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Promoter Sequence, Expression, and Fine Chromosomal Mapping of the Human Gene (*MLP*) Encoding the MARCKS-like Protein: Identification of Neighboring and Linked Polymorphic Loci for *MLP* and *MACS* and Use in the Evaluation of Human Neural Tube Defects

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The MARCKS-like protein (MLP), also known as F52, MacMARCKS, or MARCKS-related protein, is a widely distributed substrate for protein kinase C (PKC). Recent studies using gene disruption *in vivo* have demonstrated the importance of both MARCKS and MLP to the development of the central nervous system; specifically, mice lacking either protein exhibit a high frequency of neural tube defects. We isolated a genomic clone for human *MLP* and discovered a directly linked polymorphism (*MLP1*) useful for genetic linkage analysis. The *MLP* promoter was 71% identical over 433 bp to that of the corresponding mouse gene, *Mlp,* with conservation of many putative transcription factor-binding sites; it was only 36% identical over 433 bp to the promoter of the human gene, *MACS,* which encodes the MLP homologue MARCKS. This 433-bp fragment drove expression of an *MLP-β-galactosidase* transgene in a tissue-specific and developmental expression pattern that was similar to that observed for the endogenous gene, as shown by *in situ* hybridization histochemistry. In contrast to *MACS,* the *MLP* and *Mlp* promoters contain a TATA box approximately 40 bp 5′ of the presumed transcription initiation site. *MLP* was localized to chromosome 1p34 → 1pter by analysis of human–mouse somatic cell hybrid DNA and to 1p34 by fluorescence *in situ* hybridization. Radiation hybrid mapping of *MLP* placed it between genetic markers *D1S511* (LOD > 3.0) and *WI9232. MACS* was localized to 6q21 between *D6S266* (LOD > 3.0) and *AFM268vh5* by the same technique. We tested the novel *MLP1* polymorphism and the *MACS* flanking markers in a series of 43 Caucasian simplex families in which the affected child had a lumbosacral myelomeningocele. We found no evidence of linkage disequilibrium, suggesting that these loci were not major genes for spina bifida in these families. Nonetheless, the identification of linked and neighboring polymorphisms for *MACS* and *MLP* should permit similar genetic studies in other groups of patients with neural tube defects and other neurodevelopmental abnormalities.

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession Nos. L76376 and AF031640.

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INTRODUCTION

The myristoylated, alanine-rich C kinase substrate, or MARCKS protein, is a widely expressed, prominent cellular substrate for protein kinase C (PKC; Aderem, 1992; Blackshear, 1993). Encoded by *MACS,* a gene localized to human chromosome 6q21 (Harlan *et al.,* 1991; Blackshear *et al.,* 1992a), the equivalent mouse protein is required for normal development of the central nervous system and postnatal survival in the mouse (Stumpo *et al.,* 1995). A cDNA, pF52, that encodes a protein resembling MARCKS was first identified by Umekage and Kato (1991). Both proteins were very similar throughout the three evolutionarily conserved regions of MARCKS, i.e., the amino terminus, the intron splice site, and the phosphorylation site domain. In addition, both proteins were found to be myristoylated, alanine-rich cellular substrates for protein kinase C and high-affinity calmodulin-binding proteins. The protein encoded by pF52 was studied by our group (Blackshear

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et al., 1992b; Verghese *et al.,* 1994) and also by Li and Aderem (1992; this group termed the protein MacMARCKS because of its high-level expression in macrophages). The mouse gene was originally assigned the name *Mrp* (for MARCKS-related protein; Lobach *et al.,* 1993). However, because of confusion with the multidrug resistance element-related protein, also abbreviated MRP (Cole *et al.,* 1992), the mouse and human protein designations have been changed to "MARCKS-like protein" or MLP, and the gene designations to *Mlp* and *MLP,* respectively, by the Committee on Standardized Genetic Nomenclature for Mice (M. Davisson, pers. comm., Bar Harbor, 9 Oct. 1997) and the Human Gene Nomenclature Committee (S. Povey, pers. comm., London, 1 Apr. 1996), respectively.

The gene encoding the mouse protein MLP has been isolated (Lobach *et al.,* 1993) and mapped to a position on mouse chromosome 4 that lies within a linkage group of genes that corresponds to a position on human chromosome 1p32–p35. In this report we describe the cloning, characterization, and fine chromosomal localization of the human gene (*MLP*) encoding the MLP protein. We have also sequenced 433 bp of the promoter and shown that a transgene containing this extent of promoter linked to MLP coding sequence and the *β-galactosidase* (*β-gal*) gene produced normal patterns of *Mlp* expression in the developing mouse embryo. Finally, since disruption of *Mlp* has been shown recently to result in high frequencies of neural tube defects in the mouse (Wu *et al.,* 1996; Chen *et al.,* 1996), we have performed fine mapping of *MLP.* This allowed us to identify nearby polymorphisms, including one directly linked to *MLP,* and to begin genetic studies evaluating possible deficiencies in *MACS* and *MLP* in patients with neural tube defects. We have begun this analysis in families in which one affected child had lumbosacral myelomeningocele or spina bifida, a neural tube closure defect that affects up to 0.1% of births worldwide (Lemire, 1988).

MATERIALS AND METHODS

Isolation, Subcloning, and Sequencing of Human Genomic MLP Clones

A human placenta genomic library in EMBL-3 SP6/T7 (Clontech, Palo Alto, CA) was screened with 5′ (202-bp *Not*I/*Sst*I fragment) and 3′ (389-bp *Eco*RI/*Not*I fragment) ^{32}P random-primed-labeled (Feinberg and Vogelstein, 1983) probes isolated from the mouse pF52 cDNA (Umekage and Kato, 1991; a generous gift from Dr. K. Kato, Osaka University Medical Center, Osaka, Japan) using standard procedures (Lobach *et al.,* 1993; Maniatis *et al.,* 1982). Five phage clones that hybridized strongly to both the 5′ and the 3′ probes were purified by two additional rounds of screening. The phage DNA was isolated, cleaved with *Asp*718, *Xho*I, *Sst*I, and *Xba*I, and fragments that hybridized to both probes were subcloned into the appropriately restricted Bluescript KS$^{(+)}$ vector (Stratagene, La Jolla, CA). The inserts from these five plasmid clones, p4.1.1, p8.1.1, p5.2.1, p6.2.1, and p24.1.1, were 5.1, 3.8, 2.9, 2.9, and 6.5 kb, respectively. Phage clone *MLP*(4-1-1) was also digested with *Xba*I, and a 1.5-kb fragment that overlapped with the 5′-end of the 5.1-kb *Asp*718 fragment was subcloned into Bluescript and labeled p4.1.1/1.5. Double-stranded plasmid DNA was isolated from the five subclones and sequenced by the dideoxynucleotide termination method (Sanger *et al.,* 1977) using the Sequenase 2.0 kit from United States Biochemical Corp. (Cleveland, OH).

