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Lissencephaly Gene (*LIS1*) Expression in the CNS Suggests a Role in Neuronal Migration

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Miller-Dieker lissencephaly syndrome (MDS) is a human developmental brain malformation caused by neuronal migration defects resulting in abnormal layering of the cerebral cortex. *LIS1*, the gene defective in MDS, encodes a subunit of brain platelet-activating factor (PAF) acetylhydrolase which inactivates PAF, a neuroregulatory molecule. We have isolated murine cDNAs homologous to human *LIS1* and mapped these to three different chromosomal loci (*Lis1, Lis3, Lis4*). The predicted sequences of murine Lis1 protein and its human homolog LIS1 are virtually identical. In the developing mouse and human, *Lis1* and *LIS1* genes were strongly expressed in the cortical plate. In the adult mouse *Lis1* transcripts were abundant in cortex and hippocampus. The direct correlation between cortical defects in MDS patients and *Lis1* expression in the murine cortex suggest that the mouse is a model system suitable to study the mechanistic basis of this intriguing genetic disease.

Sequence data are deposited as L25108 for mouse *Lis1* cDNA and L25109 for mouse *Lis3-4* cDNA.

[Key words: cerebral cortex, cerebellum, neurogenesis, Miller-Dieker syndrome, lissencephaly, platelet-activating factor acetylhydrolase,* in situ *hybridization]

Lissencephaly is a human brain malformation characterized by a smooth cerebral surface and a disordered organization of the cortical layers believed to result from a defect in neuronal migration (Barth, 1987; Aicardi, 1989). Two types of lissencephaly have been defined (Aicardi, 1991; Kuchelmeister et al., 1993). In type I, the cortex consists of four layers whereas in type II the cortex is unlayered. In the case of type I lissencephaly, the cortex consists of the molecular layer (layer 1), layer 2 harboring neurons with a morphology normally seen in layers 3, 5 and 6. Layer 3 in type I lissencephaly is cell sparse, and layer 4 contains neurons that are misplaced and seem to be arrested in their migration (Barth, 1987; Aicardi, 1989). Examples of clinical manifestations of type I and type II lissencephaly are the Miller-Dieker syndrome (Miller, 1963; Dieker et al., 1969) and the Walker-Warburg syndrome, respectively. A gene termed *LIS1* that is involved in Miller-Dieker syndrome has recently been isolated and is located on human chromosome 17p13.3 (Reiner et al., 1993). *LIS1* encodes a polypeptide with "WD-40 repeats" found in proteins with diverse functions (Duronio et al., 1992). It has now been shown that the bovine homolog of *LIS1* is a subunit of the brain platelet-activating factor acetylhydrolase (Hattori et al., 1994). This trimeric enzyme inactivates platelet-activating factor, an alkyl-ether phospholipid implicated in various aspects of neural development (Kornecki and Ehrlich, 1988) and in neuronal function (Bito et al., 1992; Clark et al., 1992; Kato et al., 1994).

LIS1 is so far the only gene isolated whose mutation causes abnormal layering of the cortex. Therefore, the study of *LIS1* gene expression may shed light on the molecular and cellular mechanisms of neuronal migration which is required for proper cortical development. We have chosen the mouse as an experimental system to elucidate the potential function of lissencephaly genes in brain development. Toward this objective, we have isolated and characterized by map position and DNA sequence mouse cDNAs that hybridized to human *LIS1*. The expression of each cDNA was studied by Northern analysis and the distribution of *Lis1* mRNA, the mouse homolog of human *LIS1*, was studied by *in situ* hybridization in the developing mouse central nervous system. An expression analysis of *LIS1* in human fetal brain was also carried out. The studies to be reported here led us to the conclusion that the human *LIS1* and mouse *Lis1* are expressed, in part, in regions of the embryo that undergo neuronal migration.

Materials and Methods

DNA analysis. 5×10^6 plaque forming units (pfu) of a lambda ZAPII library, and 10^6 pfu of a lambda gt11 library were screened. Both libraries were generated from adult mouse brain. Sequencing was done essentially as described (Fu et al., 1993) with slight modifications. cDNA fragments were subcloned into BlueScript II KS$^+$ (Stratagene, La Jolla, CA), and plasmid DNA was prepared using Qiagen kits (QIAGEN, Chatsworth, CA). Inserts were gel-purified using Geneclean (BIO 101, La Jolla, CA), sonicated and size-selected by agarose gel electro-

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Table 1. Probes used in genetic mapping

Locus	Gene name	Probe	R.E.	Size AEJ/Gn	Size *M. spretus*[a]	Reference
A. RFLPs						
Acrb	Acetylcholine receptor β	pAchrb	*Taq*I	8.8, 1.7	4.8, 3.1, 1.7	Heidmann et al. (1986)
Evi2a	Ecotropic viral integration-2a	pBK4	*Bgl*I	18.4	13.6	Buchberg et al. (1990)
Lis	Lissencephaly	LIS-ORF	*Pst*I	9.1, 4.6, 3.4, 1.7	10.9, 1.4, 0.9[b]	Reiner et al. (1993)
Myhs	Myosin heavy chain, skeletal	p32	*Bam*HI	13.6, 4.7	13.6, 10.7	Weydert et al. (1983), Suter (1992)
Ngfb	Nerve growth factor β	pmngf6	*Hin*dIII	6.9	4.9	Scott et al. (1983)
Tpi-rs2	Triose phosphate isomerase, related sequence-2	pHTPI-5A	*Hin*dIII		3.5	Siracusa et al. (1991)
Tpi-rs3	Triose phosphate isomerase, related sequence-3	pHTPI-5A	*Hin*dIII		20	Siracusa et al. (1991)
B. SSLPs						
D3Mit22		DNA segment, Chr 3 MIT 22		240	210	Dietrich et al. (1992a,b)
D7Mit21		DNA segment, Chr 7 Mit 21		130	170	Dietrich et al. (1992a,b)
D7Mit27		DNA segment, Chr 7 Mit 27		240	220	Dietrich et al. (1992a,b)
D11Mit7		DNA segment, Chr 11 Mit 7		146	180	Dietrich et al. (1992a,b)

The table lists the loci used to generate the linkage map of each chromosome. The first three columns define the locus, the gene name, and the probe name. The fourth column, R.E., represents the restriction endonuclease used to determine the allele distribution pattern in the N2 mice. The next two columns describe the size of the restriction fragments inherited from the AEJ/Gn and *M. spretus* chromosome, respectively, in kilobases for section A and in base pairs for section B.

[a] The underlined restriction fragments listed in section A and PCR products listed in section B identify the segregating *M. spretus* alleles that were followed in the N2 progeny for their presence or absence in each mouse.

