

Neural tube defects and abnormal brain development in F52-deficient mice

MIN WU, DONG FENG CHEN, TOSHIKUNI SASAOKA, AND SUSUMU TONEGAWA*

Howard Hughes Medical Institute, Center for Learning and Memory, Center for Cancer Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139

Contributed by Susumu Tonegawa, November 15, 1995

ABSTRACT F52 is a myristoylated, alanine-rich substrate for protein kinase C. We have generated F52-deficient mice by the gene targeting technique. These mutant mice manifest severe neural tube defects that are not associated with other complex malformations, a phenotype reminiscent of common human neural tube defects. The neural tube defects observed include both exencephaly and spina bifida, and the phenotype exhibits partial penetrance with about 60% of homozygous embryos developing neural tube defects. Exencephaly is the prominent type of defect and leads to high prenatal lethality. Neural tube defects are observed in a smaller percentage of heterozygous embryos (about 10%). Abnormal brain development and tail formation occur in homozygous mutants and are likely to be secondary to the neural tube defects. Disruption of F52 in mice therefore identifies a gene whose mutation results in isolated neural tube defects and may provide an animal model for common human neural tube defects.

Neural tube defects (NTD) are among the most common and severe congenital malformations in humans. They occur worldwide with an incidence of between 1 and 9 per 1000 births (1). The disturbance of neural tube formation may lead to various defects such as anencephaly and spina bifida. NTD occur either alone (>80% of cases) or as a part of a more complex malformation syndrome (2, 3). The etiology of NTD is thought to be heterogeneous, and both genetic and environmental factors have been implicated (4). Direct genetic analysis of human NTD has been difficult because of the scarcity of useful familial cases and because of the lack of any candidate genes involved. An alternative approach is to create animal models which may allow a detailed description of the critical embryonic events in neural tube closure and the identification of the crucial genes responsible for these events. Such studies may also lead to the cloning of the relevant human genes.

More than 10 mouse mutants exhibiting various types of neural tube defects have been described. However, in most of these cases, the genes involved have not been cloned (reviewed in ref. 5). One of these mouse mutants, curly tail (*ct*) (6), has been extensively studied as the best available model for human NTD. NTD in *ct* mice closely resemble the human malformation in many respects, including their location and form (6, 7) and mode of multifactorial inheritance (6, 8).

We have generated F52 (also called MacMARCKS or Mrp)-deficient mice and have fortuitously observed defects in neural tube formation. F52 is a myristoylated alanine-rich substrate for protein kinase C (PKC). The gene encoding F52 was cloned initially from a mouse brain cDNA library (9) and subsequently from lipopolysaccharide-stimulated mouse macrophages (10). F52 shares the domain structure and many biochemical features with another myristoylated alanine-rich PKC substrate, MARCKS (11–13), as reviewed by Aderem

(14) and Blackshear (15). Both proteins have three domains: an N-terminal myristoylated domain that mediates binding to membranes, a highly conserved MH2 domain of unknown function, and a basic domain containing the PKC phosphorylation sites and a calcium/calmodulin binding site. In addition, the basic domain of MARCKS contains an actin binding site, while the existence of such a site has not been established for F52. The sites for phosphorylation by PKC and for binding to calmodulin and actin have been localized to an 18-amino acid sequence. PKC-dependent phosphorylation of MARCKS alters its ability to bind calcium/calmodulin and actin and controls its localization to different cellular compartments. Although their domain structures are similar, F52 and MARCKS differ in their subcellular distribution before and after activation by PKC and presumably have distinct functions (16).

We found severe NTD, including exencephaly, spina bifida, and tail flexion anomaly, in ≈60% of the homozygous mutants and in ≈10% of the heterozygous mutants. Those homozygous mutants without exencephaly survive despite brain abnormalities such as agenesis of the corpus callosum, which appear to occur secondarily to NTD.

EXPERIMENTAL PROCEDURES

Generation of Targeting Vector and Embryonic Stem (ES) Cell Clones. Genomic DNA clones corresponding to the F52 locus were isolated from a library of strain 129 mouse DNA. The targeting vector was constructed in a trimolecular ligation reaction using a 3.8-kb *Bam*HI–*Xho*I fragment from the 5' end of the F52 gene cloned in pBluescript (Stratagene), a 1.8-kb *Cl*a I–*Not*I fragment containing a neomycin phosphotransferase (*neo*) gene driven by the phosphoglycerate kinase (PGK) promoter, and a 5.5-kb *Eco*RI–*Xba*I fragment from the 3' end of F52, digested with appropriate restriction enzymes. The targeting vector was designed to delete a 2.8-kb fragment encompassing the entire protein-encoding region of the F52 gene.