Transient Expression in Eukaryotic Cells and Northern Blot Analysis

The *MLP* genomic plasmid clones p4.1.1, p8.1.1, p5.2.1, p6.2.1, and p24.1.1 were transiently transfected into LM/TK$^-$ cells (a gift from Dr. Richard D. Klausner, National Cancer Institute, Bethesda, MD) using the DEAE–dextran method as described previously (Stumpo *et al.,* 1989). Seventy-two hours posttransfection, total cellular RNA was isolated from the cells as described (Stumpo *et al.,* 1995). Fifteen micrograms of RNA was fractionated on a 2.2 M formaldehyde/1.2% agarose gel, transferred to Nytran, and hybridized with a 1.6-kb *Eco*RI fragment of the human MLP cDNA (P. J. Blackshear and J. S. Tuttle, unpublished data) as described (Stumpo *et al.,* 1989).

Chromosomal Mapping of MLP and MACS

Chromosomal localization of *MLP* was initially performed by Southern analysis with the ^{32}P-labeled 5.1-kb *Asp*718 insert of p4.1.1 on *Hin*dIII-digested genomic human–mouse somatic cell hybrid DNA (Shows *et al.,* 1982, 1984; Shows, 1983). Chromosomal fluorescence *in situ* hybridization was then performed to confirm the localization of *MLP.* The same probe was labeled with digoxygenin-11–dUTP (Boehringer Mannheim, Indianapolis, IN) by random priming (Feinberg and Vogelstein, 1983). *In situ* hybridization analysis on complete chromosome spreads was performed according to the method of Trask (1991). Finally, fine mapping of *MLP* was performed by polymerase chain reaction (PCR) analysis of the GeneBridge 4 radiation hybrid panel (Walter *et al.,* 1994). Statistical analysis of the data was performed using the RHMAPPER software package (D. Slonim, L. Stein, L. Kruglyak, and E. Lander, unpublished software). Oligonucleotide primers used for PCR were 5′ CCAGAAGTGGGGTGCTTATA 3′ (forward) and 5′ AAAAACCTAGACGGGGCAGT 3′ (reverse). These were based on sequences 971 to 990 and 1074 to 1093, respectively, in the human MLP cDNA clone (GenBank Accession No. X70326). The data vector for *MLP* was 220020201022010-1100000001002000000001102001210000020200002000000000002000110000020102011000200.

Mapping of *MACS* to 6q21 has been previously described (Harlan *et al.,* 1991; Blackshear *et al.,* 1992a). Fine mapping was accomplished as described for *MLP.* Oligonucleotide primers used for PCR were 5′ TGGGGAAATTGAAGTGGTGC 3′ (forward) and 5′ GTGTTGTAAACCTGCCTCAC 3′ (reverse). These were based on sequences 1430–1449 and 1539–1558, respectively, in the human MARCKS cDNA clone (Harlan *et al.,* 1991). The data vector for *MACS* was 121120210022100001000000010101000002100000120-0100010200102000100100002100110010200120000012 01.

Mlp–LacZ Transgenic Mice

Plasmid construct. A unique *Eco*RV site was created at base 1977 (the last codon of the MLP protein) in the mouse genomic *Mlp* clone 2a2a (Lobach *et al.,* 1993) using site-directed mutagenesis (Amersham, Arlington Heights, IL). This clone contains 407 bp of *Mlp* promoter region, 186 bp of 5′ UTR, the complete 600-bp protein coding sequence, and the single 784-bp intron (Lobach *et al.,* 1993). A 3073-bp *Sma*I/*Sal*I fragment of pMC1871 (Pharmacia, Piscataway, NJ) containing the *Escherichia coli LacZ* gene was isolated, blunted, and subcloned into the newly created *Eco*RV site in *Mlp* 2a2a. The presence of the correct, in-frame, *Mlp–LacZ* junction was verified by sequencing.

Generation of transgenic mice. The 6.8-kb *Mlp–LacZ* fusion gene was excised from Bluescribe with *Xba*I, purified as described previously (Harlan *et al.,* 1991), and injected into mouse pronuclei by standard procedures (Hogan *et al.,* 1986; Blackshear *et al.,* 1996). Fertilized eggs were isolated from a cross of B6SJLF$_1$/J mice (The Jackson Laboratory, Bar Harbor, ME) and were injected with approximately 2 pl of 2.0 ng/μl of *Mlp–LacZ* DNA. Genomic DNA was

isolated from tail biopsies of 10- to 12-day-old mice resulting from the transfer of microinjected eggs into the oviducts of pseudopregnant F_1 mice. Genomic DNA was digested with *Sst*I and analyzed by Southern blot hybridization with a ^{32}P random-prime-labeled 505-bp *Bam*HI fragment of the genomic *Mlp* clone 2a2a (Lobach *et al.*, 1993). This probe recognizes a 2.5-kb *Sst*I fragment from the endogenous *Mlp* gene and a 3.3-kb *Sst*I fragment from the *Mlp–LacZ* fusion gene.

Localization of β-galactosidase activity. Embryos (E8.5, E14.5, and E16.5) were dissected, fixed, stained for β-galactosidase activity, and photographed as described previously (Blackshear *et al.*, 1996). No detectable endogenous β-galactosidase activity was observed in control nontransgenic littermates.

In Situ Hybridization

In situ hybridization histochemistry was performed on fresh-frozen sections of whole embryos or tissues as previously described (Bradley *et al.*, 1992), using both sense and antisense RNA probes. *Mlp* 2a2a containing the unique *Eco*RV site at base 1977 was digested with *Eco*RI. An approximately 1.3-kb *Eco*RI fragment containing the 3′ end of the mouse gene was removed, and the remaining 2.4-kb *Mlp* 2a2a clone was religated. A 282-bp *Eco*RV/*Eco*NI fragment was excised from this plasmid, blunted, and subcloned into the *Hinc*II site of Bluescribe +. This plasmid construct containing the 282-bp *Eco*RV/*Eco*NI fragment was used to generate sense and antisense RNA probes. For antisense RNA, the plasmid was linearized with *Eco*RI, and ^{32}P-labeled RNA was made using T3 RNA polymerase following the manufacturer's protocol (Promega, Madison, WI). Sense RNA was made using T7 RNA polymerase and *Hin*dIII linearized plasmid DNA. *Macs* sense and antisense probes were generated from a plasmid containing the intron, the second exon, and the 3′ UTR of the mouse gene encoding MARCKS (pMG80K4.5; Blackshear *et al.*, 1992a). A 720-bp *Pst*I/*Bgl*II fragment containing 357 bp of the 3′ end of exon 2 and 363 bp of the 3′ UTR was excised and subcloned into Bluescribe +. Sense RNA was generated using T3 RNA polymerase and *Eco*RI linearized DNA, and antisense RNA was generated with T7 RNA polymerase and *Hin*dIII linearized DNA. Sections were washed, dried, and examined using Kodak XAR-2 film.