[b] The 10.9 kb restriction fragment identifies the *Lis1* locus, the 1.4 kb restriction fragment identifies the *Lis3* locus, and the 0.9 kb restriction fragment identifies the *Lis4* locus.

phoresis. DNA fragments sized 0.9–1.5 kb were recovered from the gel by Geneclean, and the DNA ends were made blunt ended by mung bean nuclease and T4 DNA polymerase (Pharmacia LKB Biotechnology, Piscataway, NJ). DNA fragments were ligated into an M13 vector. DNA from white plaques was isolated and sequenced with dideoxynucleotide termination reactions. Sequencing reactions were prepared on a Biomek 1000 Automated Labstation according to a cycle sequencing protocol. Reactions were analyzed on an automated DNA sequencer (ABI 373) (Applied Biosystems, Foster City, CA) with fluorescently labeled oligonucleotide primers. Sequence ambiguities were then resolved by ABI dye terminators with the use of specific primers. Sequence information was assembled with SEQUENCE ASSEMBLY MANAGER software (Molecular Biology Computational Resource, Department of Cell Biology, Baylor College of Medicine). Southern and northern blot analyses were done by standard protocols (Sambrook et al., 1989). Northern blots were from adult mouse tissues and were purchased (Clontech, Palo Alto, CA).

Generation of specific probes. For mouse probes, the probe from the open reading frame was a 285 bp fragment generated by PCR using primers 5′-CTGACGTCCCACATCTTAATAG-3′ and 5′-GCTACTGTGTGAAGACATTCAC-3′; the *Lis3-4* 3′-specific probe was a 262 bp fragment generated by PCR using primers 5′-GCACTTCTACCAGCTGAACC-3′ and 5′-GCAGCCATGAAGTAGCAGTG-3′; the probe for *Lis1* 3′-specific probe was a 296 bp fragment generated by restriction digest of *Lis1* by *Xho*I and *Nhe*I.

For human probes, the specific probe for the *LIS1* 3′ end was a 521 bp fragment generated by restriction enzyme cleavage of clone 6-1 using *Eco*RI and *Bsp*HI, subcloned in BlueScriptII KS+; the open reading frame probe used for screening mouse cDNA libraries was an *Nco*I-*Eco*RI fragment derived from clone 47.

RNA in situ hybridization. Embryo collection, sectioning and *in situ* hybridization were performed as described previously (Sundin et al., 1990). Antisense and sense RNA probes labeled with α^{35}S-UTP (1000 Ci/mmol, Amersham, Arlington Heights, IL) were synthesized with T7 or T3 RNA polymerase using *Eco*RI- or *Hin*dIII-linearized *Lis1* and *Lis3-4* ORF constructs, or *Eco*RI- and *Sal*I-linearized *Lis3-4* templates (see Fig. 2). Hybridization was done overnight at 50°C with a probe concentration of 0.24 or 0.09 ng/μl, depending on the probe. Posthybridization treatments were as follows: (1) two washes in 50% formamide, 2× SSC, 20 mM β-mercaptoethanol (FSH) at 63.5°C for 30 min, (2) digestion with 10 μg/ml RNase A in 4× SSC, 20 mM Tris-HCl (pH 7.6), 1 mM EDTA at 37°C for 30 min, and (3) two washes in FSH at 63.5°C for 45 min. Slides were dipped in Kodak NTB-2 emulsion and exposed for 10 d. Nissl staining with thionin was done as described (Putt, 1972). Early stages of mouse embryos (E10.5–E13.5) were sagittally sectioned, and later stages (E14.5–E16.5) were sectioned coronal. For stages after E16.5, brains were dissected and coronally sectioned except for the adult brains which were sectioned in the horizontal plane.

Genetic mapping. The interspecific backcross between (AEJ/Gn-*a* bp^H/a bp^H × *Mus spretus*) F1 × AEJ/Gn-*a* bp^H/a bp^H has been previously described (Marini et al., 1993). Genomic DNA extractions, restriction endonuclease digestions, agarose gel electrophoresis, and Southern blot transfers, hybridizations, and washes were as described previously (Ma et al., 1993). DNA oligonucleotides used for detecting simple sequence length polymorphism (SSLP) markers (Dietrich et al., 1992a) were made using an Applied Biosystems Model 394 DNA synthesizer. SSLP markers were detected by amplifying genomic DNA from N2 animals using the specified DNA oligonucleotide pairs and Taq DNA polymerase as described (Ma et al., 1993).

Results

Genetic mapping of LIS clones in the mouse

We mapped the murine *Lis* genomic loci and afterwards isolated cDNA clones. Genomic DNA from parents of the interspecific backcross (AEJ/Gn and *M. spretus*) was digested with several restriction endonucleases and analyzed by Southern blot hybridization. The open reading frame of the human *LIS1* cDNA was used to identify restriction fragment length polymorphisms (RFLPs), useful for establishing the map location. Three polymorphic restriction fragments were identified with *Pst*I. The segregation patterns of these three polymorphic restriction fragments were followed in random subsets of 195 N2 backcross progeny. Segregation analysis revealed that the three restriction

