Disruption of the murine nuclear factor I-A gene (*Nfia*) results in perinatal lethality, hydrocephalus, and agenesis of the corpus callosum

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The phylogenetically conserved nuclear factor I (NFI) family of transcription/replication proteins is essential both for adenoviral DNA replication and for the transcription of many cellular genes. We showed previously that the four murine NFI genes (*Nfia*, *Nfib*, *Nfic,* and *Nfix*) are expressed in unique but overlapping patterns during mouse development and in adult tissues. Here we show that disruption of the *Nfia* gene causes perinatal lethality, with >95% of homozygous *Nfia*$^{-/-}$ animals dying within 2 weeks after birth. Newborn *Nfia*$^{-/-}$ animals lack a corpus callosum and show ventricular dilation indicating early hydrocephalus. Rare surviving homozygous *Nfia*$^{-/-}$ mice lack a corpus callosum, show severe communicating hydrocephalus, a full-axial tremor indicative of neurological defects, male-sterility, low female fertility, but near normal life spans. These findings indicate that while the *Nfia* gene appears nonessential for cell viability and DNA replication in embryonic stem cells and fibroblasts, loss of *Nfia* function causes severe developmental defects. This finding of an NFI gene required for a developmental process suggests that the four NFI genes may have distinct roles in vertebrate development.

The nuclear factor I (NFI) family of site-specific DNA-binding proteins plays a dual role in adenoviral DNA replication and in the transcription of viral and cellular genes. Early studies showed that NFI proteins are essential for adenovirus DNA replication both *in vitro* (1–4) and *in vivo* (5, 6) and suggested that NFI may function in cellular DNA replication. More recently, expression of the four NFI genes has been shown to be developmentally regulated (7–9), and NFI proteins have been implicated in the regulation of transcription of genes expressed in many tissues, including liver (10, 11), mammary gland (12, 13), and brain (14–16). To assess the role of individual NFI family members in cell growth and development, we have begun a genetic analysis of NFI function in mice.

The NFI gene family has four members in vertebrates (*NFI-A, NFI-B, NFI-C,* and *NFI-X*) (17, 18), a single member in the nematode *Caenorhabditis elegans* (*nfi-1*) (19), and no homologues in any of the sequenced prokaryotic or simple eukaryotic genomes. A single NFI gene in the cephalochordate *Amphioxus* suggests that the four vertebrate genes were generated by duplications during vertebrate evolution (19). The vertebrate NFI genes share a conserved 220-aa N-terminal domain that is sufficient for dimerization, DNA-binding, and stimulation of adenovirus DNA replication *in vitro* (20–22). Products of the four NFI genes form homo- and heterodimers that bind to the canonical NFI binding site [TTGGC(N)$_5$GCCAA] with apparently identical affinities (23–26). Transcripts of all four NFI genes are alternatively spliced, yielding as many as nine different proteins from each gene (26, 27). This multitude of NFI isoforms has complicated the analysis of the role of specific NFI genes in transcription and development.

While all NFI proteins appear to bind to the same DNA sequence, differences in function among NFI gene products have been seen in a number of systems. NFI-C/CTF proteins (CTF, CAAT-box transcription factor) differ in their abilities to activate transcription in transfected *Drosophila* cells and yeast, depending on the sequence of their C-terminal domains (20, 27). In addition, gene-, cell-type-, and promoter-specific differences in transcriptional modulation have been demonstrated for various NFI isoforms (28–31). These different activities, together with the finding that the four NFI genes are expressed in distinct but overlapping patterns during mouse development (7), suggests that each NFI gene may play a unique role in mouse development. To address the role of the *Nfia* gene in development we have disrupted the gene by homologous recombination in embryonic stem (ES) cells. Mice with a disruption of the *Nfia* gene die shortly after birth and have neuroanatomical defects, indicating an essential role for NFI-A-regulated transcriptional pathways in the development of the brain.

Materials and Methods

Disruption of the *Nfia* Gene. Genomic clones of the *Nfia* gene were isolated from a 129/Sv library by using the *Nfia* gene-specific primer described previously (7), and a combination of restriction mapping, PCR, DNA sequencing, and Southern analysis with exon 1a- and exon 2-specific probes was used to map the locations of exons 1a and 2. The targeting construct pNFI-A5′3′ was prepared by cloning a 7.2-kb *Eco*RI fragment containing exon 1a of *Nfia* and a 2.4-kb *Eco*RV–*Spe*I fragment containing the 3′ half of exon 2 of *Nfia* and adjacent intron sequences separated by a 1.8-kb PGK-neo expression cassette from pNT (32) (Fig. 1*A*). A PGK-TK negative selection cassette from pNT was cloned upstream of the *Nfia* gene segments (Fig. 1*A*). E14–1 cells (33) were electroporated with the targeting vector and neomycin- and ganciclovir-resistant clones were isolated (34). Four of 91 clones had a correctly targeted disruption of *Nfia* as shown by Southern blot and PCR analyses with probes and primers outside of the targeting vector. *Nfia*$^{-}$ ES cells were injected into C57BL/6 blastocysts and chimeric mice were bred to Black Swiss mice to transmit the disrupted allele. The *Nfia*$^{-}$ allele was transmitted from chimeras from two different ES cell clones, and both lines exhibited identical perinatal lethal phenotypes in homozygous *Nfia*$^{-/-}$ animals.