Production of F52-Deficient Mice. D3 ES cells were transfected with linearized targeting vector by electroporation. G418 selection was applied at a concentration of 200 μg/ml 24 hr after transfection. G418-resistant colonies were picked and amplified further for frozen stocks and DNA isolation. Positive clones were identified by Southern analysis of genomic DNA digested with *Bam*HI, using a ³²P-labeled 1.3-kb *Hind*III–*Xho*I fragment from the F52 gene 3' flanking region.

Chimeric mice were produced as described by Bradley (17). Chimeric mice were bred to C57BL/6 mice, and germline transmission of the mutant allele was detected by Southern analysis of tail DNA from F₁ offspring with agouti coat color. Heterozygous F₁ mice were interbred to homozygosity.

Histological Analysis. Animals were deeply anesthetized with avertin and sacrificed by intravenous perfusion with 4%

paraformaldehyde. Frozen sections of brain were cut at 30 μ m with a freezing microtome. Sections were stained by the cresyl violet–Nissl or bodian silver procedure (18, 19).

RNA Analysis. Total RNA was purified with Tri Reagent (Molecular Research Center, Cincinnati). Northern blot hybridization was performed as described (20). The hybridized membrane was analyzed with a Bio-Image analyzer (Fuji).

Whole-Mount *in Situ* Hybridization and Embryo Sections. Whole-mount *in situ* hybridizations were performed with embryos at 8.5–10.5 days postcoitum (dpc), according to a protocol from Andrew McMahon's laboratory modified from Wilkinson (21).

Serial sections of 5 μ m were prepared from embryos embedded in paraffin. Tissue sections were dewaxed and dehydrated through an ethanol series after collection onto glass slides and were mounted in 80% glycerol.

RESULTS

Generation of F52-Deficient Mice. Overlapping mouse genomic clones that contained the F52 gene and its flanking sequences were isolated. In the targeting vector, a 2.8-kb sequence containing the entire coding region was replaced with a *neo* gene (Fig. 1A). One hundred ninety-six ES cell clones resistant to G418 were isolated after transfection, of which 8 contained the intended DNA sequence replacement. Four of these ES cell clones were used to generate chimeric mice. Offspring heterozygous at the F52 locus were bred to homozygosity as confirmed by Southern analysis of tail DNA (Fig. 1B).

The lack of an intact F52 gene was confirmed by Northern analysis of brain RNA. F52 mRNA was not detectable in homozygous mutant mice and was reduced by about half in heterozygous mutant mice as compared with wild-type littermates (Fig. 1C).

F52 Deficiency Is Lethal in Some Mice. Southern analysis of tail DNA isolated from 3-week-old mice derived from heterozygote crosses indicated that the number of homozygous mutants was significantly lower than expected from the 1:2:1 Mendelian segregation: 142 wild type mice, 247 heterozygotes, and only 28 homozygotes were obtained.

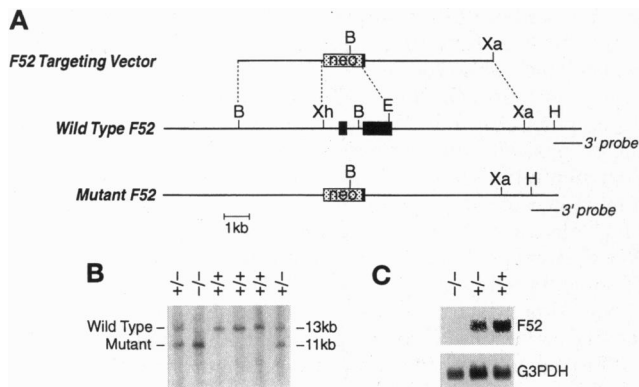


FIG. 1. Generation of F52-deficient mice. (A) The F52 locus and targeting construct. A 2.8-kb fragment of the gene was deleted and replaced with a *neo* gene. The 3' flanking probe used to screen ES cell clones and mice is indicated. B, *Bam*HI; E, *Eco*RI; H, *Hind*III; Xa, *Xba*I; Xh, *Xho*I. (B) Southern blot analysis of representative tail DNA from heterozygotic intercrossings. Genomic DNA was digested with *Bam*HI and hybridized with the 3' flanking probe. The expected sizes of hybridizing restriction fragments in the wild-type and mutant F52 allele are indicated. (C) Northern blot analysis of brain RNA from homozygous and heterozygous mutants and wild-type mice. Ten micrograms of total RNA was loaded in each lane. The same filter was hybridized successively with an antisense F52 and a glyceraldehyde-3-phosphate dehydrogenase (G3PDH) RNA probe. The bands that correspond to the F52 and G3PDH mRNA are indicated.