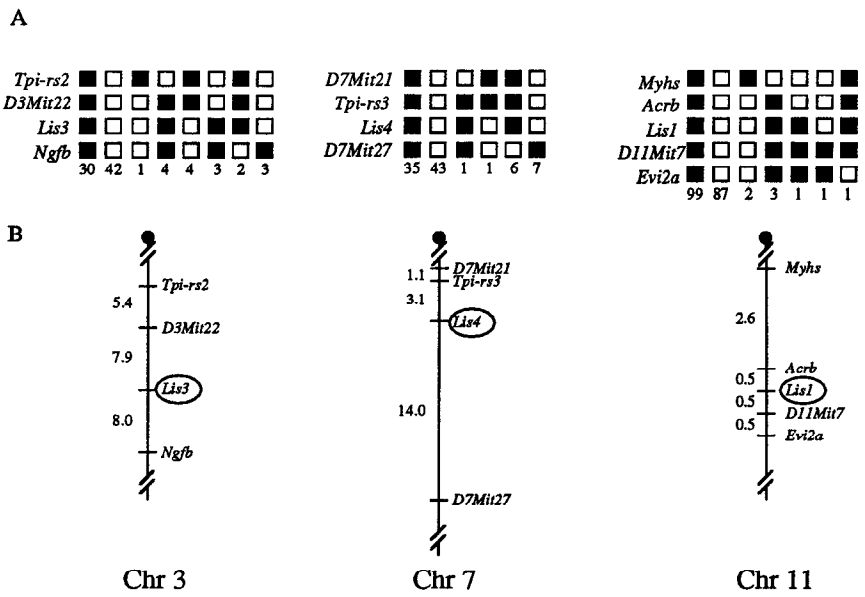

Figure 1. Genetic mapping in the mouse. *A*, Summary of the results of the interspecific backcross analysis for chromosomes 3, 7, and 11, respectively. Genes mapped in the analysis are listed on the left. Each column represents the chromosome identified in the N2 progeny inherited from the (AEJ/Gn × *M. spretus*) F1 parent. The solid boxes represent the AEJ/Gn allele and the open boxes represent the *M. spretus* allele. The number of each type of chromosome identified in the backcross progeny is listed at the bottom. Haplotype analysis of chromosomes 3, 7, and 11 were performed using a subset of the N2 offspring in which each locus was scored. The order of the loci and the ratio of the number of recombinants to the total number of N2 offspring examined for each locus are as follows: chromosome 3, *Tpi-rs2* − 5/92 − *D3Mit22* − 7/88 − *Lis3* − 12/150 − *Ngfb*; chromosome 7, *D7Mit21* − 1/93 − *Tpi-rs3* − 5/161 − *Lis4* − 13/93 − *D7Mit27*; chromosome 11, *Myhs* − 5/194 − *Acrb* − 1/194 − *Lis1* − 1/194 − *D11Mit7* − 1/194 − *Evi2a*. *B*, Placement of the genes on their respective chromosomes (*Chr*, chromosome). Genetic localization of *Lis3*, *Lis4*, and *Lis1* (*circled* on each chromosome). The figure represents the regions of mouse chromosomes 3, 7, and 11 analyzed in the interspecific backcross. The genes mapped are listed on the right of each chromosome and the genetic map distance (in cMs) between adjacent loci, are listed on the left of each chromosome. The genetic distances between the loci in cM ± SE are as follows: chromosome 3, *Tpi-rs2* − 5.4 ± 2.4 − *D3Mit22* − 7.9 ± 2.9 − *Lis3* − 8.0 ± 2.2 − *Ngfb*; chromosome 7, *D7Mit21* − 1.1 ± 1.1 − *Tpi-rs3* − 3.1 ± 1.4 − *Lis4* − 14 ± 3.6 −*D7Mit27*; chromosome 11, *Myhs* − 2.6 ± 1.1 − *Acrb* − 0.5 ± 0.5 − *Lis1* − 0.5 ± 0.5 − *D11Mit7* − 0.5 ± 0.5 − *Evi2a*.

fragments segregated independently. Comparison of the segregation patterns with locations of known markers in this mapping panel (Table 1) indicated that these loci mapped to chromosomes 3, 7 and 11 (Fig. 1). The gene order was resolved by minimizing the number of multiple recombinants along the length of each chromosome. It thus appears that the mouse genome contains several *Lis* genes, reminiscent of the situation in the human genome that contains at least two *LIS* genes, one on chromosome 17 (*LIS1*) and the other one on chromosome 2 (*LIS2*, O. Reiner et al., unpublished observations) (Reiner et al., 1993). Hybridizing the 3′ untranslated region of the *Lis1* cDNA (Fig. 2*B*) and using several different restriction endonucleases, we detected a single polymorphic restriction fragment that concordantly segregated with the chromosome 11 locus. Thus, this locus was designated *Lis1*. The localization of *Lis1* to mouse chromosome 11 between *Acrb* and *Evi2a* is consistent with its position on human chromosome 17. Based on both sequence comparison (see below) and linkage analysis, *Lis1* most likely represents the murine homolog of human *LIS1*. The restriction polymorphisms detected by the 3′ untranslated region of the *Lis3-4* cDNA (Fig. 2*B*) identified chromosome 3 and 7 loci. Since the 3′ end of the *Lis3-4* cDNA does not share homology with either *LIS1* or *LIS2*, these loci were designated *Lis3* and *Lis4*, respectively, and potentially represent new members of the *Lis* gene family.

Isolation of mouse Lis *clones*

Next, two adult mouse brain cDNA libraries were screened with a probe containing the open reading frame of the human *LIS1* cDNA. Forty cDNAs were isolated and partial sequencing allowed classification into three groups. Group 1 cDNAs were designated *Lis1* (Fig. 2*B*) because they hybridized to the *Lis1* locus (see previous section). Group 2 cDNAs were termed *Lis3-4* (Fig. 2*B*) and originated from either the *Lis3* or the *Lis 4* locus. A third group of cDNAs consisted of clones that contained a short 3′ untranslated region with a polyA track located 107 bp after the stop codon (data not shown). These cDNAs were possibly generated by use of alternative polyadenylation sites, thus resembling the previously described short human *LIS1* cDNA (47) (Fig. 2*A*). One of the group 1 cDNAs was sequenced and exhibited high nucleotide sequence similarity to the 5′ untranslated region, the open reading frame region, and the 3′ untranslated region of the human *LIS1* cDNA (*LIS1* clone 71, Fig. 2*A,B*). Since the deduced amino acid sequence of this cDNA was identical to human *LIS1* (except for a methionine substituted by a valine), it was given the name *Lis1*. *Lis3-4* and *Lis1* diverged 71 bp prior to the stop codon, and the predicted amino acid sequence of *Lis3-4* was shorter by 19 amino acids. Moreover, the C-terminal five amino acids of *Lis3-4* differed from those of *Lis1*. The 3′ untranslated sequence of *Lis3-4* had no similarity to the other *Lis* clones and contained a mouse repetitive sequence of the B2 family (Bladon and McBurney, 1991). The evolutionary relationship between the human and mouse genes are the focus of continuing studies.

Northern blot analysis in adult mouse tissues

For Northern analysis of adult mouse RNA two probes specific for each locus were used (marked in Fig. 2*B*). One probe was derived from the *Lis1* cDNA ("*Lis1* probe"), and the second is

