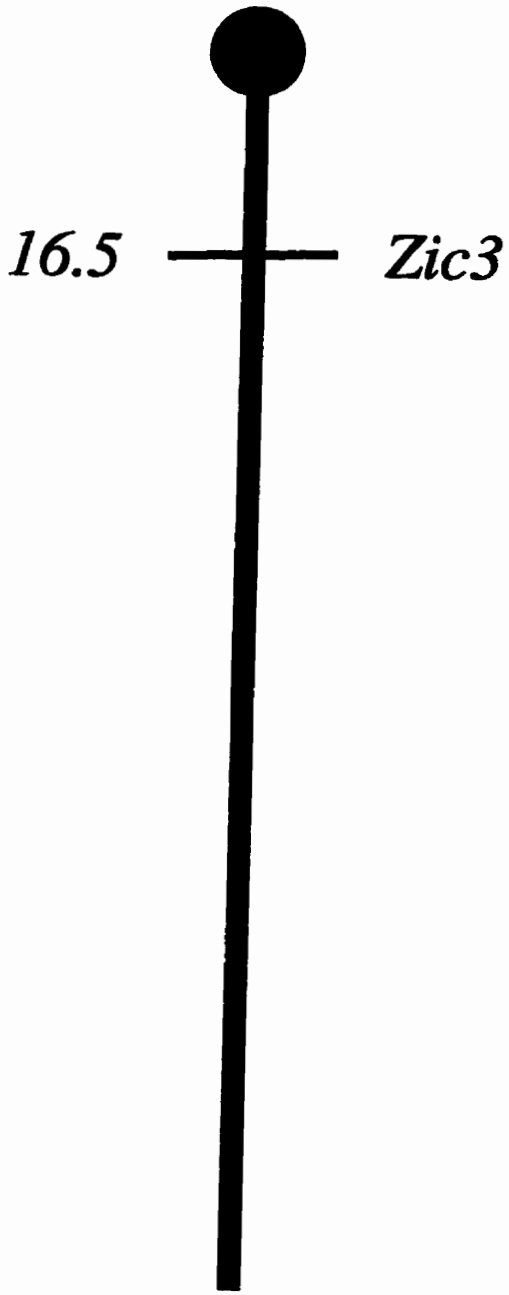


Table 12. A Summary of all Gene Loci with at Least a 90% Probability of Linkage that Affect Cerebellar Fissure Number.

Locus	Chromosome	Marker	Effect	Candidate Gene
Cfp1	4	*D4Mit16*	Pattern-specific	*Pou3f1* *Pax7* *Lmyc*
Cfp4	5	*Mpmv13*	Pattern-specific	*En2*
Cfp5	8	*Cph1*	Pattern-specific	
Cfp6	X	*Pmv6*	Pattern-specific	*Zic3*
Cfp7	3	*D3Mit7*	General	
Cfp8	7	*Iapls3-49*	General	
Cfp9	13	*Xmv13*	Pattern-specific	

Appendix 1. Glossary of Genetic Terms

1. Locus: A defined site on a chromosome that may be occupied by a gene or part of a gene. It may be thought of as a "parking spot".[1]

2. Allele: One of the different forms of a gene that can exist at a single locus.[2] In the thesis the allele from the A/J strain has been designated "n^a", and the allele from the B6 strain has been designated "n^b".[2]

3. Penetrance: The proportion of individuals with a specific genotype who manifest that genotype at the phenotype level.[3]

4. Overdominance or Superdominance: A phenotypic relation in which the phenotypic expression of the heterozygote is greater than that of either homozygote.[4]

5. Transgression: The appearance in a segregating generation (F2, backcross, etc.) of one or more genotypes (individuals) which fall outside the limits of variation defined by the parents and the F1 of the cross in respect to one or more characters.[5]

6. Heterosis: The superiority of heterozygous genotypes with respect to one or more characters in comparison with the corresponding homozygotes. The phenotypic result of gene interaction in heterozygotes and is thus confined to that state. It can be disrupted by inbreeding and restored by interbreeding of the inbred lines.[6]

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[1] *Glossary of Genetics*, Rieger, R., Michaelis, A., Green, M.M. 5th ed. New York: Springer-Verlag, 1991: p. 301.

[2] *An Introduction to Genetic Analysis*, Griffiths, A.J.F., Miller, J.H., Suzuki, D.T., Lewontin, R.C., Gelbart, W.M. 5th ed. New York: W.H. Freeman and Company, 1993: p.783.

[3] *Ibid.* p.794.

[4] *Ibid.*

[5] *Glossary of Genetics, op.cit.* p. 487.

[6] *Ibid.* p.248.

Appendix 2. Linkage Analysis

Part 1.

A total of 269 marker loci was available for the AXB and BXA RI set. The following is the strain distribution pattern (SDP) for one of the 269 marker loci, *D4Mit16*, which is located on the distal end of Chromosome 4. The cells containing an "A" represent the allele derived from the A/J background, n^a. The cells containing a "B" represent the allele derived from the B6 background, n^b. Note: "U" means undetermined.