Analysis of *Nfia*-Deficient Mice. Mice heterozygous for the *Nfia*$^{-}$ allele (*Nfia*$^{+/-}$) were bred to generate homozygous *Nfia*$^{-/-}$ animals. Embryos were harvested by cesarean section at different times of gestation and RNA was prepared from samples frozen in liquid nitrogen (Trizol, Life Technologies). Reverse

Abbreviations: NFI, nuclear factor I; ES, embryonic stem; RT, reverse transcription; GFAP, glial fibrillary acidic protein; PLP, myelin phospholipid protein; E*n*, embryonic day *n*.

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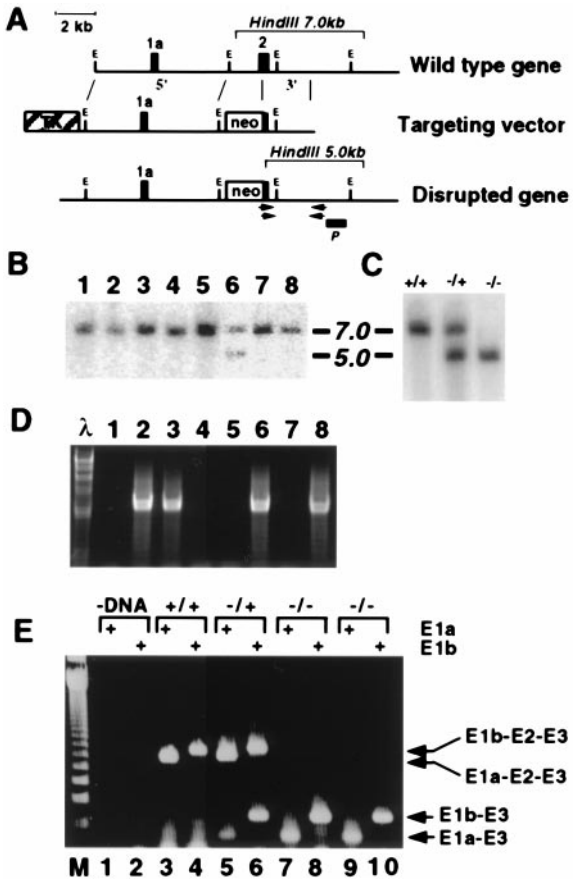

Fig. 1. Disruption of the murine *Nfia* gene and demonstration of ''exon-skipping.'' (*A*) The structure of a portion of the wild-type *Nfia* gene is shown with exons 1a and 2 as black boxes, *Eco*RI sites marked E, and the 7.0-kb *Hin*dIII fragment shown with a bracket. The targeting vector was constructed as described in *Materials and Methods,* and recombination within the 5′ and 3′ regions with the wild-type gene would yield the disrupted *Nfia* gene missing most of exon 2. (*B*) Clones of ES cells electroporated with the targeting vector were lysed and DNA was isolated, digested with *Hin*dIII, transferred to membranes, and probed with the probe shown as *P* in *A*. This probe lies outside of the targeting vector and detects a 7.0-kb band from the wild-type *Nfia* allele and a 5.0-kb band from the disrupted allele. Lanes 1–5, 7, and 8 show a wild-type pattern, while lane 6 shows both the wild-type and disrupted alleles. (*C*) Genomic DNA from the tails of +/+, +/−, and −/− animals was digested with *Hin*dIII and analyzed as in *B*. (*D*) The nested primers shown as arrows below the disrupted gene in *A* were used to amplify genomic DNA of the progeny of a mouse heterozygous for the disrupted *Nfia*$^{-}$ allele. Lanes 2, 3, 6, and 8 are positive for the 2.3-kb PCR fragment, while lanes 1, 4, 5, and 7 are negative. Size markers of *Hin*dIII-digested λ DNA are shown in the leftmost lane, marked λ. The rightmost set of nested primers from *A* lies outside of the targeting construct. (*E*) RNA from the brains of wild-type (lanes 3 and 4), heterozygous (+/−, lanes 5 and 6), and homozygous *Nfia*$^{-}$ mice (−/−, lanes 7–10) was isolated, reverse transcribed, and PCR amplified by using primers in exon 3 and either exon 1a or 1b of *Nfia* as denoted above the lanes. The products were analyzed on a 2% agarose gel along with 123-bp ladder markers (lane M). The arrows E1b-E2-E3 and E1a-E2-E3 show the positions of correctly spliced NFI-A mRNAs, while the arrows E1b-E3 and E1a-E3 show the positions of the products lacking exon 2. Lanes 1 and 2 contained no reverse transcribed product.