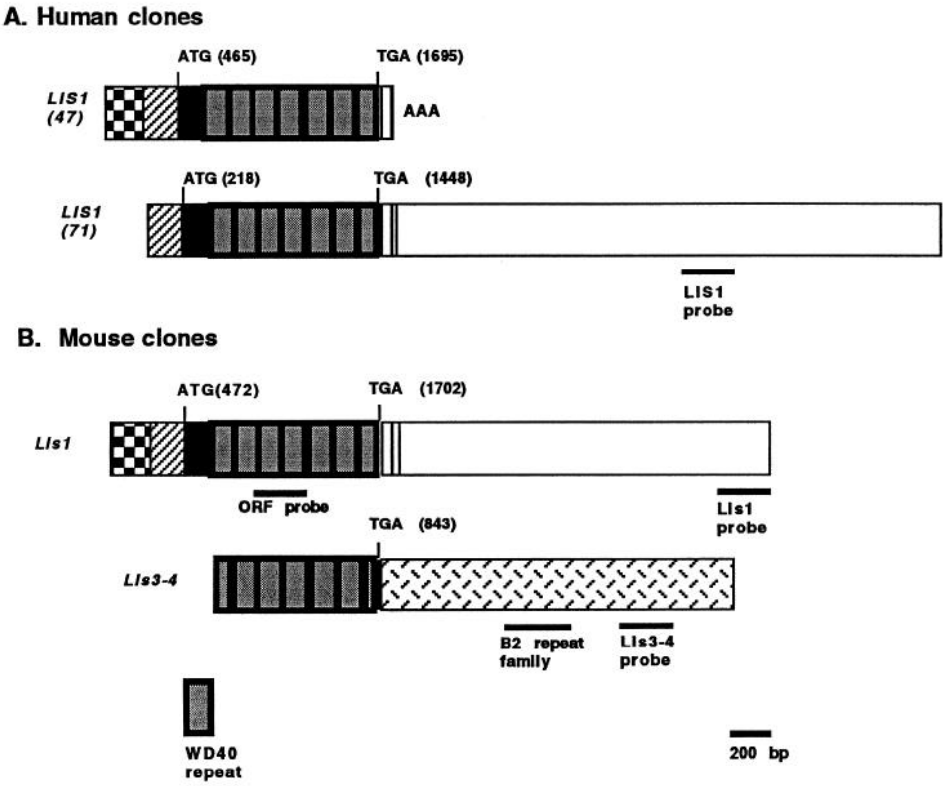

Figure 2. Comparison of the structure of human and mouse *Lis* cDNAs. Identical shading patterns indicate largely similar nucleotide sequences. The overall nucleotide sequence similarity between mouse *Lis1* and human *LIS1* (71) is 89.5%, whereas the overall similarity between these *Lis1* and *LIS1* (47) is 93.3%. *Lis3-4* and *LIS1* (71) are 95% identical within an open reading frame region extending between nucleotides 575–1376 of *LIS1*. The positions of the starting methionine codon (ATG) and termination codon (TGA) are indicated. For the *Lis3-4* cDNA N-terminal sequences and 5′ untranslated sequences have not yet been determined. Mouse B2 repetitive elements are found in the *Lis3-4* cDNAs between nucleotides 1036–1289. The WD40 motif repeats are shown. The *Lis* probes used for Northern blots and *in situ* hybridization are indicated.

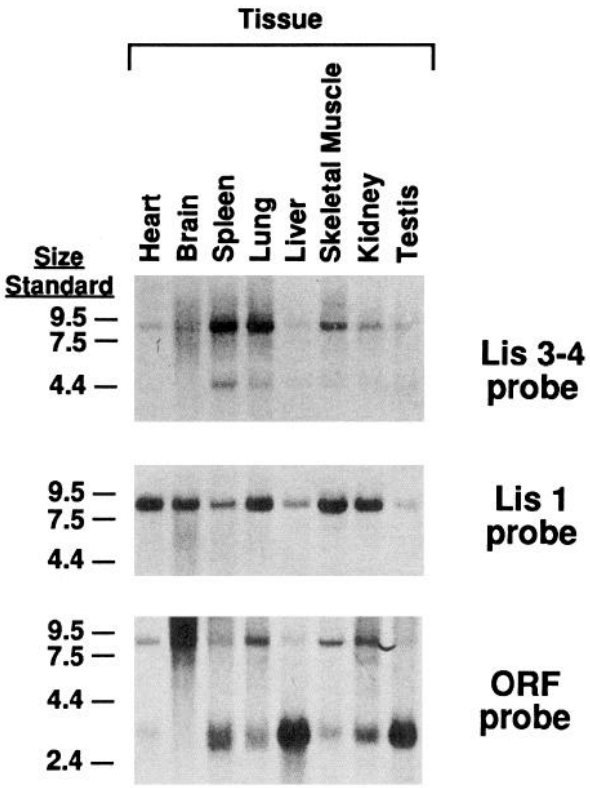

Figure 3. Northern blot analysis of adult mouse tissues. Two micrograms of polyA$^+$ RNA transferred to nylon membrane (Mouse Multiple Tissue Northern, Clontech) were hybridized to three probes (marked in Fig. 2*B*), a probe specific for *Lis1*, a probe specific for *Lis3-4* and the ORF probe that is common to both cDNAs. *Lis3-4* probe detects transcripts of 8 and 4.4 kb in length, while the *Lis1* probe detects mainly an 8 kb message. The ORF probe detects messages of 3 and 8 kb in size.

specific for *Lis3-4*. Additionally hybridized was a third probe from an open reading frame sequence ("ORF probe") that was shared by all the known murine *Lis* cDNAs. The analysis demonstrated expression in all tissues tested for each of the specific probes (Fig. 3). The probe specific for *Lis3-4* detected two main transcripts of 8 and 4.4 kb in length with the highest level of expression detected in spleen and lung. In contrast, *Lis3-4* expression was low in brain. Interestingly, in skeletal muscle two additional bands of 6 and 10 kb were seen. *Lis1* transcripts are 8 kb in length and are expressed in all tissues examined with somewhat lower levels in spleen, liver and testis. The ORF probe detected mainly 3 and 8 kb messages, with fainter bands migrating at 4.4 and 6 kb in some tissues (e.g., kidney). The 3 kb transcript was expressed in all tissues except for the brain, with highest levels in liver and testis. This transcript arises by alternative splicing and polyadenylation (Reiner, unpublished data).

Expression pattern of Lis1 *in the developing nervous system*

Sequence comparison and genetic mapping to syntenic chromosomal positions indicated that the *Lis1* gene is the mouse homolog of human *LIS1* involved in Miller-Dieker lissencephaly. Therefore, expression analyses were carried out by *in situ* hybridization using a *Lis1*-specific riboprobe ("*Lis1* probe" in Fig. 2*B*). Control hybridizations using sense riboprobe gave no signal (Fig. 6*A*). In E10.5–E14 embryos *Lis1* mRNA was very abundant in the developing central and peripheral nervous systems, including the neuroepithelium of the fore-, mid-, and hindbrain, the spinal cord, the dorsal root ganglia (Fig. 4*A,B*), and the cranial ganglia. In addition, *Lis1* was broadly expressed at low levels in mesodermal tissues such as lateral plate-derived structures (Fig. 4*A*), and craniofacial- and limb bud mesenchyme (not shown).