The homozygous mutant mice that reached adulthood (6 weeks or older) were generally healthy. One homozygote and one heterozygote were found to have hydrocephalus and died soon after weaning. Attempts to mate homozygous males with homozygous females were rarely successful, with one or two pups being born on occasions. When homozygous mutant males were crossed with heterozygous females, both heterozygous and homozygous mutant offspring were produced. However, among the offspring, the ratio of homozygotes to heterozygotes was 30:86, which is much lower than the expected ratio of 1:1. Female homozygotes bred poorly with both homozygous and heterozygous males.

NTD in F52-Deficient Mutant Mice. To investigate the reason why some homozygous mutant embryos died, timed matings were set up between homozygous males and heterozygous females. Embryos were examined from 9.5 to 15.5 dpc. The genotypes of embryos were determined by Southern blot analysis of DNA isolated from visceral yolk sac.

Most homozygous embryos (63%) developed NTD. The defects consisted of open cranial neural tubes (exencephaly), open spinal neural tubes (spina bifida), and tail flexion (Fig. 2). The type and severity of the defects varied among mutant embryos. Exencephaly alone occurred in 72% of all affected mutant embryos, while spina bifida (including flexed tail) occurred alone in 11%, and exencephaly and spina bifida occurred together in 17% of affected embryos (Table 1). NTD were also observed in 11% of heterozygous embryos (Table 1), suggesting that 50% reduction of F52 gene expression may be sufficient to perturb neurulation.

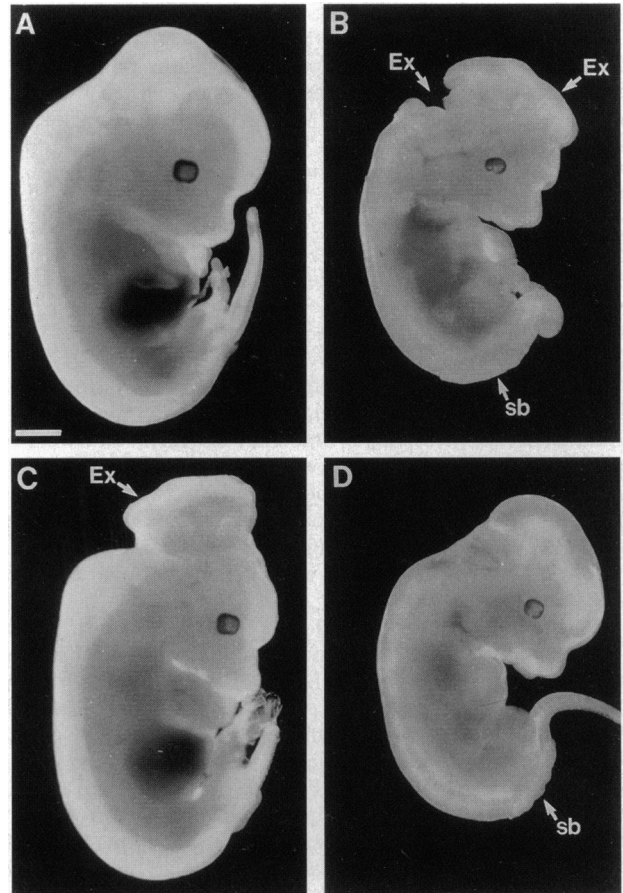


FIG. 2. NTD in F52-deficient embryos. Homozygous embryos at gestation day 12.5 exhibiting NTD: exencephaly (ex) together with spina bifida (sb) (B), exencephaly (C), and spina bifida (D). A day 12.5 wild-type embryo is also shown (A). Embryos were dissected and fixed in 2% paraformaldehyde. Note the curly tails in B and D.