Strain Distribution Pattern for *D4Mit16* for the AXB RI Set	
AXB RI Strain No.	Marker Locus: *D4Mit16*
1	B
2	A
3	U
4	A
5	A
6	A
7	U
8	B
9	U
10	A
11	B
12	A
13	B
14	B
15	B
17	U
18	B
19	B
20	B
21	U
23	A

Strain Distribution Pattern for *D4Mit16* for the BXA RI Set	
BXA RI Strain No.	Marker Locus: *D4Mit16*
1	A
2	A
4	B
7	A
8	B
9	A
11	B
12	A
13	A
14	B
17	B
18	U
20	U
22	U
23	U
24	A
25	A
26	U

Part 2.

The relative frequencies of the AXB, BXA RI strains for *D4Mit16* at the UVS fissure (Table 10, sixth column) are presented. This was carried out for the remaining 268 loci for the UVS. Each of the seven variable fissures and fissure number were treated similarly; in total, this procedure was carried out 2152 times (269 x 8). (Note: The following values are relative frequencies; therefore, "1" indicates 100%.)

Relative frequency of the UVS fissure at *D4Mit16* for the AXB, BXA RI Strains with the "A" allele, n^a.

RI Strain with the "A" allele, n^a	Relative frequency at *D4Mit16*
AXB-2	1.0
AXB-4	0.0
AXB-5	1.0
AXB-6	1.0
AXB-10	.4
AXB-12	1.0
AXB-23	.3
BXA-1	.9
BXA-2	1.0
BXA-7	1.0
BXA-9	1.0
BXA-12	0.0
BXA-13	1.0
BXA-24	.9
BXA-25	.8

Relative frequency of the UVS fissure at *D4Mit16* for the AXB, BXA RI Strains with the "B" allele, n^b.

RI Strain with the "B" allele, n^b	Relative frequency at *D4Mit16*
AXB-1	1.0
AXB-8	.1
AXB-11	.5
AXB-13	0.0
AXB-14	0.0
AXB-15	.1
AXB-18	0.0
AXB-19	0.0
AXB-20	0.0
BXA-4	1.0
BXA-8	.8
BXA-11	1.0
BXA-14	.6
BXA-17	.4

Part 3.

In order to conduct statistical tests, the sample size, mean and standard deviation needed to be determined for the two populations. One population consisted of cells containing the "A" allele or the first table in part 2. The other group consisted of all the cells containing the "B" allele or the second table in part 2. (Again, the example shown below was for the marker locus *D4Mit16*.)

	"A" Allele			"B" Allele		
Locus	*n*	Mean	SD	*n*	Mean	SD
D4Mit16	15	0.747	0.374	14	0.393	0.418

Part 4.

The next statistical test was a *F*-test. The *F*-test is a test of the equality of the variances of two populations to determine which *t*-test to use, one assuming equal variance, the other assuming unequal variance. If the *F*-test was greater than 2.7, then unequal variance was used. If the *F*-test was less than 2.7, then equal variance was used. For the example above, the *F*-test was:

F-test: 0.374/0.418=.895

Part 5.

The next step was to conduct a *t*-test. Any *t*-test with a value of .007 was considered statistically significant and possibly associated to a gene that influences cerebellar pattern formation. The table below is a summary of p-values with associated linkage probabilities (From Neumann 1992).

P-value	Probability of Linkage
.007	50
.001	85
.0004	90
.0003	95

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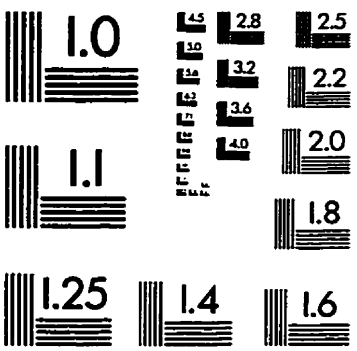

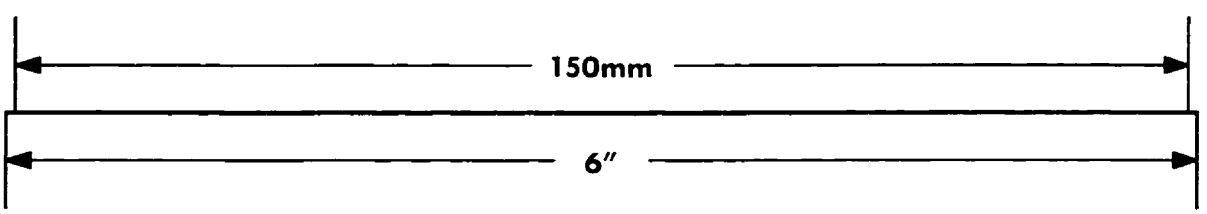

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