Identification of a Novel Polymorphic Marker Linked to MLP

A Southern blot containing various restriction digests of phage DNA from our *MLP* clones was prepared and hybridized with a 5′ end-labeled $(CA)_{15}$ oligonucleotide essentially as described (Stumpo *et al.*, 1989), except that the blot was washed for 1 h at 60°C in 1× SSC/0.1% SDS and then for 30 min at 60°C in 0.2× SSC/0.1% SDS. A 2.4-kb *Sst*I fragment from the original phage clones *MLP*(5-2-1) and *MLP*(6-2-1) hybridized to the $(CA)_{15}$ oligonucleotide. Subsequent Southern analysis of this 2.4-kb *Sst*I fragment demonstrated that the sequence hybridizing to the $(CA)_{15}$ oligonucleotide was localized to a 285-bp *Alu*I fragment. The 285-bp *Alu*I fragment was subcloned into Bluescribe + (Stratagene) and sequenced using M13 forward and M13 reverse sequencing primers (Sanger *et al.*, 1977). Based on the sequence flanking this CA repeat, we designed PCR primers to type this locus: HGMLP(CA)f, 5′ GTGACAGAGGGAGATTCTGTCTCA 3′, and HGMLP(CA)r, 5′ AAATCCTCCAGTGTGGTCTGCACC 3′.

We calculated allele frequencies for this polymorphic marker, designated *MLP1*, on a series of 53 unrelated Caucasians (n = 106 chromosomes; Haynes *et al.*, 1997) using standard allele counting techniques (Speer, 1995). These frequencies and their associated CEPH standards are available via anonymous ftp at site dnadoc.mc.duke.edu in the /pub/ALLELE_FREQ directory.

The localization of *MLP1* was confirmed by genetic mapping in 15 CEPH (Dausset *et al.*, 1990) reference pedigrees. The polymorphic marker was genotyped in these pedigrees as previously described (Vance *et al.*, 1996). Genotypes for markers which were within 15 cM of the most likely location of *D1S511* in the Cooperative Human Linkage Center (CHLC) version 3 integrated map were extracted from the CHLC database (http://www.chlc.org). The most likely location for this marker within the available CHLC map and two-point lod scores was determined as previously described (Straub *et al.*, 1993) using the CRIMAP program.

Assessment of Disease Pedigrees for MACS and MLP

Blood samples were obtained following informed consent from 43 simplex pedigrees of American Caucasian descent in which the proband was affected with a lumbosacral myelomeningocele. DNA was extracted from whole blood using standard procedures. Genotyping was performed using a semiautomated fluorescence scanning system (Vance *et al.*, 1996).

The multiallelic extension of the transmission disequilibrium test (TDT) (Spielman *et al.*, 1996; Kaplan *et al.*, 1997) was applied to the data to test for linkage disequilibrium with an allele at the newly identified *MLP1* locus and the spina bifida phenotype. Since no polymorphisms within the *MACS* locus have been identified, we genotyped the flanking markers *D6S266* and *AFM268vh5*, estimated to be within 2 cM of one another. The resulting statistic was compared to a χ^2 distribution with degrees of freedom equal to the number of alleles−1. Alleles that were observed in fewer than five parents were pooled.

RESULTS

Isolation, Sequencing, and Expression of Human MLP Genomic Clones

Screening of a human placenta genomic library with 5′ and 3′ fragments of the mouse pF52 cDNA resulted in the isolation of five plasmids whose inserts were derived from five separate phage clones, as detailed under Materials and Methods. Sequence analysis showed that the inserts in the 2.9-kb p5.2.1 and p6.2.1 clones were identical. These five plasmids were transfected into mouse LM/TK$^-$ fibroblasts (these cells contain no endogenous MLP mRNA or protein; Lobach *et al.*, 1993), and total cellular RNA was isolated and analyzed for the presence of the full-length MLP mRNA. Transfection of three clones, p4.1.1, p5.2.1, and p6.2.1, resulted in roughly equivalent expression of MLP mRNA (Fig. 1A). Transfection of either p8.1.1 (Fig. 1A) or p24.1.1 (data not shown) did not result in the expression of an mRNA species that hybridized to the MLP probe. These clones may represent pseudogenes, which were frequently isolated during the cloning of mouse *Mlp* (Lobach *et al.*, 1993), or perhaps true *MLP* clones that lack the appropriate 5′ promoter sequences or enough of the 3′ untranslated region necessary for proper processing of the RNA. Partial sequence analysis of p24.1.1 indicated that this clone was a pseudogene, as evidenced by frequent frameshifts and internal stop codons within the putative protein coding region.

The original phage clone from which p4.1.1 was derived, *MLP*(4-1-1), was digested with *Xba*I, and a 1.5-kb *Xba*I fragment, which hybridized to the 5′ cDNA probe and overlapped with the 5.1-kb *Asp*718 insert of p4.1.1, was isolated, subcloned, and sequenced. Based on comparisons with the sequences of the mouse (Umekage and Kato, 1991) and human (GenBank Accession No. X70326) MLP cDNAs, we concluded that this fragment contained 433 bp of promoter sequence, 182 bp of 5′ UTR, 87 bp of protein coding sequence, and 789 bp of one intron (GenBank Accession No.