transcription (RT)-PCRs (Superscript II, Life Technologies) were analyzed by using primers specific for exon 1a (NFI-AE1, TGTATTCTCCGCTCTGTCTCAC) or exon 1b (NFI-AE1b, CTCCCGTGAGTTAGCATCAGATG) and exon 3 (NFI-AE3C, TGGCTGGGACTTTCAGATTGAC). RT-PCRs were analyzed by electrophoresis on 0.8% and 2% agarose gels. Some embryos and fetuses were fixed in 4% paraformaldehyde, Histochoice (AMRESCO), or snap frozen in OCT and cryosectioned or embedded in paraffin and sectioned. Immunostaining was performed as described previously (35) by using monoclonal antibodies to glial fibrillary acidic protein (GFAP) (G-A-5; Sigma) and myelin phospholipid protein (PLP) (M2; ref. 35).

Results

Disruption of the *Nfia* Gene. The *Nfia* gene is on mouse chromosome 4 flanked by the *Jun* and *Cyp4a10* genes (19). The *Nfia* gene was disrupted by homologous recombination of the flanking arms of the pNFI-A5′3′ vector with the wild-type allele, deleting the 3′ splice acceptor site and 219 bp of exon 2 (Fig. 1*A*). Disruption was confirmed by Southern blotting (Fig. 1 *B* and *C*) and PCR analyses with probes outside of the targeting vector (Fig. 1*D*). Because exon 2 of the *Nfia* gene encodes essentially the entire N-terminal NFI DNA-binding domain, any proteins produced from transcripts lacking this exon would lack DNA-binding or dimerization activity. Also, because the splice donor and acceptor sites of exon 2 of *Nfia* are spliced in different reading frames (19), transcripts lacking exon 2 would generate a missense polypeptide containing alternate first exons fused to an out-of-frame 37-aa polypeptide stopping at a termination codon in exon 3. To confirm that the disrupted *Nfia* allele generates a transcript lacking exon 2, RT-PCRs were performed on whole embryos homozygous for the *Nfia* disruption by using primers in exons 1a, 1b, and 3 (Fig. 1*E*). Exon 1a is the first exon of most NFI-A cDNAs, whereas exon 1b is the first exon in a subset of NFI-A cDNAs from brain and olfactory epithelium (ref. 14; data not shown). In brain RNA from homozygous wild-type embryos (*Nfia*$^{+/+}$), RT-PCR products consistent with the expected NFI-A transcripts were seen (Fig. 1*E*, lanes 3 and 4, upper arrows). In *Nfia*$^{-/-}$ mice, only PCR products of a size consistent with transcripts lacking exon 2 were seen (Fig. 1*E*, lanes 7–10, lower arrows), whereas in heterozygotes both transcripts were detected (Fig. 1*E*, lanes 5 and 6). Both restriction enzyme digestion and direct sequencing of the PCR products confirmed the predicted splicing of exons 1, 2, and 3 at the wild-type allele and direct splicing of alternate exons 1 and 3 of the mutant allele (not shown). Thus the disruption of exon 2 generates a functionally null allele of *Nfia*.

Homozygous *Nfia*$^{-}$ ES Cells and Fibroblasts Are Viable. Since NFI proteins are required for adenoviral DNA replication and have been implicated in the replication of other DNA viruses and of cellular DNA, we determined whether ES cells lacking NFI-A were viable. ES cells containing a single targeted *Nfia*$^{-}$ allele were cultured in the presence of high concentrations of G418 to select for cells containing only the targeted allele (36). Several clones were obtained which by both Southern blotting and PCR analysis contained only the targeted *Nfia*$^{-}$ allele and no wild-type allele (data not shown). These apparently homozygous *Nfia*$^{-}$ ES cell lines grew at a rate comparable to that of the heterozygous ES cells (data not shown). Fibroblasts from *Nfia* −/−, +/−, and +/+ embryos (see below) have also been prepared and show no obvious growth differences in culture (data not shown). These data indicate that the *Nfia* gene products are not required for DNA replication in the cell types examined.

Phenotype of *Nfia*$^{-}$ Mice. Heterozygous *Nfia*$^{+/-}$ mice were bred to generate animals homozygous for the *Nfia* disruption. In the random-bred Swiss background in which the mice were generated, ≈95% of the *Nfia*$^{-/-}$ animals died within 2 weeks of birth, with most animals dying on the day of birth (Table 1). There was also a loss of heterozygous *Nfia*$^{+/-}$ animals, suggesting potential haploinsufficiency at the *Nfia* locus. Consistent with haploinsufficiency, far fewer than 50% of the animals generated from breeding wild-type males with *Nfia*$^{+/-}$ females contained the

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Table 1. Loss of *Nfia*$^-$ homozygous and heterozygous mice from wild-type (WT)/*Nfia*$^-$ × WT/*Nfia*$^-$ crosses