Table 1. Embryos obtained from crossings between homozygous and heterozygous F52 mutants

Genotype	No. of embryos with phenotype					Total
	Exencephaly alone	Spina bifida alone	Exencephaly and spina bifida	Flexed tail	Normal	
-/-	26	2	6	2	21	57
+/-	4	1	1	0	45	51

Embryos from one homozygous female were examined at day 10.5 of pregnancy. Only three live embryos were found, two of which were exencephalic. An additional five embryos were absorbed at earlier stages of development. The high incidence of early embryonic death in addition to exencephaly explains the poor fertility of homozygous females.

A Few F52-Deficient Mice Have Tail Flexion Defects. Out of 59 adult homozygous mutants, 4 (7% of the total) exhibited a curl or a bend in the middle or base of the tail, indicating that this phenotype is partially penetrant.

Brain Development Is Abnormal in F52-Deficient Mice. Brain sections from adult mutants and wild-type littermates were stained with cresyl violet and examined by light microscopy. The corpus callosum was absent in 9 of 9 homozygous mutant mice and 1 of 9 heterozygotes analyzed. Ten wild-type littermates failed to show this trait. The axons that failed to form corpus callosum accumulated abnormally in the dorsolateral portion of the cerebral hemisphere, forming Probst's bundles (Fig. 3). The ventral hippocampal commissure and anterior commissure were intact in the mutants. The agenesis of the corpus callosum in the mutant animals was also demonstrated with silver stain (Fig. 3).

Nissl stain of coronal sections revealed a number of other abnormalities of the brains from homozygous mutant mice. First, the overall brain size was reduced, although the body weight was not significantly lower than that of wild-type littermates. Second, the six cortical layers were intact, but the cortex was thinner and its staining appeared to be more intense (Fig. 3). Finally, all four ventricles were enlarged (Fig. 3). No other anatomical differences were observed between brains of homozygous mutants and wild-type littermates. The abnormalities found in homozygous mutants suggest that F52 plays multiple roles in the development of the brain, particularly in the development of cortex-related structures. This is supported by the observation that F52 mRNA remained at fairly high levels well after neural tube formation in the developing brain, but not in other parts of the embryo (see below).

F52-Deficient Mice Have Abnormal Retinas. Nissl stain of retina sections from homozygous mutants indicated that the mutant retinas were thinner and compressed when compared with the retinas from wild-type animals, although the overall layered structure was not altered (data not shown). These abnormalities in mutant retina were strikingly similar to those observed in the cortex.

F52 Is Highly Expressed Along the Entire Length of the Neural Tube During Embryonic Development. Northern blot analysis was performed with total RNA isolated from C57BL/6 embryos at 8.5–17.5 dpc. F52 mRNA was found to be expressed at high levels from 8.5 dpc through 14.5 dpc. At 17.5 dpc, mRNA levels remained high in the brain but not in other parts of the embryo (Fig. 4). Thus, F52 mRNA is abundantly expressed during the period of neurulation.

The spatial distribution pattern of F52 transcripts in developing mouse embryos was analyzed by whole-mount *in situ* hybridization. At 8.5 dpc, F52 mRNA was detected in the neural tube from the procephalon to the future posterior neuropore (Fig. 5), being most abundant in the anterior neural plates. This is consistent with the high incidence of cranial NTD observed in F52 mutants. At 9.5 and 10.5 dpc, F52 transcripts were present in the neural tube along the entire

anterior–posterior axis of the embryos (Fig. 5). The other areas of expression included optic vesicle, otic pit, branchial arches, somites, and fore- and hindlimb buds (Fig. 5). No signals were detected in homozygous mutant embryos that were incubated with an antisense F52 RNA probe or in wild-type embryos incubated with the sense F52 RNA probe.

The expression of F52 in the neural tube was further analyzed by examining transverse sections of an embryo at 10.5 dpc. Transcripts were found in the neural epithelium of the neural tube (Fig. 5) and in many other tissues (data not shown). The expression of F52 in neural epithelium varied among cells at various differentiation stages, depending on their position along the neural tube. For instance, in the hindbrain, much stronger signals were detected in neural epithelial cells of the subventricular zone than in cells of the ventricular zone (Fig. 5). In contrast, F52 transcript was quite uniform in all neural epithelial cells of the spinal neural tube (data not shown).

DISCUSSION

We report here that a deficiency of F52 in mice leads to NTD that closely resemble human NTD.