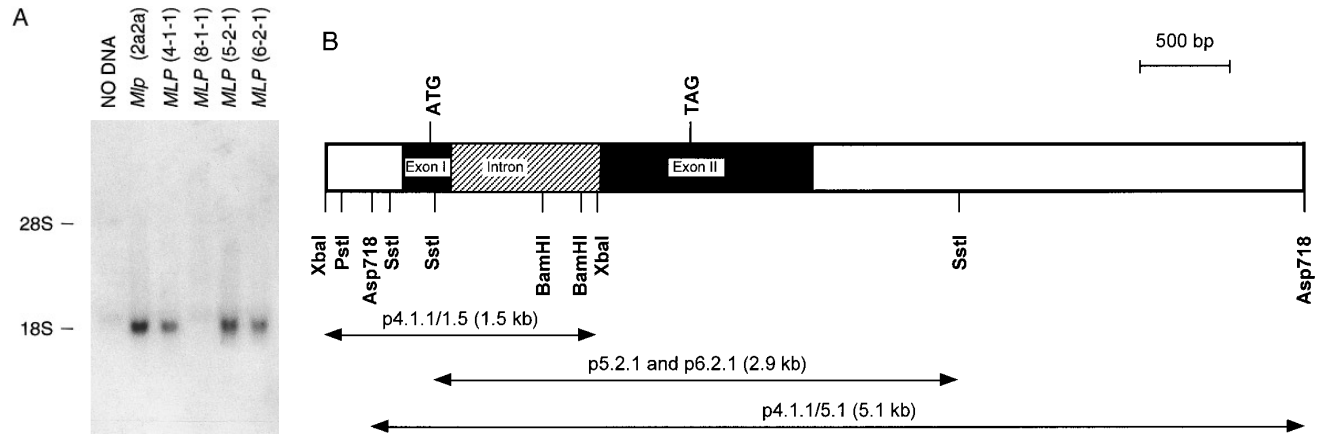

FIG. 1. (**A**) Expression of MLP mRNA in LM/TK$^-$ cells. Cells were seeded at a density of 1.85×10^6 cells/100-mm dish 24 h prior to transfection. Ten micrograms of plasmid DNA was transfected into the cells as described under Materials and Methods. Controls included no DNA and *Mlp*(2a2a), the mouse genomic clone encoding MLP (Lobach *et al.*, 1993). Four potential human *MLP* clones were tested, p4.1.1, p8.1.1, p5.2.1, and p6.2.1. Total cellular RNA was prepared 48 h posttransfection, and 15 μg was examined by Northern blot analysis with a human MLP cDNA probe. The markers indicate the position of the major ribosomal RNA species on the gel. (**B**) Organization of *MLP*. A schematic representation of *MLP* is shown, including the positions of several restriction enzyme sites, the two exons, the ATG site, the single intron, and the stop codon. The orientations of the four plasmid clones that span this locus are depicted at the bottom of the figure.

L76376) (Fig. 1B). Sequence analysis of the identical inserts in clones p5.2.1 and p6.2.1 revealed that these inserts began within the protein coding region of the first exon and ended in the 3′-flanking region (Fig. 1B). Despite the absence of 5′-flanking sequences in a traditional promoter location, both of these clones expressed mRNA when transfected into LM/TK$^-$ cells. Previous examples of this phenomenon have been noted in this cell type (Pitha *et al.*, 1982); it is possible that promoter elements within the coding sequence, large (~800 bp) intron, or 3′-flanking region are responsible for this expression. Sequence analysis of the 5.1-kb *Asp*718 insert of p4.1.1 revealed that the 5′ end of this insert started at base 274 of the 1.5-kb *Xba*I fragment (Fig. 1B). This 5.1-kb insert of plasmid clone p4.1.1 was capable of directing the synthesis of MLP mRNA (Fig. 1A), indicating that the 5′-most 274 bp of the 433-bp putative promoter from the 1.5-kb *Xba*I fragment (p4.1.1/1.5) are not necessary for expression of MLP mRNA in LM/TK$^-$ fibroblasts under these conditions.

Comparison of MLP with Mlp and MACS

The sequence identity between 433 bp of the putative promoter regions of *MLP* and *Mlp* (Lobach *et al.*, 1993) was 71% (Fig. 2A). Both sequences were very GC-rich (70% over 433 bp for *MLP* and 63% over 407 bp for *Mlp*). Both the *MLP* and the *Mlp* promoters contained a potentially active TATA box at about 40 bp 5′ of the start of the mouse cDNA (Umekage and Kato, 1991). The sequence found in *MLP* has been found to represent an active TATA sequence by Wobbe and Struhl (1990). The sequence found in *Mlp* has not been shown to be active; however, two similar sequences, CATAAA and TACAAA, were able to mediate TFIID-dependent transcription (Wobbe and Struhl, 1990). Neither *MACS* nor *Macs* contains an equivalent TATA sequence (Blackshear *et al.*, 1992a; Harlan, *et al.*, 1991).

The degree of sequence identity between the *MLP* and the *MACS* promoters (36% over 433 bp; Fig. 2B) was similar to that observed between the *Mlp* and the *Macs* promoters (37%; Lobach *et al.*, 1993). However, both *MLP* and *MACS* from both human and mouse contain a single intron at identical sites (data not shown; Blackshear, 1993). An identical 10-amino-acid motif surrounding this intron splicing site is present in both proteins (data not shown; Blackshear *et al.*, 1992b).

Analysis of the 433 bp of putative promoter by the program TF sites (Ghosh *et al.*, 1990) revealed many potential transcription factor-binding sites. Several of these potential sites are conserved between the human *MLP* and the mouse *Mlp* promoter regions (Fig. 2A) and include two Sp1 sites, an Sp1/AP2 site, a CTF site, and a TFII-1 site.

FIG. 2. (**A**) Alignment of *MLP* and *Mlp* promoter regions. The promoter regions from the human and mouse (Lobach *et al.*, 1993) genes were aligned using the GAP program (Genetics Computer Group, Madison, WI). Vertical lines indicate base identities. Several potential transcription factor-binding sites that are conserved between the two sequences are indicated. These include several SP1 sites, a CTF site, an AP2 site (overlapping one of the SP1 sites at base 369 in the human sequence), and a TFII-1 site. The putative TATA box in both sequences is underlined. Both sequences end at the putative transcription initiation site based on the extreme 5′-end of the mouse cDNA (Umekage and Kato, 1991). H, human *MLP;* M, mouse *Mlp.* (**B**) Alignment of MLP and MACS promoter regions. The sequences of the promoters for *MLP* and *MACS* (Harlan *et al.*, 1991) are shown; both sequences end at the putative transcription initiation sites. The alignment was performed using the GAP program. The vertical lines represent base identities. The putative TATA box for *MLP* is underlined; no equivalent TATA box sequence is present in *MACS*.

A

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H   1 GTATAATTCAGGACCAGCCATCTGACGAGCCTGGTCGTGTGAGCCCTTCG 50
M   1 GTGTAACTCAGCACCAGCCGTCTGACGAGTCTGGACATGTGAGCCCTTCC 50

  51 AAGGGTTCAAGCGGGAGACTGCCAGCGGCTGCAGGACCAAGGCGTTGGGG 100
  51 AAGAGTTCAAG...........GAGCCAGTGTAGAACCAGGGACT..GGG 87

 101 GCCGCTCCAGGCTGCGGGGGCGCCCACCGGAGAGGTGGAGCGGAGAGGAT 150
  88 GCCTCTCCACTGTGAAGTATGAGTCAGGCTGAAAGTGGGCTGGGGATGAC 137

 151 GGAATGACTGCCCGTCTCCTTCCTCCAGGCCCCCCGCCCCGCCCCGCCCA 200
 138 TGAGTG.........CTTCTTCATCGACGGCCTCCTCCCAGTCCATCTCC 178

 201 GCTCGCGGGAGGACTGGCCTCGCTCCTGCCCACACCGGACTCTACCTGCC 250
 179 CGGACCGACCGGA...ACCTCTCTCATTCCCACACTGAACCCTACTTGAC 225

 251 CCTGGGATTCGGGTGGGGTGGGGGGTACCCGACCGC.CAGCGTCGCCTGC 299
 226 CCCTGGTCGTCCCAAGTGATGGGGGAACCAGACAGCTTGGCACTGCCCAC 275
                                SP1                CTF
 300 TGCGCAAGTGGGGCCTGCGAGCCAACCCCGCCTCCGCCCCTCGATTGGGC 349
 276 TGCACGAGAGGAACCCG.GGCCCGACCCCGCCTCCACCCC.GGATTGGGT 323
                          SP1/AP2
 350 GCGCATTTAAATGTCCGGCCCCGCCCCGAGCTCCGGGGCGCTTATATATAG 399
 324 GCTCATTTAAATGTCCGGCCCCGCCCCGAGCCTCGGGCGCGTACATATAG 373
                     SP1              TFII-1
 400 GCAGCTCGGCGGGGCGGCGGGCGGCATTCTGGCGC 433
 374 GCGGCTCGGCGGGCGGCGGGCGGCATTCTGGCGC 407
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B