	Number of progeny with genotype		
Progeny	WT/WT	WT/*Nfia*$^-$	*Nfia*$^-$/*Nfia*$^-$
Male	17	17	8
Female	18	23	5
Total	35	40	13*
(Expected)	(35)	(70)	(35)

Heterozygous WT/*Nfia*$^-$ mice were bred and the number of progeny of each genotype was scored. These numbers exclude any lost mice for which DNA was not obtained (<5%). The expected values are calculated assuming that the WT/WT homozygotes all survive and should represent 25% of the progeny. The apparent loss of heterozygotes (40/70) is not seen in male WT/*Nfia*$^-$ × female WT/WT crosses and may represent a maternal effect phenotype.

*Only 4 *Nfia*$^-$/*Nfia*$^-$ mice survived more than 6 weeks after birth (4/102 total pups from nine litters). Most *Nfia*$^-$/*Nfia*$^-$ pups die within 1 day of birth with a few surviving up to 2 weeks and <5% surviving 6 weeks.

Nfia$^-$ allele (Table 2). Surprisingly, no loss of *Nfia*$^{+/-}$ animals was seen when *Nfia*$^{+/-}$ males were bred to wild-type females, indicating a maternal effect in loss of *Nfia*$^{+/-}$ animals. When postnatally the *Nfia*$^-$ heterozygotes are lost and the nature of the maternal effect require further investigation. Loss of >90% of *Nfia*$^{-/-}$ mice has also been seen in N5 back-crosses into the inbred C57BL/6 strain. Of the rare *Nfia*$^{-/-}$ survivors, all were runted for the first 2 weeks of life (Fig. 2*A*, −/− vs. +/+) and had cranial defects suggesting brain abnormalities. The most obvious phenotype was a "dome head" frequently seen in mice with hydrocephalus as compared with the roughly wedge-shaped heads of wild-type mice (Fig. 2*B*, −/− vs. +/+). These obvious cranial defects diminished with time, and adult animals appeared relatively normal but possessed a tremor indicating neurological defects. Adult *Nfia*$^{-/-}$ males appeared sterile, whereas the females had very low fecundity but were able to give birth and to suckle young when bred to wild-type males (not shown). It is unclear whether the sterility in males was behavioral or physiological, since histological examination of the testes revealed no obvious abnormalities.

Genotyping of embryos at different times of gestation indicated that few or no *Nfia*$^{-/-}$ or *Nfia*$^{+/-}$ animals were lost prior to birth (data not shown). To determine whether major morphological defects present in embryos might contribute to the perinatal death, embryos were fixed, sectioned, and stained, and some embryos were used for skeletal and cartilage preparations. No obvious morphological defects were detected in such preparations, indicating similar skeletal and cartilage formation in the *Nfia*$^{-/-}$ and wild-type animals (data not shown). However, several clear brain defects were seen upon serial sectioning of the brains of embryonic day 18.5 (E18.5), newborn, and adult *Nfia*$^{-/-}$ animals. The rare adult *Nfia*$^{-/-}$ animals examined (8 total) had severe hydrocephalus, as noted by dilation of the lateral and third ventricles (Fig. 2 *C* vs. *D*, arrows denote expanded ventricles). Both intraventricular dye injections and serial sectioning of adult brains indicated that the hydrocephalus was communicating, with no obstructions detected in either the foramens of Munro or the aqueduct (data not shown). The cause of the hydrocephalus is not yet known.