The cerebral cortex is the tissue most severely affected in

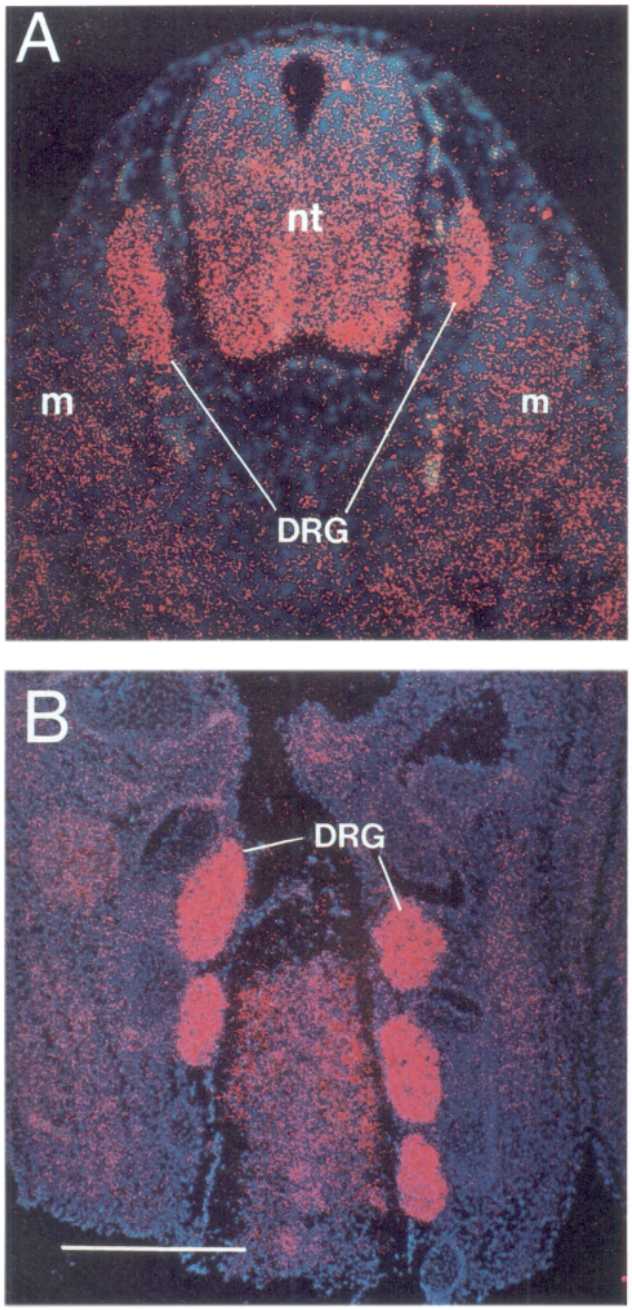

Figure 4. Expression of *Lis1* in the mouse neural tube and in dorsal root ganglia. Micrographs show double exposures with the red color representing signal from *in situ* hybridization and blue showing nuclei stained with Hoechst 33258 dye. *A*, Transverse section through a 12.5 p.c. (E12.5) embryo. Highest expression is observed in the ventral part of the neural tube (*nt*) and in the dorsal root ganglia (*DRG*). Lower signal is seen in the premuscle mesodermal condensation (*m*). *B*, Coronal section through the cervical region of an E14 embryo. *Lis1* mRNA is abundant in the DRGs. Scale bar, 500 μm.

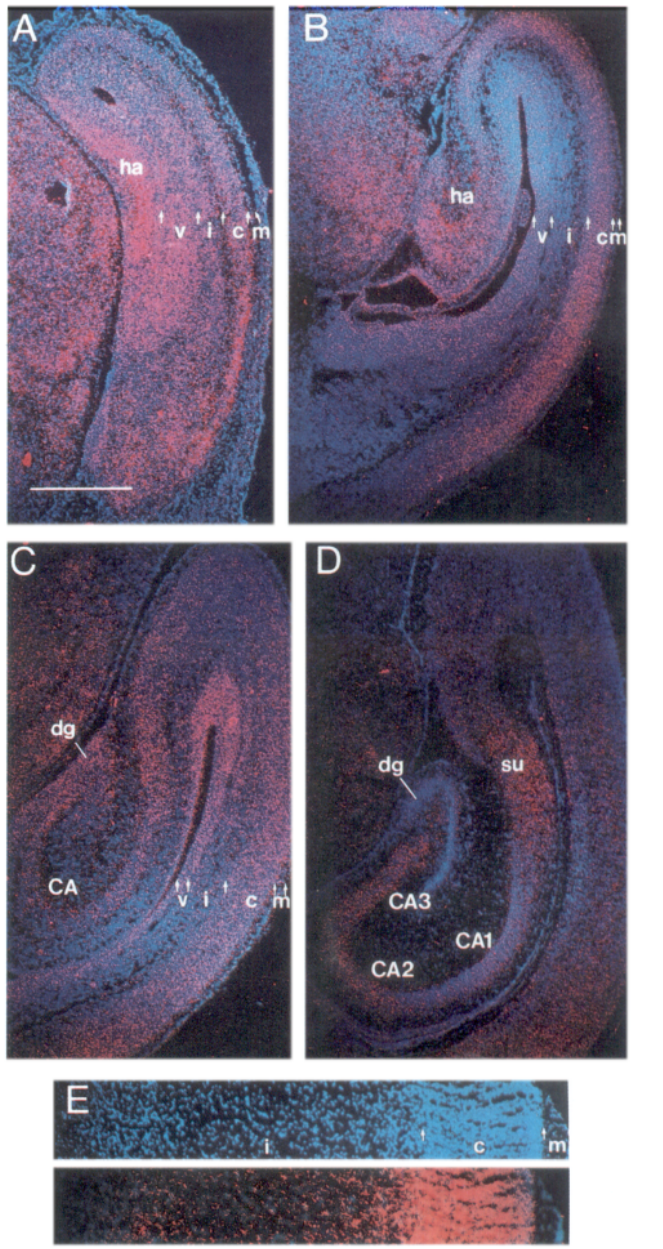

Figure 5. Expression of *Lis1* in the developing mouse cortex and hippocampus, and expression of *LIS1* in human fetal cortex. Coronal sections through the left ventricle of the mouse forebrain (*A–D*); rostral is on top. *A*, At E15.5 the developing brain shows broad *Lis1* expression with elevated levels in the hippocampal anlage (*ha*). *B*, At E17 *Lis1* expression is observed in the hippocampal anlage, the ventricular zone (*v*) and the cortical plate (*c*). Low signal is observed in the intermediate zone (*i*). *C*, At E18 the dentate gyrus (*dg*) is apparent and is *Lis1* positive. *D*, Expression in the dentate gyrus is attenuated by postnatal day 5. At this stage the *cornu ammonis* (*CA*) is positive for *Lis1* in regions 2 and 3, and *Lis1* mRNA is also detected in the subiculum (*su*). Note that the size of the ventricular zone (*v*) decreases during development while the cortical plate (*c*) increases in size (boundaries marked by *arrows*). *E*, Coronal section through the cortex of a 19 week old human fetus at the level of the striatum. The *upper panel* shows a Hoechst stain. The *lower panel* shows that the cortical plate (*c*) is strongly *LIS1* positive, whereas the intermediate zone (*i*) shows substantially less signal and the molecular layer (*m*) is negative. Scale bar, 500 μm.