F52-Deficient Mice as a Mouse Model of NTD. Several mouse mutants that exhibit NTD have been reported and the relevant genes have been cloned (22–25). Unlike NTD patients, all of these mutants exhibit additional defects. For instance, mouse mutants *splotch* and *extra toe* exhibit an array of abnormalities that are more similar to those found in human Waardenburg's syndrome type I (26, 27) and Greig's cephalopolysyndactyly (28), respectively. In contrast, the defects observed in our F52 mutants are very reminiscent of common human NTD. Both exencephaly and spina bifida occur in the mutants without other unrelated malformations. Hydrocephalus, which is sometimes associated with common human NTD, is also observed in the F52 mutant mice. The agenesis of the corpus callosum and other abnormalities in brain development are most likely secondary to NTD. Agenesis of the corpus callosum and other brain abnormalities have been documented in some patients with NTD (29, 30). The human homologue of the F52 gene has been cloned (X. H. Block, C. Maerteb, and L. M. L. Frensen, GenBank accession no. 38435). It is very likely that mutations in this gene are the cause of some, possibly many, human NTD.

Nutritional factors are known to be important etiological factors in human NTD. In particular, diet supplementation with folic acid has been shown to reduce the risk of NTD (31, 32). However, folic acid has not proven effective in previously reported mouse models (33). It will be interesting to determine the influence of these factors on F52-deficient mice.

Is *ct* a Mutation in the F52 Gene? The NTD observed in *ct* mice resemble those of F52 mutants. Moreover, the F52 gene and *ct* have been mapped to the distal part of chromosome 4 (8, 34), raising the possibility that mutations in F52 cause *ct*. Arguing against this possibility are the frequencies of mutant phenotypes in homozygous mutant F52 and *ct* mice. For F52 mutants, about 60% are exencephalic and about 10% have spinal NTD, whereas these frequencies are 5% and 60%, respectively, for *ct* mice (6, 7). The semidominant nature of the F52 and *ct* mutations complicates the interpretation of crosses between the two strains. While our results from crosses between F52 homozygous and *ct* homozygous