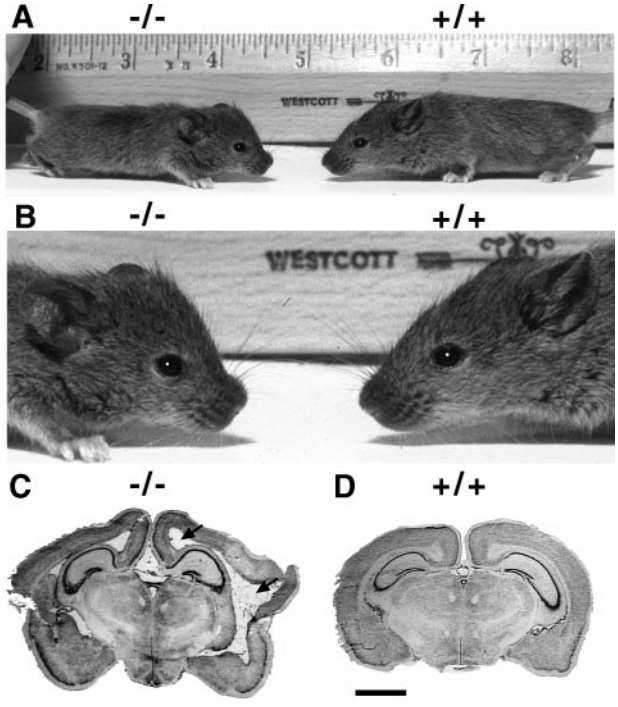

Fig. 2. Hydrocephalus in homozygous *Nfia*$^-$ mice. (*A*) A homozygous *Nfia*$^-$ pup (−/−) and a wild-type littermate (+/+) are shown. Note the smaller size and foreshortened head of the −/− animal. (*B*) Enlargement of *A* showing the characteristic "dome head" of the *Nfia*$^-$ (−/−) animal compared with the more wedge-shaped head of wild-type animal. (*C* and *D*) Cresyl violet stained coronal sections through the brains of 6-month-old *Nfia*$^{-/-}$ (−/−) and wild-type (+/+) animals. Note the dilation of the ventricles (arrows in *C*). (Bar in *D* = 2 mm.)

Table 2. Loss of heterozygous *Nfia*$^-$ progeny from heterozygous *Nfia*$^-$ mothers

	Number of progeny with genotype			
	Heterozygous *Nfia*$^-$ male × WT female		Heterozygous *Nfia*$^-$ female × WT male	
Progeny	+/+	−/+	+/+	−/+
Male	60	46	33	26
Female	41	40	35	13
Total	101	86	68	39*
(Expected)	(101)	(101)	(68)	(68)

Heterozygous *Nfia*$^-$ males were crossed with wild-type (WT) females and heterozygous *Nfia*$^-$ females were crossed with wild-type males. The genotype and sex of the progeny of the different crosses were recorded. All animals were in the outbred Black Swiss background. The reduction in the total number of heterozygous *Nfia*$^-$ animals compared to expected in the latter cross is significant (*, $P < 0.01$ by χ^2) and may be caused by preferential loss of heterozygous female progeny.

The second major defect seen in adult *Nfia*$^{-/-}$ animals was the absence of a corpus callosum. The corpus callosum is a major fiber tract formed during embryogenesis that connects the two hemispheres of the brain. This surprising finding of agenesis of the corpus callosum was noted during examination of serial sections of adult brains for the investigation of the hydrocephalic phenotype. Since the corpus callosum develops between ≈16 and 18 days post coitus (d.p.c.) in the mouse (37, 38), we examined serial sections of the brains of E18 C57BL/6 fetuses. By 18 d.p.c., the corpus callosum was well developed in wild-type and *Nfia*$^{+/-}$ animals but was absent from *Nfia*$^{-/-}$ animals (Fig. 3 *A* vs. *B*, expanded box in 3 *C* vs. *D*, −/− vs. +/+, labeled cc). Slight expansion of the lateral ventricles was frequently detected in coronal sections of brains of *Nfia*$^{-/-}$ animals suggesting that hydrocephalus begins to develop by E18, near the time of the development of the corpus callosum. While the corpus callosum was absent from the *Nfia*$^{-/-}$ mice, the anterior and hippocampal

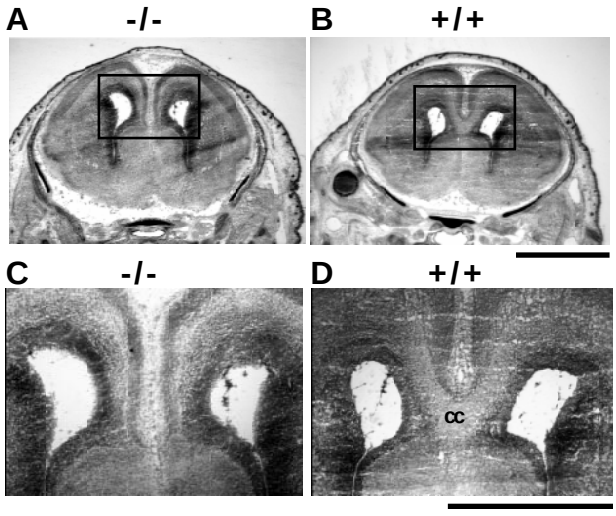

Fig. 3. Absence of corpus callosum in *Nfia*$^{-/-}$ mice. Cresyl violet-stained coronal sections thorough the brains of C57BL/6 fetuses 18 days post coitus. (*A* and *B*) Wide-field pictures of the brains of $-/-$ and $+/+$ littermates with the region surrounding the corpus callosum boxed. (*C* and *D*) Expansion of *A* and *B* showing the corpus callosum in the $+/+$ animal (labeled cc in *D*) and the absence of the corpus callosum in the $-/-$ animal. Serial sections throughout this region showed a complete absence of callosal development in $-/-$ animals. (Bars at the bottom right of *B* and *D* equal 2 and 1 mm, respectively.)