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MLP     1 ...............................GTATAATTCAGGACCAGCC 19
MACS  101 CTGTAAAACCAATATGGAAAAAAGAAAGAAAACATGCTGCAAACACACAC 150

       20 ATCTGACGAGCCTGGTCGTGTGAGCCCTTCGAAGGGTTCAAGCGGGAGAC 69
      151 AAATCGCAACCATTGTCTTTAACACTCGCATACACACACATACACATAAA 200

       70 TGCCAG..CGGCTGCAGGACCAAGGCGTTGGGGGCCGCTCCAGGCTGCGG 117
      201 CACACGGAAAGATGGGGGAGGGGAGCGAGTGGAGAGAGAGAGACACACGG 250

      118 GGGCGCCCACCGGAGAGGTGGAGCGGAGAGGATGGAATGACTGCCCGTCT 167
      251 ATATAGACCGACGAGAGTCAATGCGAGTCAAGTTAACGCTTTCTCCGCCC 300

      168 CCTTCCTCCAGGCCCCCCGCCCCGCCCCGCCCAGCTCGCGGGAGGACTGG 217
      301 CGATGCACAAGTACCCCCCTCCCGTGTTAATTAATTCCAAACAAAGCAAG 350

      218 CCTCGC.............TCCTGCCCACACCGGACTCTACCTGCCCCTG 254
      351 CCAATCAAGAAATGCATTATTATTTTCAAGCAGAGAGAGACGAGTGCCAT 400

      255 GGATTCGGGTGGGGTGGGGGGTACCCGACCGCCAGCGTCGCCTGCTGCGC 304
      401 GTATTTTTTAATTGATTTATTTATTTGGAGGACATTAATTCTCGGTCTGA 450

      305 AAGTGGGGCCTGCGAGCCAACCCCGCCTCCGCCCCTCGATTGGGCGCGCA 354
      451 GGCTGATTTTCAAACCGTTTGCAAAGCGCGGCTCCATTGTTCGCCAGGCG 500

      355 TTTAAATGTCCGGCCCCGCCCCGAGCTCCGGGCGCTTATATATAGGCAGC 404
      501 CGCAGACCCCGCCCCCTGCTTTGTGTGCGGCGCGCGGATTGGCCGACGCG 550

      405 TCGGCGGGCGGCGGGCGGCATTCTGGCGC..................... 433
      551 CGCGGGGGCCGCGTCAGCGCCCGCGAGCCCATTCATCTGTGCACTTGGGC 600
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TABLE 1

Segregation of *MLP* with Human Chromosomes in *Hin*dIII-Digested Human–Mouse Cell Hybrid DNA

Chromosome	Concordant number of hybrids (+/+)	Concordant number of hybrids (−/−)	Discordant number of hybrids (+/−)	Discordant number of hybrids (−/+)	% Discordancy
1	8	18	0	0	0
2	7	14	2	5	25
3	9	9	0	8	31
4	6	13	2	6	30
5	8	12	1	7	29
6	7	14	2	5	25
7	8	12	0	7	26
8	7	10	2	9	39
9	3	18	5	1	22
10	7	7	2	12	50
11	4	14	1	5	25
12	7	11	2	8	36
13	8	12	1	7	29
14	6	9	2	10	44
15	7	12	1	7	30
16	7	16	2	3	18
17	5	5	3	13	62
18	6	9	3	10	46
19	5	17	4	2	21
20	5	10	4	9	46
21	7	5	2	14	57
22	6	14	3	4	26
X	4	7	2	10	52

Note. Genomic Southern analysis of *Hin*dIII-digested DNA from 28 human–mouse cell hybrids was performed using the 5.1-kb Asp718 insert from p4.1.1. The table is a summary of the segregation of the Southern blot human *MLP* bands present (+) or absent (−) in each hybrid compared to the presence (+) or absence (−) of human chromosomes in each hybrid. The (+/+) and (−/−) band/chromosome represent concordant segregation, and the (+/−) and (−/+) represent discordant segregation. Chromosome translocations with no intact chromosome present were not tabulated for percentage discordancy. A 0% discordancy indicates a matched segregation of the specific human *MLP* bands with a chromosome. The *MLP* DNA mapped to human chromosome 1.

Chromosomal Localization of MLP

Chromosomal localization of *MLP* was initially performed by Southern analysis of genomic DNA from somatic cell hybrids (Shows *et al.*, 1982, 1984; Shows, 1983). The 5.1-kb *Asp*718 insert from p4.1.1 was used to probe *Hin*dIII-digested DNA from 28 human–mouse cell hybrids. The hybrids were characterized for human chromosome content by karyotype analysis and mapped by enzyme markers (Shows *et al.*, 1982, 1984; Shows, 1983). Table 1 is a summary of the discordance analysis from the 28 human–mouse cell hybrids. The *MLP* DNA mapped to human chromosome 1. Two hybrids, 20L-28, positive for *MLP* with the translocation 17qter → 17p13::1p34 → 1pter, and 20L-37, negative for *MLP* with the translocation 1qter → 1p34::17p13 → 17pter, further localized *MLP* to 1p34 → 1pter (data not shown).

Confirmation of the chromosomal localization of *MLP* was performed by chromosomal fluorescence *in situ* hybridization using the 5.1-kb *Asp*718 fragment of p4.1.1. The DNA was labeled with digoxygenin-11–dUTP (Boehringer Mannheim) by random priming (Feinberg and Vogelstein, 1983), and *in situ* hybridization analysis was carried out on complete metaphase chromosome spreads (Trask, 1991). Double fluorescent signals were found at 1p34 in 12 of 15 metaphase spreads (80%) examined (Fig. 3). Seven of fifteen spreads showed double fluorescent signals on both chromosomes.