individuals with Miller-Dieker lissencephaly. Cerebral cortical neurons are generated from the ventricular zone of the telencephalon. Postmitotic neurons exit the ventricular zone and eventually form the marginal zone (layer 1; see Fig. 6*B*). Later born neurons migrate radially to form the cortical plate. The later arriving neurons migrate subsequently through layer 6 and form layer 5. The later developing layers 2–4 are formed by the same inside-first mechanism, thus the younger layers are positioned

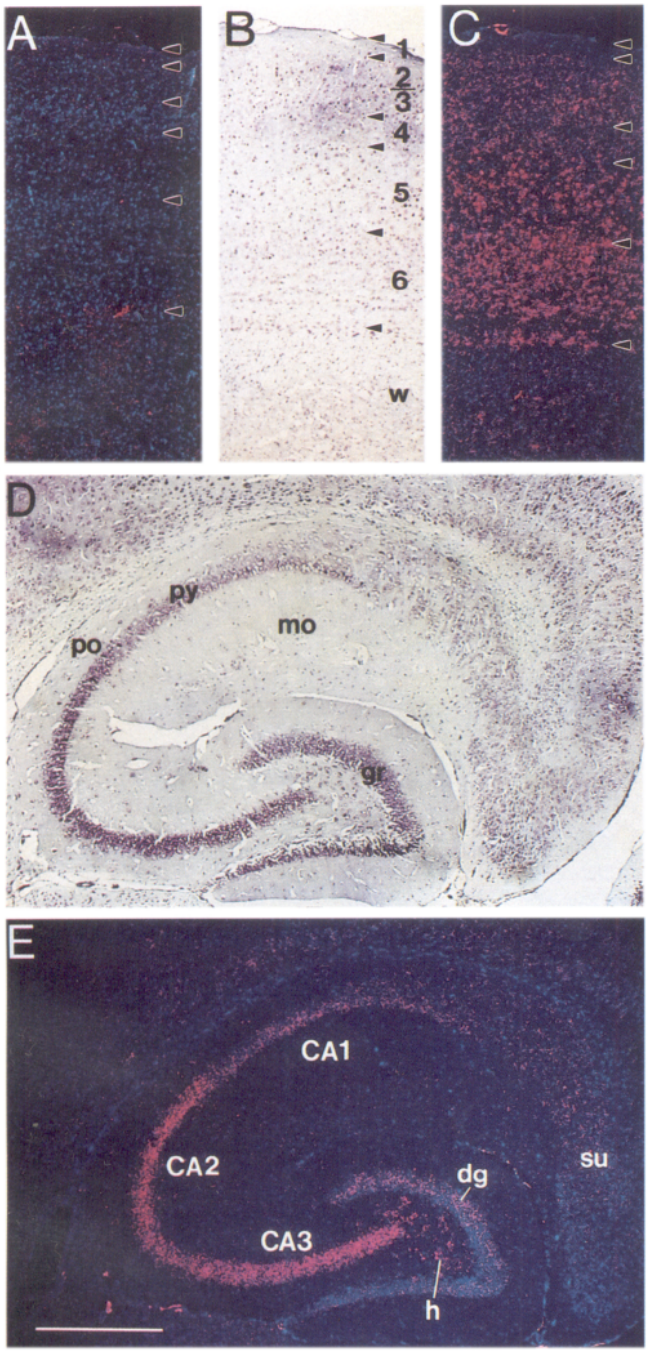

Figure 6. Expression of the *Lis1* gene in the adult mouse brain. All figures display horizontal sections. *A–C* show the cerebral cortex in adjacent sections. *A*, Section hybridized with sense riboprobe. Nissl-staining in *B* reveals the six cortical layers. *C*, Expression of *Lis1* transcript is detected in layers 2–6 with particularly higher levels in layers 5 and 6. *D* and *E*, Hippocampal formation. *D*, Nissl-stained section adjacent to the section shown in *E*. *Lis1* expression is detected in the pyramidal cells of regions CA2 and CA3 of the hippocampus and in the hilus (*E*). *CA*, cornu ammonis; *dg*, dentate gyrus; *h*, hilus; *mo*, molecular layer; *po*, polymorphic cell layer; *py*, pyramidal cell layer; *su*, subiculum; *w*, white matter. Magnification in *A–E* is identical. Scale bar, 500 μm.

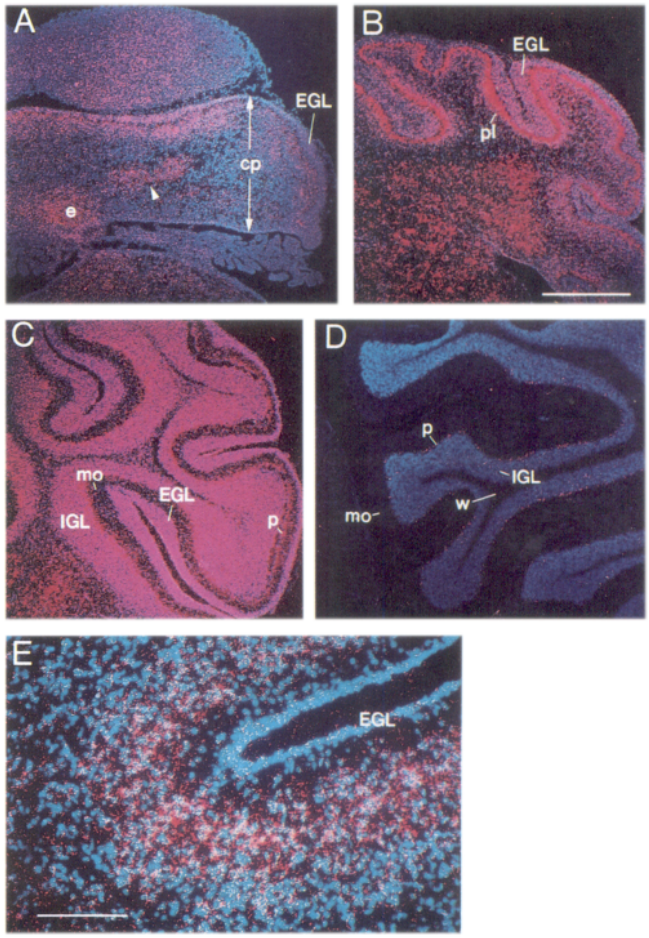

Figure 7. Expression of the murine *Lis1* gene and human *LIS1* gene in the developing cerebellum of mouse and human. *A–D*, Coronal sections through mouse cerebellum. *A*, Cerebellar plate (*cp*) of a mouse one day before birth (E18). A distinct expression pattern is seen in the ependymal (ventricular) zone (*e*) and the external granular layer (*EGL*). *B*, At P5 strongest expression is seen at the border of the EGL and the future Purkinje cell layer (*pl*). *C*, At P10, the granule cells which have migrated through the Purkinje cell layer show strong expression in the internal granular layer (*IGL*). The Purkinje cells (*p*) and the later migrating granule cells, still located in the EGL, also strongly express *Lis1*. In the adult (*D*) expression in the IGL is very low but is still detected in the Purkinje cells. *E*, Section through the developing cerebellum of a 19 week old human fetus. *LIS1* transcripts are found in a broad zone containing granule cells and Purkinje cell precursors. Scale bars: *A–D*, 500 μm; *E*, 80 μm.