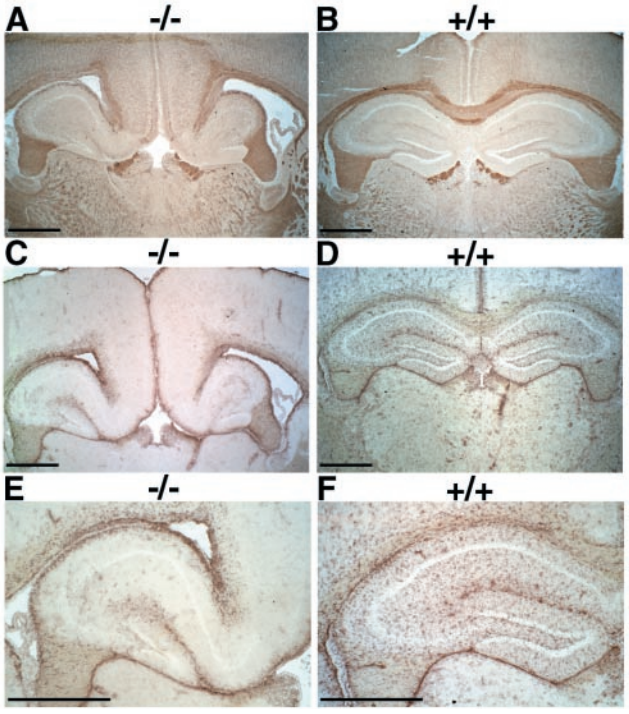

Fig. 4. Reduced GFAP expression in *Nfia*$^{-/-}$ mice. Coronal cryostat sections of the brains of 3-month-old Black Swiss *Nfia*$^{-/-}$ ($-/-$) and wild-type mice ($+/+$) were fixed and stained with antibodies against PLP and GFAP. (*A* and *B*) PLP expression in *Nfia*$^{-/-}$ (*A*, $-/-$) and wild-type (*B*, $+/+$) mouse brains. Note expanded ventricles indicating hydrocephalus and absence of the corpus callosum (tract lying directly above the hippocampus spanning the two hemispheres) in *A* vs. *B*. Expression of PLP appears normal in both animals in the tracts of the fimbria (dark sac-like regions on lower left and right of the hippocampus). (*C* and *D*) GFAP expression in *Nfia*$^{-/-}$ (*C*, $-/-$) and wild type (*D*, $+/+$) mouse brains. Note the severe reduction in GFAP staining in the cortex and hippocampus (as assessed by reduced levels of punctate cellular brown precipitate), the expanded ventricles, and the absence of corpus callosum in *C* vs. *D*. Loss of GFAP staining is regional with reduction of staining in the cortex and hypothalamus but retention of staining with some altered morphology in the fimbria (sacs below the hippocampus) and thalamic nuclei (not shown). (*E* and *F*) Higher magnification of GFAP expression in the left dentate gyrus regions of *C* and *D*. Note distorted dentate gyrus appearance (probably secondary to hydrocephalus) and reduced GFAP expression in the dentate gyrus in *E* vs. *F*. (Bars = 1 mm.)

commissures appeared normal in these animals (data not shown).

Since binding sites for NFI proteins have been implicated in the expression of GFAP and PLP in the brain (39, 40), we examined the expression of these proteins in adult *Nfia*$^{-/-}$ mice by immunocytochemistry. Wild-type mice showed normal patterns of expression of both PLP (Fig. 4*B*) and GFAP (Fig. 4*D*) with strong PLP expression in the fiber tracts of the corpus callosum (Fig. 4*B*, horizontal band above hippocampus) and fimbria (Fig. 4*B*, tracts below to the left and right of the hippocampus). GFAP expression was seen in astrocytes throughout the cortex and hippocampus (Fig. 4*D*). *Nfia*$^{-/-}$ mice showed relatively normal expression of PLP (Fig. 4*A*; note, however the expanded ventricles and absence of the corpus callosum vs. 4*B*) but had reduced levels of GFAP expression in both the cortex and hippocampus (Fig. 4 *C* vs. *D*; higher magnification of dentate gyrus in 4 *E* vs. *F*). Immunostaining with another astrocyte marker, S100, indicated similar numbers of total astrocytes in the *Nfia*$^{-/-}$ and *Nfia*$^{+/+}$ animals (data not shown), suggesting a down-regulation of GFAP expression in astrocytes in specific regions of the brain (see *Discussion*).

Discussion

These studies demonstrate that *Nfia* plays an important role in normal postnatal survival in the mouse and in the development of the corpus callosum in the developing brain. The ≈95% penetrance of lethality in the random-bred Swiss background suggests that while *Nfia* is important for normal postnatal survival, a small fraction of animals can survive to adulthood without *Nfia*, but they suffer severe neurological consequences. Given the previously described expression of *Nfia* at day 9 of embryonic development (7), it was somewhat surprising that *Nfia*-deficient mice survived embryonic development only to die perinatally (Table 1). The lack of major morphological defects in *Nfia*$^{-/-}$ embryos (excluding the brain) and the characteristic deformities in surviving *Nfia*$^{-/-}$ mice indicate that *Nfia* is not essential for development of most tissues and body structures (Figs. 2 and 3 and data not shown).