Fine mapping was performed by PCR analysis of the GeneBridge 4 radiation hybrid panel (Walter *et al.*, 1994). This placed *MLP* on chromosome 1 within 1.92 cR_{3000} from *D1S511* (LOD > 3.0); it is between *D1S511* and *WI9232* (Schuler *et al.*, 1996). *MACS* had been localized previously to chromosome 6q21 by analysis of somatic cell hybrids and studies of cells from a patient with a deletion at 6q21 (Harlan *et al.*, 1991; Blackshear *et al.*, 1992a). In the present study, radiation hybrid mapping of *MACS* placed this gene on chromosome 6 within 4.2 cR_{3000} of *D6S266* (LOD > 3.0); it is most likely between *D6S266* and *AFM268vh5*.

MLP–β-Galactosidase Fusion Protein Expression in Transgenic Mice

To determine whether the 407 bp of *Mlp* promoter was sufficient to drive normal developmentally regulated and tissue-specific expression of *Mlp*, the β-galactosidase staining from transgenic mice expressing the MLP–β-galactosidase fusion protein was compared to the results of *in situ* hybridization analysis of endogenous MLP mRNA expression. As in the case of MARCKS, MLP mRNA was expressed in the developing neural tube as early as E8.5 (Fig. 4). It remained most highly expressed in the developing brain and spinal cord during later development; however, it could also be detected readily in the lung, adrenal gland, gut, and kidney, particularly the kidney cortex. As in the case of MARCKS, MLP mRNA expression in the liver was essentially undetectable by this analysis (Fig. 4).

We next compared the pattern of endogenous mRNA expression with the pattern of Mlp–β-galactosidase expression seen in the transgenic mice. As shown in Fig. 5A, at E14.5 MLP mRNA expression was greatest in neural tissues, particularly in the developing brain and spinal cord. Expression was also readily seen in many other tissues, as described above, with the exception of the fetal liver. Essentially identical patterns of expression of the MLP–β-galactosidase fusion protein were seen at E14.5 (Fig. 5B), although the apparent expression in the brain and spinal cord was greater than that in the nonneural tissues.

Comparison of MARCKS and MLP mRNA Expression in Mouse Brain

In situ hybridization histochemistry was used to compare distribution patterns for MARCKS and MLP

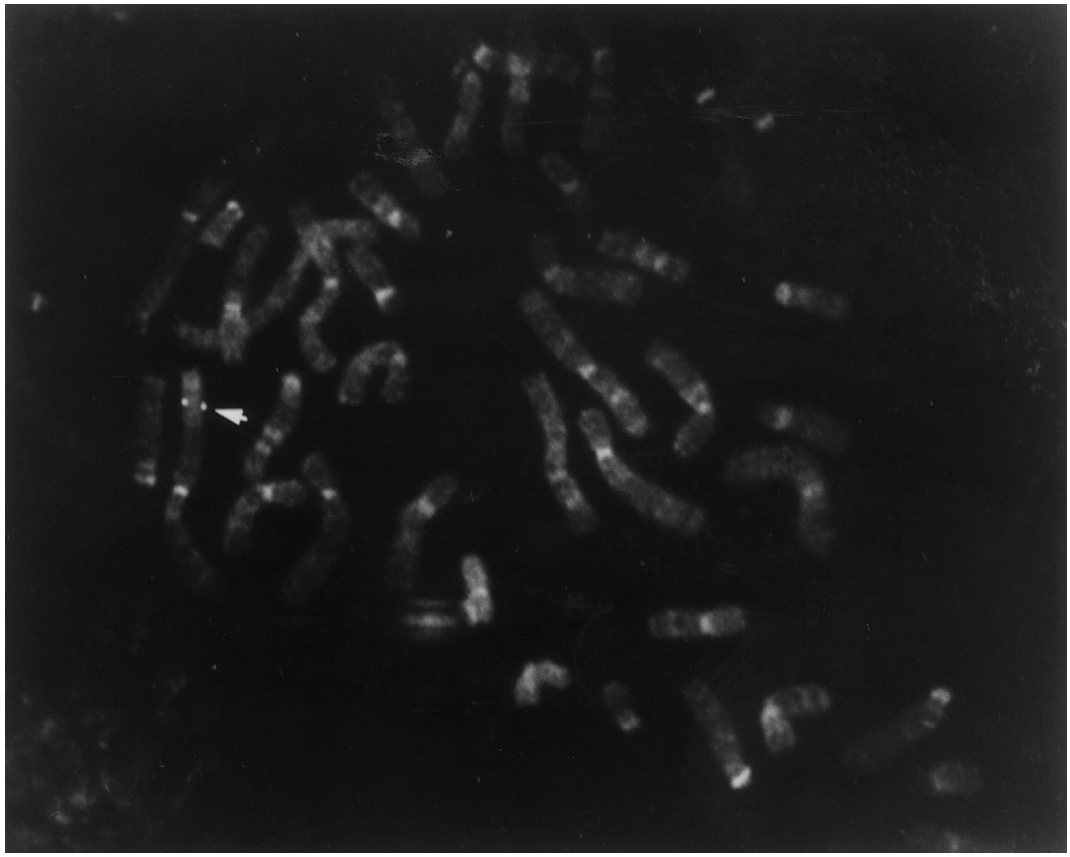

FIG. 3. Fluorescence *in situ* hybridization analysis of *MLP*. Metaphase chromosome spreads were probed with the 5.1-kb Asp718 insert of p4.1.1, labeled with digoxygenin-11–dUTP by random priming. Digital images were obtained using a Nikon epifluorescence microscope coupled to a cooled CCD camera (Photometrics). The images were merged and enhanced using the IP Lab Spectrum image analysis software. This is a partial spread showing signals (arrow) only at 1p34.

mRNA in brain from newborn mice (Fig. 6). Both messages were very highly expressed in the retina, the olfactory epithelium, and essentially the entire brain parenchyma at this age. However, MARCKS mRNA expression was readily detectable in the skin and other soft tissues (Figs. 6b and 6d), whereas MLP expression was not (Figs. 6e and 6g).

Identification of a Polymorphic Marker Linked to MLP

Southern analysis of *MLP* phage DNA using a 5′ end-labeled $(CA)_{15}$ oligonucleotide probe led to the identification of a putative CA repeat in a 2.4-kb *Sst*I fragment of the 13-kb insert from the original phage clone *MLP(5-2-1)*, from which p5.2.1 was derived. We sequenced approximately 390 bp surrounding the putative repeat and confirmed its presence (Fig. 7; GenBank Accession No. AF031640). PCR analysis of DNA from a series of 53 unrelated Caucasians was performed to determine the informativeness of this poly(CA) sequence, which we have labeled *MLP1*. The allele sizes and frequencies are presented in Table 2. The CEPH standards are 1331/01 134,134, and 1331/02 134,134, and the heterozygosity value is 0.52. We also identified a CA repeat in the mouse *Mlp* gene (Lobach *et al.*, 1993). A poly(CA) sequence of $(CA)_8AA(CA)_{28}$ was located approximately 800 bp 5′ of the potential transcription start site (D. J. Stumpo and P. J. Blackshear, unpublished data). This sequence has not been investigated for polymorphisms in mice. Based on Southern analysis, the CA repeat present in *MLP* does not appear to be located in the same region as this *Mlp* microsatellite.