outside of the older ones (Berry and Rogers, 1965; Shoukimas and Hinds, 1978; Miller, 1985).

In the cerebral cortex and the hippocampus, *Lis1* was strongly expressed throughout development. By E15.5, *Lis1* was expressed in the ventricular and intermediate zones, and the cortical plate, but not in the marginal zone (Fig. 5*A*). By E16 and E17 a more restricted signal was detected encompassing the ventricular zone and the cortical plate with the emerging intermediate zone being only weakly positive (Fig. 5*B*). As development of the brain proceeded, *Lis1* expression declined in the ventricular zone along with reduction in size of this structure and became restricted to the regions forming the cerebral cortex (Fig. 5*B,C*). The murine adult cortex consists of six layers, as visualized by Nissl staining (Fig. 6*B*), with five of the six layers showing *Lis 1* hybridization (Fig. 6*C*). *Lis1* mRNA was most

abundant in layers 5, 6, and the subplate (Fig. 6*C*), which are established prior to layers 2–4 during development. Layers 2–4 also showed pronounced hybridization, but at somewhat lower levels than the deep layers. Low levels of expression were detectable in the white matter (Fig. 6*C*). In layer 1 (marginal zone) no hybridization was observed.

To complement the studies in the mouse, we have examined the developing cerebral cortex of a 19 week old normal human fetal brain for *LIS1* expression. As in the corresponding stages of mouse (E16; see O'Rahilly and Muller, 1992; Bayer et al., 1993), the cortical plate, the marginal and intermediate zones are present and distinct by week 19. Reminiscent of the mouse (Fig. 5*A,B*), we found that *LIS1* was highly expressed in the cortical plate and at lower levels in the intermediate zone of human fetal brain (Fig. 5*E*).

The hippocampal anlage of the mouse was apparent by embryonic day 15. Elevated expression of *Lis1* was detected in the hippocampal primordium by E15.5 (Fig. 5*A*). During the following days, the involution of the lateral cortical plate and the emergence of mature features of hippocampal structures becomes evident. The prospective *cornu ammonis*, which is a direct continuation of the cortical plate, showed distinct expression of *Lis1* (Fig. 5*B*). *Lis1* expression in the dentate gyrus was visible by E18 (Fig. 5*C*), but later, expression in the gyrus appeared to be markedly reduced especially with reference to CA2 and CA3 regions (Figs. 5*D*, 6*E*). The subiculum, a transitional zone between the neocortex and the archicortex of the hippocampus, expressed *Lis1* by P5 (Fig. 5*D*) but expression was substantially reduced at later times (Fig. 6*E*). In the adult brain, the pyramidal cell layer of regions CA2 and CA3 was strongly positive for *Lis1*, whereas in the CA1 region markedly reduced levels of expression were observed (Fig. 6*E*). Punctate hybridization was detected in the hilus of the dentate gyrus, but only a low level of *Lis1* signal was detected in the dentate gyrus itself.

Most of the cerebellar neurons (e.g., Purkinje cells) are generated from the ventricular zone and migrate outward to their appropriate positions. However, the granule cells arise from a distinct proliferative region, the external granular layer (EGL). EGL cells, in turn, derive from a migratory population of cells that are born in and leave the ventricular zone of the alar plate of the fourth ventricle and move around the rhombic lip to eventually cover the entire surface of the cerebellum (Miale and Sidman, 1961; Altman and Bayer, 1978). Once the EGL is formed, cells in it proliferate, leave the cell cycle, and migrate inward through the Purkinje cell layer and along Bergmann glial fibers to give rise to the internal granule cell layer (IGL) (Rakic, 1971; Chuong, 1990; Millen et al., 1994; Sotelo et al., 1994).

Expression of *Lis1* in the cerebellum was transient. The first distinct pattern of expression in the cerebellum was seen by E16 in the ventricular zone and in the EGL (not shown). By E17 and E18, a more pronounced expression was seen in both the ventricular zone and the EGL (Fig. 7*A*). Furthermore, there were distinct patches of *Lis1*-positive cells between the ependymal zone and the EGL, possibly representing outwardly migrating neuronal precursors (Fig. 7*A*, arrowhead). By P3–P5, when cells in the EGL are mitotically active and beginning to generate granule neurons, strong *Lis1* expression was detected at the boundary between Purkinje cell layer and molecular layer (Fig. 7*B*). At P10, *Lis1* expression was very pronounced in the Purkinje cells which can now be detected in a characteristic single layer (Fig. 7*C*). Expression was also apparent in the EGL and in postmigratory granule neurons that had migrated inward through the Purkinje layer and into the internal granule layer (IGL). Also, note the emerging molecular layer, located between the Purkinje cell layer and the residual EGL. This molecular zone was largely devoid of *Lis1* expression: the low levels seen may derive from migrating granule cell neurons (Fig. 7*C*). Between P20 and P25, developmental stages at which the external granule layer has disappeared, *Lis1* expression was greatly reduced (not shown). In the cerebellum of the adult mouse, the *Lis1* gene was expressed in a punctate manner in Purkinje cells (Fig. 7*D*), and more weakly in the IGL.

LIS1 was distinctly expressed in the cerebellum of a 19 week old human fetus (Fig. 7*E*). By 19 weeks of development, the EGL is established and EGL neurons have begun to migrate inward (O'Rahilly and Muller, 1992). There is a distinct layer of cells located on the pial surface which display mitotic figures and represent the proliferating external granule cell layer (Fig. 7*E*). By that time of development, EGL cells have already begun their inward migration. Due to the absence of a distinct histological stratification at this developmental stage, it was not possible to determine which cells contained *LIS1* mRNA, but in analogy to the mouse, expressing cells may include inward-migrating granule cells and Purkinje cells.