Even though the four NFI genes are expressed in broadly overlapping patterns during embryogenesis and adult life (7), there are apparently unique functions of the *Nfia* gene that cannot be compensated for by the other three NFI genes. It is of interest that the levels of expression of the other three NFI genes (*Nfib, Nfic,* and *Nfix*), as assessed by using quantitative real-time PCR (ABI Prism 7700), are about the same (<1.4-fold difference, data not shown) in brain RNA from adult wild-type and *Nfia*$^{-/-}$ mice. Thus we have no evidence for compensation for loss of *Nfia* by increased levels of the other three gene products in the brain. It is possible that increased levels of the other three NFI genes in other tissues may account for the lack of phenotype seen in these organ systems. Since products of the four NFI genes bind to the same DNA sequence but differ in their promoter-specific activation and repression of transcription, the proposed defect in gene expression caused by loss of *Nfia* gene products may be due either to loss of the *Nfia* gene product function per se, or conversely to increased binding of other NFI family members at promoters normally occupied by NFI-A proteins. Because NFI proteins bind DNA as either homo- or heterodimers (41), it is also possible that heterodimers of NFI-A

proteins with other NFI proteins, which would be missing in the *Nfia*$^{-/-}$ mouse, may be important in postnatal development. Since NFI proteins differ in their abilities to both activate and repress transcription from reporter genes (30, 31), it will be important to determine to what extent the *Nfia*$^{-/-}$ ES cells or fibroblasts exhibit changes in NFI-dependent gene expression. Such studies, together with analysis of global gene expression changes in tissues of the *Nfia*$^{-/-}$ mice, may allow us to determine the changes in NFI-A-responsive transcriptional pathways that lead to the morphological phenotypes seen.

One indication of the cause of the postnatal lethality is the phenotype of the rare *Nfia*$^{-/-}$ survivors. A tremor seen in all of the *Nfia*$^{-/-}$ survivors indicates neurological defects. The ''domehead'' phenotype of young *Nfia*$^{-/-}$ animals (Fig. 2*B*) is lost as the animals age, and it is unclear whether it plays an important role in later phenotypes, including sterility and tremor. Also, agenesis of the corpus callosum does not in general cause postnatal lethality; indeed, several inbred stains of mice show hypoplasia or agenesis of the corpus callosum (37, 42–44). However, the combination of callosal agenesis and hydrocephalus bears some resemblance to the CRASH syndrome in humans (corpus callosum hypoplasia, mental retardation, adducted thumbs, spastic paraplegia, and hydrocephalus), which results in a number of developmental defects in the brain. Since mutations in the L1 gene have been found in many patients with CRASH syndrome (45–47) and mutations in the mouse L1 gene cause similar neuroanatomical defects (48, 49), it would be of interest to determine whether expression of the L1 gene is affected in the *Nfia*$^{-}$ mice. However, hydrocephalus and/or agenesis of the corpus callosum is seen in several targeted and spontaneous mouse mutants [e.g., E2F-5 (50), Otx2 (51), Sek4 (52), βAPP (44), and CREB (53)], which suggests that a complex multigenic developmental pathway is disrupted in the *Nfia*$^{-/-}$ mice. The observed runting that the rare *Nfia*$^{-/-}$ survivors undergo during the first several weeks of postnatal life may also contribute either directly or indirectly to the phenotypes seen in the adult *Nfia*$^{-/-}$ animals. Thus it will be important to establish the molecular pathways that lead to each of these diverse phenotypes (perinatal lethality, hydrocephalus, agenesis of the corpus callosum, reduced GFAP expression, tremor, male sterility) and to determine whether early runting contributes to later defects.

Agenesis of the corpus callosum can occur through a number of mechanisms (54), including defects in (*i*) generation or survival of the cells whose axons form the callosum, (*ii*) axonal outgrowth, (*iii*) axonal pathfinding, (*iv*) axonal crossing of the midline, and others. Some clues as to which process(es) may be affected by loss of *Nfia* come from the embryonic expression pattern of *Nfia*, the brain-specific promoters that contain NFI binding sites, and the final neuroanatomy of the callosal defect. *Nfia* is expressed from embryonic day 9 in the developing nervous system, and strong expression continues in the cerebral cortex and other regions up to birth (7). Since the neurons that eventually extend processes across the midline to generate the corpus callosum originate in regions of the neocortex, it is possible that loss of *Nfia* gene products affects the growth, differentiation, or viability of these neurons. However, the precise number and location of the cell bodies of neurons that pioneer the corpus callosum is still under investigation, and markers specific for this subset of neurons are not yet available (37, 38, 55). Furthermore, in some acallosal strains of mice it has been suggested that the inability to cross the midline may result from deficiencies in the substrates found by axonal growth cones at the midline (56). Thus the aberrant expression of genes in nonneuronal cells that are important for growth cone guidance could generate this acallosal phenotype in the *Nfia*$^{-/-}$ mice. Given the previously reported high level of expression of *Nfia* at embryonic days 15–18 throughout the regions of the cerebral cortex known to contribute to the corpus callosum (7), the acallosal phenotype of the *Nfia*$^{-}$ mice suggests an important role for NFI-A proteins in either developing neurons or glial cells in this region of the brain. However, it will be necessary to assess cell generation and death rates, as well as the expression levels of the large number of molecules known to influence axon guidance and axon outgrowth to determine the specific (and likely multiple) transcriptional pathways affected by the loss of *Nfia* gene products in the brain.