Two-point linkage analysis with the CEPH reference families (Dausset *et al.*, 1990) demonstrated significant evidence for linkage between the new CA repeat polymorphic marker *MLP1* and *D1S511* with a lod score of 3.42 ($\theta = 0.05$). This marker, *MLP1*, also exhibited significant linkage to a number of genetic markers near *D1S511*, including *D1S513* and *D1S201*, which yielded lod scores of 18.43 ($\theta = 0.02$) and 16.95 ($\theta = 0.01$), respectively. We determined that the most likely position for *MLP1* relative to the CHLC version 3 map is in the 2-cM interval spanned by *D1S233* and *D1S201; D1S511* is not unequivocally localized on this map, and this analysis could not localize *MLP1* relative to *D1S511*. We did, however, attempt to develop a genetic map that incorporated both *D1S511* and *MLP1* to determine their relative order. No order that was more than 100 times more likely than a competing order could be developed, since the two markers were relatively uninformative.

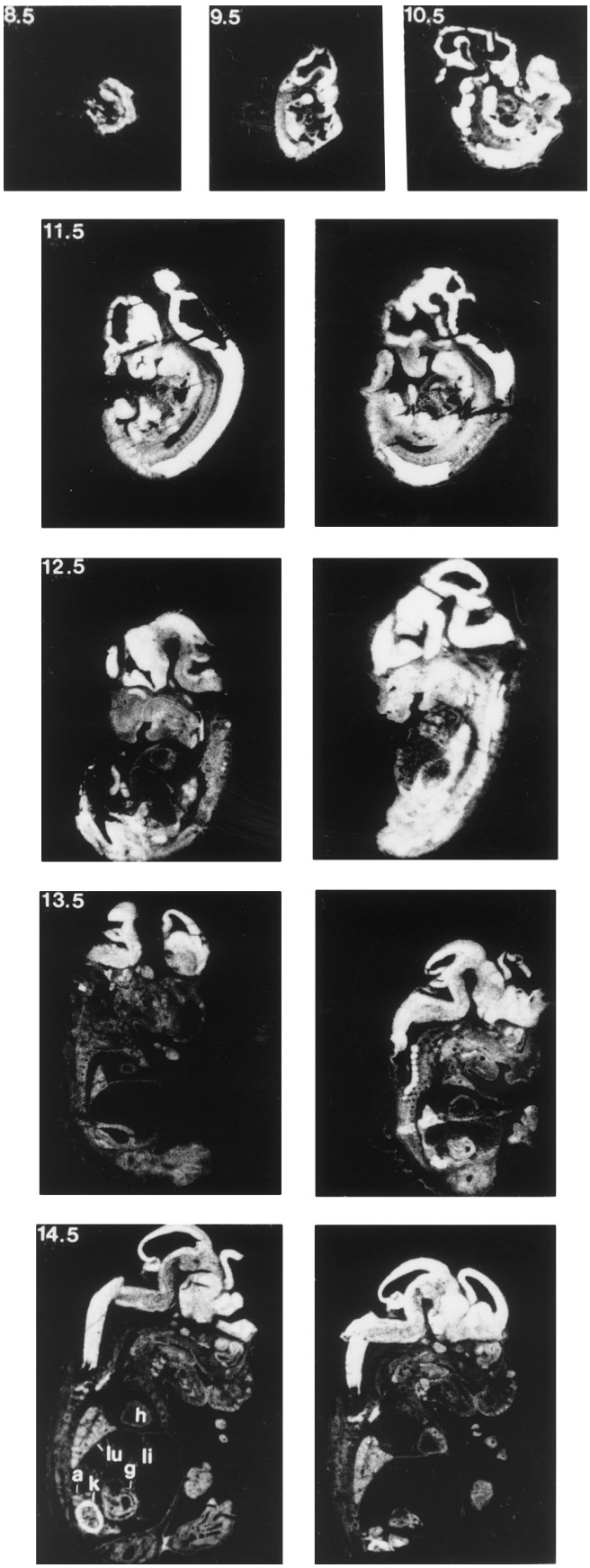

FIG. 4. *In situ* hybridization histochemistry of MLP mRNA in normal mouse embryos. Shown are sagittal sections of developing embryos between embryonic day 8.5 and 14.5, as indicated. Embryos were fresh-frozen and sectioned as described under Materials and Methods. Sections (one each from E8.5, 9.5, and 10.5; two each for E11.5–14.5) were analyzed using both antisense and sense (data not shown; see Fig. 6) MLP RNA probes; the signal appears white in this darkfield photograph. h, heart; lu, lung; li, liver; g, gut; a, adrenal; k, kidney.

Results of Genotyping of Families with a Member Affected with Lumbosacral Myelomeningocele

Because of the high frequency of cranial and caudal neural tube defects in mice lacking either MARCKS (Stumpo *et al.*, 1995) or MLP (Wu *et al.*, 1996; Chen *et al.*, 1996), we investigated the possibility of linkage disequilibrium between *MLP* and *MACS* in patients with sporadic spina bifida. There was no evidence for linkage disequilibrium between the alleles at *MLP1* and the spina bifida phenotype ($\chi^2_5 = 8.11$; $P = 0.15$) in these pedigrees. Our power to detect evidence for linkage disequilibrium at this locus is low, however, due to the presence of the frequent 134-bp allele in the population. Similarly, we found no evidence for linkage disequilibrium for loci surrounding *MACS*, at *D6S266* ($\chi^2_7 = 10.98$; $P = 0.14$) or for *AFM268vh5* ($\chi^2_6 = 7.53$; $P = 0.38$).

DISCUSSION

We have described the sequence, expression, and chromosomal localization of the human gene, *MLP*, encoding the MARCKS-like protein. Comparison of the *MLP* 433-bp putative promoter with the *Mlp* promoter (Lobach *et al.*, 1993) revealed sequence identity of 71%. Both promoters contained several potential transcription factor-binding sites and a potential TATA box at about 40 bp 5′ of the start of the mouse cDNA. The sequence identity between the *MLP* and the *MACS* promoter regions was only 36% over 403 bp; however, both genes contain only one intron, and the protein sequence surrounding the intron splice sites is identical.