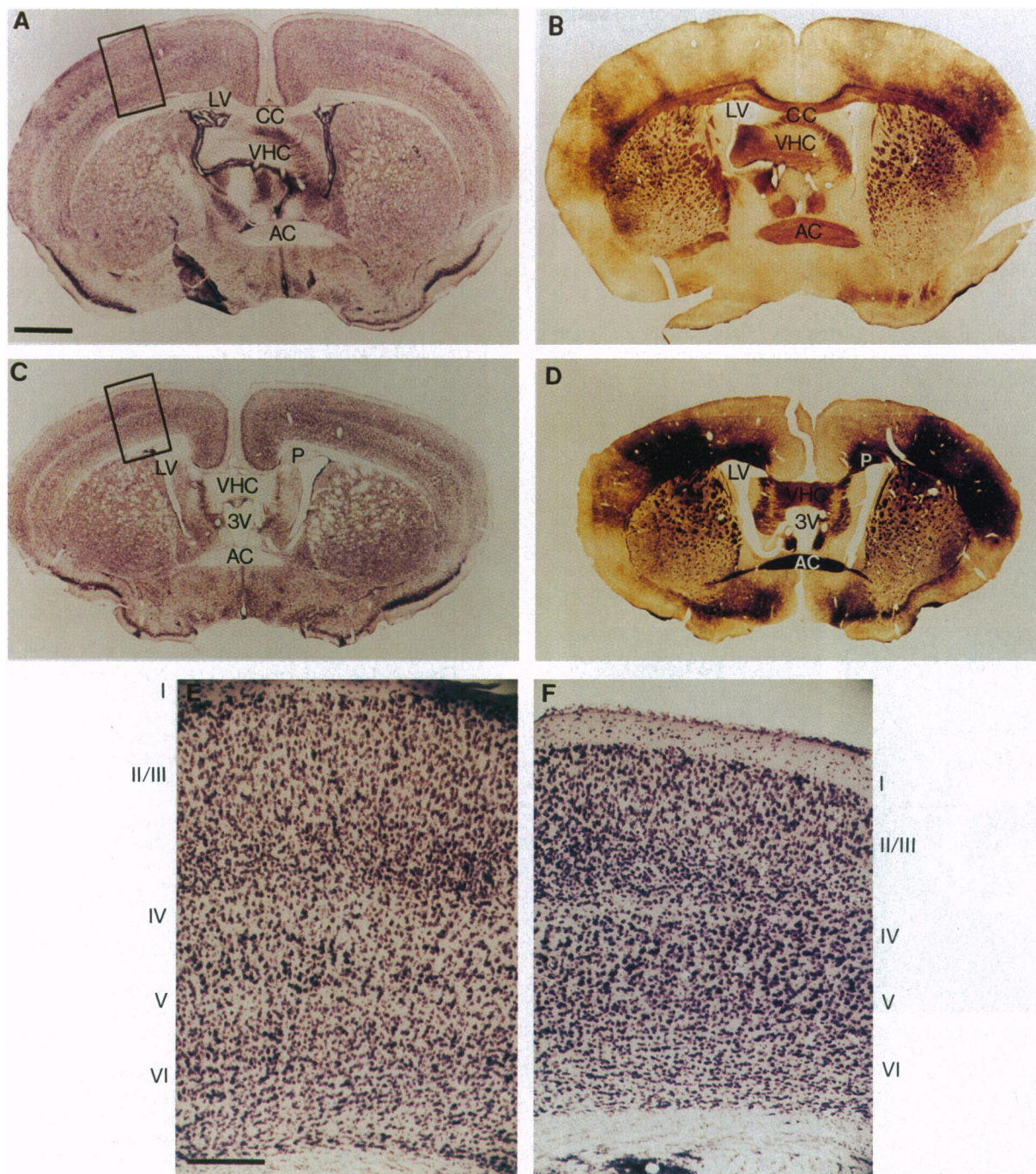


FIG. 3. Histological analysis of F52 homozygous mutant brain. Coronal sections of the brain of a homozygote (*C*, *D*, and *F*) and a wild-type littermate (*A*, *B*, and *E*), stained with cresyl violet (*A* and *C*) and silver (*B* and *D*). Note the presence of the corpus callosum (CC) in *A* and *B* and complete agenesis in the mutant mice in *C* and *D*. Also note the enlarged ventricles in *C* and *D*. The areas enclosed by boxes are shown at higher magnification in *E* and *F*, illustrating the intact but more intensely stained layers of the mutant cortex (*F*). The cortical layers are indicated as I–VI. Abbreviations: AC, anterior commissure; CC, corpus callosum; LV, lateral ventricle; 3V, third ventricle; P, Probst's bundles; VHC, ventral hippocampal commissure. (Bars: *A–D*, 1 mm; *E* and *F*, 200 μ m.)

mutants or between male *ct* homozygous and female F52 heterozygous mutants are not definitive, they are consistent with the possibility that the mutations are not allelic. Furthermore, we did not detect any nucleotide sequence differences within the protein-encoding regions between F52 cDNA derived from *ct* and wild-type mice, although mutation(s) may exist in a different part of the gene. Thus, most likely, F52 and *ct* mutations are not allelic.

What Is the Function of F52? The present work shows that F52 is necessary for normal development of the neural tube, the primordium of the central nervous system. However, the molecular and cellular functions of F52 remain to be elucidated.

Morphogenesis, including neurulation, requires cellular processes such as cell proliferation, cell shape change, and cell migration (35). All of these processes involve reorganization of the cytoskeleton as well as cell–cell signaling. F52, MARCKS, and PKC substrates such as GAP43 may mediate cytoskeletal change by integrating extracellular and intracellular signals and thereby regulate morphogenesis in different tissues. MARCKS- and GAP43-deficient mice have been produced; both mutants die either prenatally or in the early postnatal period (36, 37). MARCKS deficiency affects a wide range of developmental processes in the brain as well as other organs.

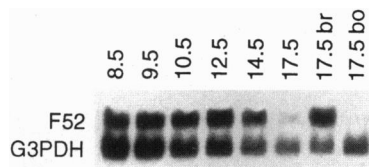


FIG. 4. Expression of the F52 gene during embryonic development. Northern blot analysis of RNA isolated from total embryos at gestation days indicated at the top. The same filter was successively hybridized with an F52 and a glyceraldehyde-3-phosphate dehydrogenase (G3PDH) antisense RNA probe. The RNA bands that correspond to F52 and G3PDH are indicated. Br, brain; Bo, fetal body without brain.

In particular, all forebrain commissures are absent and some mutants have exencephaly (36). Neuronal pathfinding is abnormal in GAP43 homozygous mutant mice (37). In particular, retinal axons are unable to cross the optic chiasm. The inability of axons to cross the midline at either the interhemisphere or the optic chiasm is a common feature of all three mutants.