While the promoter regions of several nervous system-specific genes are known to contain NFI-binding sites [e.g., MBP (14), NF(M) (57), and 5HT3R (15)], many of these genes are expressed in tissues only at times later than callosal formation (15–18 days post coitus) and thus are unlikely to play a role in the process. Furthermore, the apparent absence of Probst bundles in the *Nfia*$^{-/-}$ mice suggests that a relatively early step in the formation of the corpus callosum is affected (54). Probst bundles are aggregations of fibers that result from a failure of axons to cross the midline after their initial extension to the medial hemispheric walls. No Probst bundles were seen in serial sections of E18 brains, even in very rostral sections where bundles are seen at this stage in some other acallosal mice (52, 58). In adult brains the distortion of the ventricular walls due to extensive hydrocephalus made identification of Probst bundles problematic, although bulging of the medial ventricular wall was noted in some but not all older animals (data not shown). Such bulges may be compensatory changes caused by the hydrocephalus or indicate a relatively late onset of Probst bundle formation. Thus, while the absence of embryonic Probst bundles suggests a defect in a step prior to this accumulation of aberrant axons, future studies must focus on the expression of genes known to be involved in neuronal proliferation, survival, outgrowth, and pathfinding, either by genetic analysis of mutant mice defective in these processes or by *in vitro* analysis of these processes in callosal neurons from the *Nfia*-deficient mice.

The down-regulation of GFAP in the *Nfia*$^{-}$ mice is intriguing because in some congenital hydrocephalic disorders mild to strong astrogliosis has been noted (59), with GFAP being found in the cerebrospinal fluid of some hydrocephalic patients (60, 61). Also, in the congenital hydrocephalic HTX rat, reactive astrocytes are found in the more severely hydrocephalic rats relative to normal or mildly affected animals (62). Thus the absence of reactive gliosis in the *Nfia*$^{-/-}$ mice may reflect either an adaptation by astrocytes to the hydrocephalic neuropathology or possibly a direct role for *Nfia* gene products in GFAP expression. Indeed, binding sites for NFI proteins have been identified in the GFAP promoter, and mutation of one of these sites reduces GFAP gene expression (39, 63). It would therefore be of interest to determine whether the reactive astrogliosis seen in some mouse models of hydrocephalus and multiple sclerosis is abrogated in *Nfia*$^{-/-}$ mice (64).

The apparent loss of heterozygous *Nfia*$^{+/-}$ progeny only when the *Nfia*$^{-}$ allele is transmitted from the female parent suggests an unusual maternal effect phenotype. Such specificity of loss of *Nfia*$^{+/-}$ progeny could arise by a number of mechanisms, including (*i*) a defect in the *Nfia*$^{-}$ female parent that influences the survival only of *Nfia*$^{-/-}$ pups (true haploinsufficiency in both female parent and progeny); (*ii*) the *Nfia* alleles may be differentially expressed from the maternal and paternal genomes (imprinting); (*iii*) loss of maternally inherited *Nfia* gene products causes partially penetrant postnatal lethality (maternal effect phenotype); or (*iv*) a combination of the above. We have initiated long-term studies of fostering pups of *Nfia*$^{-}$ females and assessment of *Nfia* levels in *Nfia*$^{+/-}$ animals containing either maternal or paternal *Nfia*$^{-}$ alleles to determine the cause of the perinatal lethal phenotype. Indeed, since all heterozygous *Nfia*$^{-}$ animals examined have normal corpus callosums, a detailed analysis of the perinatal lethality in the heterozygotes may yield important insights into the proximate cause of death in the

absence of potential physiological compensation for callosal agenesis and hydrocephalus.

The rare survival of *Nfia*$^{-/-}$ mice may provide insights into genes or conditions that can modify the lethal phenotype in *Nfia*$^{-}$ mice. Survival may be because of either a stochastic process in which a small fraction of homogeneous animals survive, or genetic heterogeneity in the random-bred Black Swiss strain used in these studies. We are currently breeding the *Nfia*$^{-}$ allele into various inbred strains of mice to determine whether the degree of lethality is affected by genetic background. If a strain is found where lethality is significantly decreased, it should be possible to map the genes that affect lethality in *Nfia*$^{-}$ mice. Such genes would be good candidates for cellular factors that interact with *Nfia* or other NFI gene products and affect the transcription or expression of NFI-dependent genes.

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