Discussion

The LIS *gene family*

Mapping of the mouse *Lis* cDNA clones revealed that these genes constitute a gene family. Two mouse cDNAs were cloned (*Lis1* and *Lis3-4*), and three genetic loci were identified (*Lis1*, *Lis3* and *Lis4*). In human, two cDNAs were previously isolated and mapped to human chromosomes 17 and 2 (*LIS1* and *LIS2*) (Reiner et al., 1993). Murine *Lis1* is likely to represent the homolog of human *LIS1* because sequences of the coding and noncoding regions are very similar, and furthermore the genes map to syntenic chromosomal positions. In addition, expression patterns are similar. Amino acid sequence identity of >99% between the human *LIS1* and the murine and bovine homologs (Hattori et al., 1994) demonstrate a unique protein type with little species variation. This finding suggests that these proteins must perform an important, conserved function. While such identity was seen with other proteins it must be stated that this high degree of similarity is unusual. Additionally, conservation of sequences in the 5′ and 3′ untranslated regions of *LIS1* and its mouse homolog suggests common regulatory mechanisms.

The independent discovery of various aspects of *LIS1* is fascinating. The initial characterization of this disease-associated gene (Reiner et al., 1993) identified protein sequence motifs found in protein/protein interactions (Duronio et al., 1992; Neer et al., 1993, 1994). A clearly demonstrated example of LIS1 protein participating in a complex is provided by brain platelet-activating factor acetylhydrolase, an enzyme in which LIS1 protein is a subunit of a ternary complex (Hattori et al., 1994). This enzyme inactivates platelet-activating factor (PAF) which has both neuronal differentiation properties (Kornecki and Ehrlich, 1988; Ved et al., 1991) and neuronal messenger activity (Bito et al., 1992; Clark et al., 1992; Kato et al., 1994).

LIS1 is a hemizygous locus. In view of its gene product functioning as a subunit in an enzyme, hemizygosity is not unexpected since reducing the cellular concentration of LIS1 protein by half would reduce the level of assembled, functional enzyme. PAF acetylhydrolase may not be the only multisubunit protein containing LIS1 protein. In this case, a reduction in the cellular concentration of LIS1 protein may have multiple effects, and the

inability to inactivate PAF being only one facet of the Miller-Dieker syndrome. An additional aspect to consider is that *Lis3-4* is also expressed in the brain, albeit at much lower levels than *Lis 1*. Nonetheless, the amino acid sequence conservation of the two gene products raises the possibility that they can mutually substitute each other.

Lis1 *expression: functional implications and relationship to Miller-Dieker lissencephaly*

Miller-Dieker lissencephaly patients exhibit severe mental retardation, hypotonia, spastic paralysis, and infantile spasms. The cortex of these individuals consists of four layers (see Stewart et al., 1975). In the mouse, expression in the fetal and postnatal cerebrum was most distinct in the ventricular zone, the cortical plate, and in the developing cortex. Likewise, the human gene was highly expressed in the cortical plate. In the adult mouse, *Lis1* transcripts were most abundant in neurons of layers 5 and 6 of the cortex. Based on histologic investigations the prevailing view is that Miller-Dieker lissencephaly is the result of neuronal migration defects (e.g., Barth, 1987; Dobyns 1987). The broad expression of *Lis1* during embryogenesis is consistent with this idea; regions in which neurons migrate express this gene. It is worth noting that *Lis1* was expressed in the neuroepithelium and later in its derivative, the ventricular zone. There is evidence for cell migration within the ventricular zone (Fishell et al., 1993).

How could a loss of PAF acetyl hydrolase activity result in abnormal neuronal migration? PAF is a phospholipid signaling molecule for which a specific G-protein linked transmembrane receptor has been isolated (Honda et al., 1991). Therefore, PAF appears to be an extracellular signal binding to the extracellular domain of PAF receptor. Treatment of neuroblastoma cells with PAF results in an increased intracellular calcium level (Kornecki and Ehrlich, 1988; Yue et al., 1993). An increase in calcium may affect the organization of the cytoskeleton which could alter the migratory behavior of cells (Komuro and Rakic, 1992, 1993; Rakic et al., 1994). How a reduction of PAF acetylhydrolase activity in Miller-Dieker patients can affect such a process is unclear. The paradox which needs to be resolved is that the PAF acetylhydrolase containing Lis1 protein resides inside cells but PAF binds to the extracellular side of its receptor.

Lis1 was expressed in the CA2 and CA3 regions of the hippocampus and at lower levels in CA1, the subiculum and the dentate gyrus. Miller-Dieker lissencephaly patients suffer from seizures possibly reflecting hippocampal defects (Lothman et al., 1992; Sutula et al., 1992; Swann et al., 1992). This could involve abnormal levels of PAF. This signaling molecule mobilizes intracellular calcium in hippocampal neurons (Bito et al., 1992), enhances hippocampal excitatory synaptic transmission (Clark et al., 1992), and participates in long-term potentiation in the CA1 region (Kato et al., 1994).

In the developing cerebellum of both mouse and human, a distinct zone of *Lis1* expression encompassed the inward-migrating external granule cell neurons and possibly also the Purkinje cell precursors. *In situ* hybridization data in human do not provide sufficient spatial resolution to accurately determine which cells in the cerebellum expressed *LIS1* and therefore, it is not clear whether migrating cells and/or the cells surrounding them expressed this gene. Abnormalities in the cerebellum of lissencephaly patients have been noted (Miller, 1963; Stewart et al., 1975). Stewart et al. (1975) have described a lissencephaly patient whose cerebellum was reduced in size and showed mild defects in the convolutional folding. More importantly the external granular layer was absent and the internal granular layer greatly reduced. Since *Lis1* is transiently expressed in granule cells a function of the *Lis1* gene product in granule cell migration can be suggested.

In human, mutations in *LIS1* seem to be the major cause for Miller-Dieker lissencephaly, since at least 90% of patients have deletions in this region. In addition to *LIS1*, mutations in other loci can also cause lissencephaly (reviewed in Dobyns et al., 1993). *reeler*, an autosomal recessive mouse mutant, also affects the organization of the cerebral cortex (Caviness and Rakic, 1978). Since *reeler* maps to mouse chromosome 5 it is not related to the known lissencephaly loci. Our understanding of the pathophysiological mechanisms underlying lissencephaly is rudimentary at this time. However, the present study reveals a clear correlation between tissues affected by the genetic defect and sites of expression in mouse and human. This suggests that the mouse is a suitable organism for development of an animal model and for future functional studies of the LIS proteins. This unique disorder provides an important tool for molecular dissection of components involved in this disease and possibly for the study of mechanisms of neuronal specification and migration which appear abnormal in lissencephalic patients.

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