Among known proteins, F52, MARCKS, and GAP43 are unique in that they have a single domain that responds to extracellular signals (via PKC) and affects internal cytoskeletal events (via actin). Our finding of NTD in the F52-deficient mice was serendipitous, but in retrospect, it makes mechanistic sense. The elucidation of the signal transduction pathways

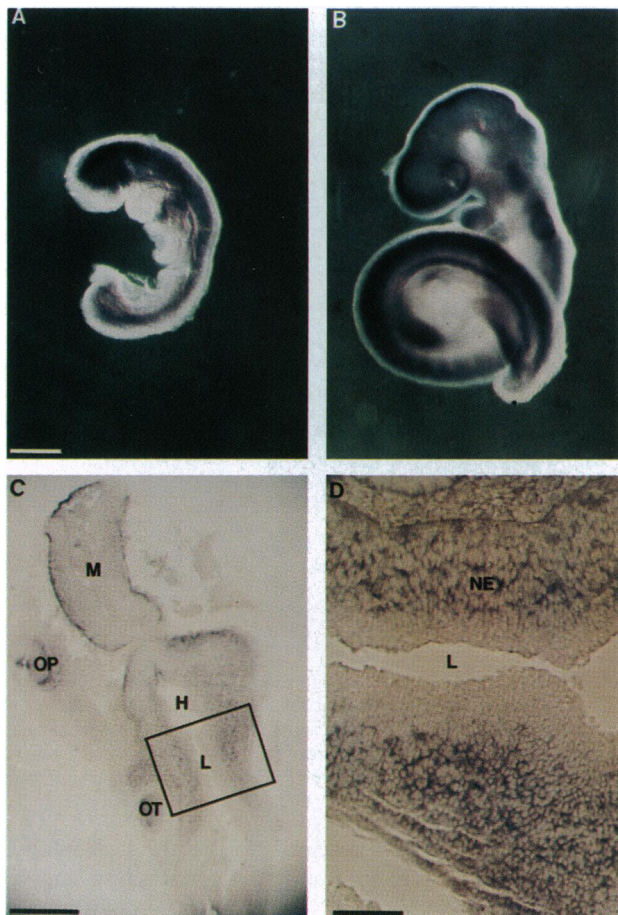


FIG. 5. *In situ* hybridization of mouse embryos. Embryos at gestation day 8.5 (A) and 9.5 (B) after hybridization with a F52 antisense RNA probe. A transverse section of a day 10.5 embryo through the midbrain and hindbrain (C) and a higher magnification of the indicated area showing the differential expression of F52 in neural epithelium of the hindbrain (D). Abbreviations: H, hindbrain; L, lumen; M, midbrain; NE, neural epithelium; OP, optic vesicle; OT, otic pit. (Bars: A and B, 1 mm; C, 200 μ m; D, 50 μ m.)

proceeding through F52 and identification of the relevant cellular events will increase our understanding of the molecular and cellular basis of neurulation and brain development.

We wish to thank L. Van Kaer and E. Li for advice on ES cell technology; B. Williams and C. W. L. Lovett for technical assistance; J. Yang for advice with embryo isolation; J. McMahon for advice on *in situ* hybridization; E. Freeman for advice on tissue sectioning; W. Quinn, W. Haas, H. Prosser, D. Gerber, J. Delaney, and L. Graziadei for critical reading of the manuscript; E. Basel for administrative work; and J. Li and the members of the Tonegawa lab for discussions. M.W. was supported by a postdoctoral fellowship from the Irvington Medical Institute, and T.S. is supported by a postdoctoral fellowship from the Stanley Foundation. This work was funded by the National Institutes of Health and the Howard Hughes Medical Institute.

1. Laurence, K. M. (1990) in *Principles and Practice of Medical Genetics*, eds. Emery, A. E. H. & Rimoin, D. L. (Churchill Livingstone, New York), pp. 323–346.
2. Khoury, M. J., Erickson, J. D. & James, L. M. (1982) *Am. J. Hum. Genet.* **34**, 980–987.
3. Hall, J. G., Friedman, J. M., Kenna, B. A., Popkin, J., Jawanda, M. & Arnold, W. (1988) *Am. J. Hum. Genet.* **43**, 827–837.
4. Copp, A. J., Brook, F. A., Estibeiro, J. P., Shum, A. S. & Cockroft, D. L. (1990) *Prog. Neurobiol.* **35**, 363–403.
5. Copp, A. J. & Bernfield, M. (1994) *Curr. Opin. Pediatr.* **6**, 624–631.
6. Grüneberg, H. (1954) *J. Genet.* **52**, 52–67.
7. Embury, S., Seller, M. J., Adinolfi, M. & Polani, P. E. (1979) *Proc. R. Soc. London B* **206**, 85–94.
8. Neumann, P. E., Frankel, W. N., Letts, V. A., Coffin, J. M., Copp, A. J. & Bernfield, M. (1994) *Nat. Genet.* **6**, 357–362.
9. Kato, K. (1990) *Eur. J. Neurosci.* **2**, 704–711.
10. Li, J. & Aderem, A. (1992) *Cell* **70**, 791–801.
11. Stumpo, D. J., Graff, J. M., Albert, K. A., Greengard, P. & Blackshear, P. J. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 4012–4016.
12. Seykora, J. T., Ravetch, J. V. & Aderem, A. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 2505–2509.
13. Erusalimsky, J. D., Brooks, S. F., Herget, T., Morris, C. & Rozengurt, E. (1991) *J. Biol. Chem.* **266**, 7073–7080.
14. Aderem, A. (1992) *Cell* **71**, 713–716.
15. Blackshear, P. J. (1993) *J. Biol. Chem.* **268**, 1501–1504.
16. Allen, L. A. & Aderem, A. (1995) *EMBO J.* **14**, 1109–1120.
17. Bradley, A. (1987) in *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, ed. Robertson, E. J. (IRL, Oxford), pp. 113–151.
18. Gallyas, F. (1979) *Neurol. Res.* **1**, 203–209.
19. Hess, D. T. & Merker, B. H. (1983) *Neurosci. Methods* **8**, 95–97.
20. Wu, M., Allis, C. D. & Gorovsky, M. A. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 2205–2209.
21. Wilkinson, D. G. (1992) in *In Situ Hybridization: A Practical Approach*, ed. Wilkinson, D. G. (IRL, Oxford), pp. 75–83.
22. Epstein, D. J., Vekemans, M. & Gros, P. (1991) *Cell* **67**, 767–774.
23. Schimmang, T., Lemaistre, M., Vortkamp, A. & Ruther, U. (1992) *Development (Cambridge, U.K.)* **116**, 799–804.
24. Hui, C. C. & Joyner, A. L. (1993) *Nat. Genet.* **3**, 241–246.
25. Homanics, G. E., Smith, T. J., Zhang, S. H., Lee, D., Young, S. G. & Maeda, N. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 2389–2393.
26. Tassabehji, M., Read, A. P., Newton, V. E., Harris, R., Balling, R., Gruss, P. & Strachan, T. (1992) *Nature (London)* **355**, 635–636.
27. Baldwin, C. T., Hoth, C. F., Amos, J. A., da-Silva, E. O. & Milunsky, A. (1992) *Nature (London)* **355**, 637–638.
28. Vortkamp, A., Gessler, M. & Grzeschik, K. H. (1991) *Nature (London)* **352**, 539–540.
29. Ruggiero, R., Mongini, T., Colangelo, M., Schiffer, D. & Ambrosio, A. (1987) *J. Neuroradiol.* **14**, 287–292.
30. Gilbert, J. N., Jones, K. L., Rorke, L. B., Chernoff, G. F. & James, H. E. (1986) *Neurosurgery* **18**, 559–564.
31. Wald, N., Sneddon, J., Densem, J., Frost, C., Stone, R. & Medical Research Council Vitamin Study Research Group (1991) *Lancet* **338**, 131–137.
32. Czeizel, A. E. & Dudas, I. (1992) *N. Engl. J. Med.* **327**, 1832–1835.
33. Seller, M. J. (1994) *Ciba Found. Symp.* **181**, 161–173.

34. Lobach, D. F., Rochelle, J. M., Watson, M. L., Seldin, M. F. & Blackshear, P. J. (1993) *Genomics* **17**, 194–204.
35. Schoenwolf, G. C. & Smith, J. L. (1990) *Development (Cambridge, U.K.)* **109**, 243–270.
36. Stumpo, D. J., Bock, C. B., Tuttle, J. S. & Blackshear, P. J. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 944–948.
37. Strittmatter, S. M., Fankhauser, C., Huang, P. L., Mashimo, H. & Fishman, M. C. (1995) *Cell* **80**, 445–452.