Gene targeting studies have shown that both MARCKS and MLP are essential for normal development of the mouse central nervous system (Stumpo *et al.*, 1995; Blackshear *et al.*, 1997). MARCKS-deficient mice died in the perinatal period; 25% of the *Macs* −/− mice had exencephaly, and 19% had omphalocele (normal frequencies, <1%). Other abnormalities noted included decreased brain size, increased ventricular volume, agenesis of the corpus callosum and other forebrain commissures, nonfused cerebral hemispheres, severe neuronal ectopia in the outer layers of the forebrain, and abnormal laminations in the cortex and retina (Stumpo *et al.*, 1995; Blackshear *et al.*, 1997). Two groups (Wu *et al.*, 1996; Chen *et al.*, 1996) have published disparate findings concerning MLP-deficient mice. In the first report, Wu *et al.* (1996) reported the survival of some MLP-deficient mice. Severe neural tube defects were observed in about 60% of the *Mlp* −/− mice and in about 10% of the *Mlp* +/− mice. Exen-

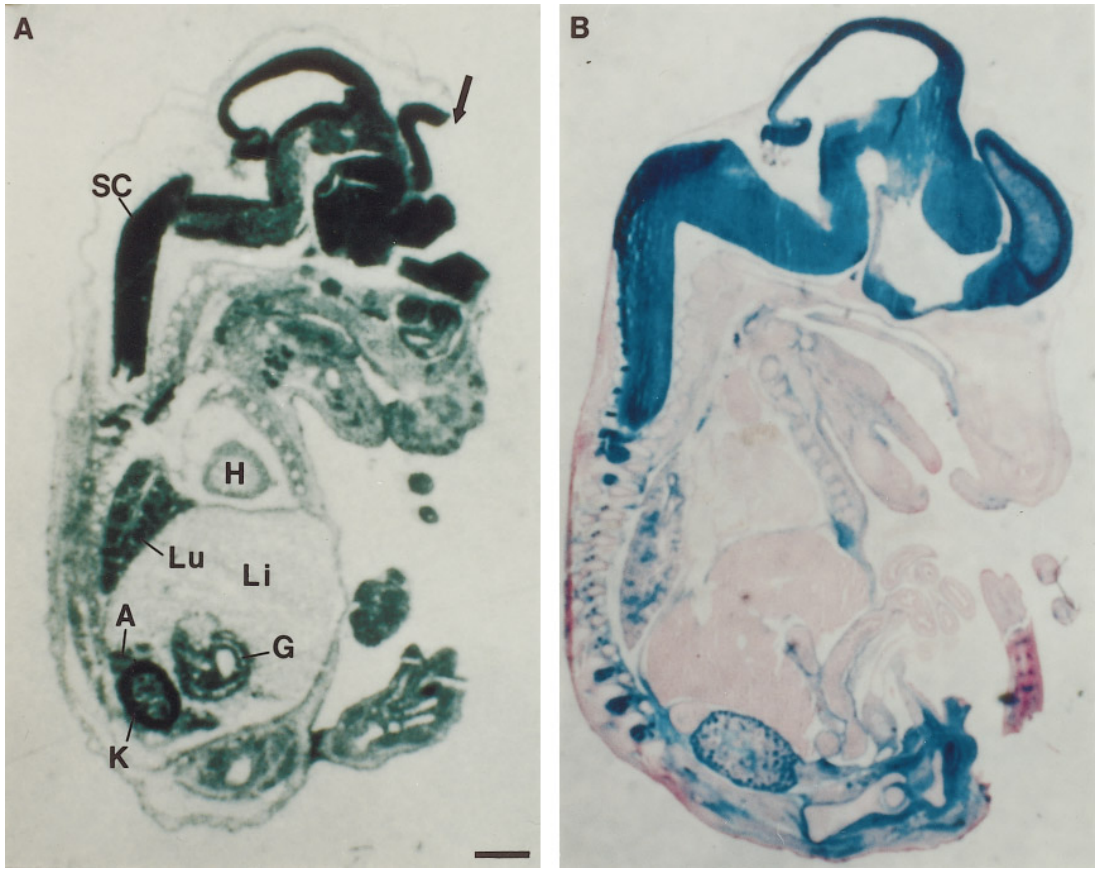

FIG. 5. Comparison of endogenous MLP mRNA expression with *Mlp-β-gal* transgene expression. (**A**) Endogenous MLP mRNA expression at E14.5, assessed by *in situ* hybridization analysis. In this photograph, dark staining represents the signal. The arrow indicates where a portion of the forebrain has broken off. H, heart; Lu, lung; Li, liver; G, gut; A, adrenal; K, kidney; SC, spinal cord. Bar, 1 mm. (**B**) MLP-β-galactosidase fusion protein expression. Embryos at E14.5 were fixed and stained for β-galactosidase activity (blue) and photographed.

cephaly occurred in 72% of all affected *Mlp* −/− embryos and spina bifida (including flexed tail) occurred in 11%. *Mlp* knockout mice without exencephaly survived even though they displayed brain abnormalities such as reduced size, enlarged ventricles, and absent corpus callosum. In contrast, Chen *et al.* (1996) demonstrated that disruption of *Mlp* resulted in 100% exencephaly with no survival after birth. The cause(s) of these differences in the MLP-deficient phenotype is unclear, since both groups used similar targeting vectors that essentially involved replacing the coding region of *Mlp* with the neomycin gene under control of the promoter of the mouse phosphoglycerate kinase-1 gene.

We have examined the expression of *Mlp* during mouse development by using transgenic mice expressing a fusion protein that contained 407 bp of *Mlp* promoter, the protein coding region, and the single intron linked to the *E. coli LacZ* gene. These transgenic mice exhibited very similar patterns of expression of the MLP–β-galactosidase fusion protein to that of endogenous MLP mRNA. We observed that MLP mRNA was expressed as early as E8.5 in the developing neural tube and that expression remained high after neural tube closure and during development of the brain, spinal cord, and cranial and peripheral nerves. Thus, the 407-bp *Mlp* promoter appears to be sufficient to confer normal developmentally regulated and tissue-specific expression of *Mlp*. Both *Mlp* and *Macs* are expressed during embryogenesis at times and locations consistent with a role in neural tube closure (Wu *et al.*, 1996; Chen *et al.*, 1996; Blackshear *et al.*, 1996; this report).

Neural tube defects are malformations of the central nervous system that result from abnormal neurulation. Neural tube defects in humans occur most often as anencephaly and spina bifida with a prevalence, worldwide, of 1 in 1000–1500 live births (Copp and Bernfield, 1994). The etiology of these defects is not well understood, but it may involve interactions between genetic and environmental factors (Copp and Bernfield, 1994). Mutant genes predisposing individuals to the major types of human neural tube defects have not been identified. Since MLP and MARCKS deficiencies both cause neural tube defects in mice, we examined the analogous human genes in preparation for studies of their possible involvement in human neural tube defects.

Radiation hybrid mapping placed *MLP* on chromosome 1 between genetic markers *D1S511* and *WI9232*, with *D1S511* being the closest polymorphic marker. This localization was confirmed by genetic mapping in the CEPH reference pedigrees. We also identified a new

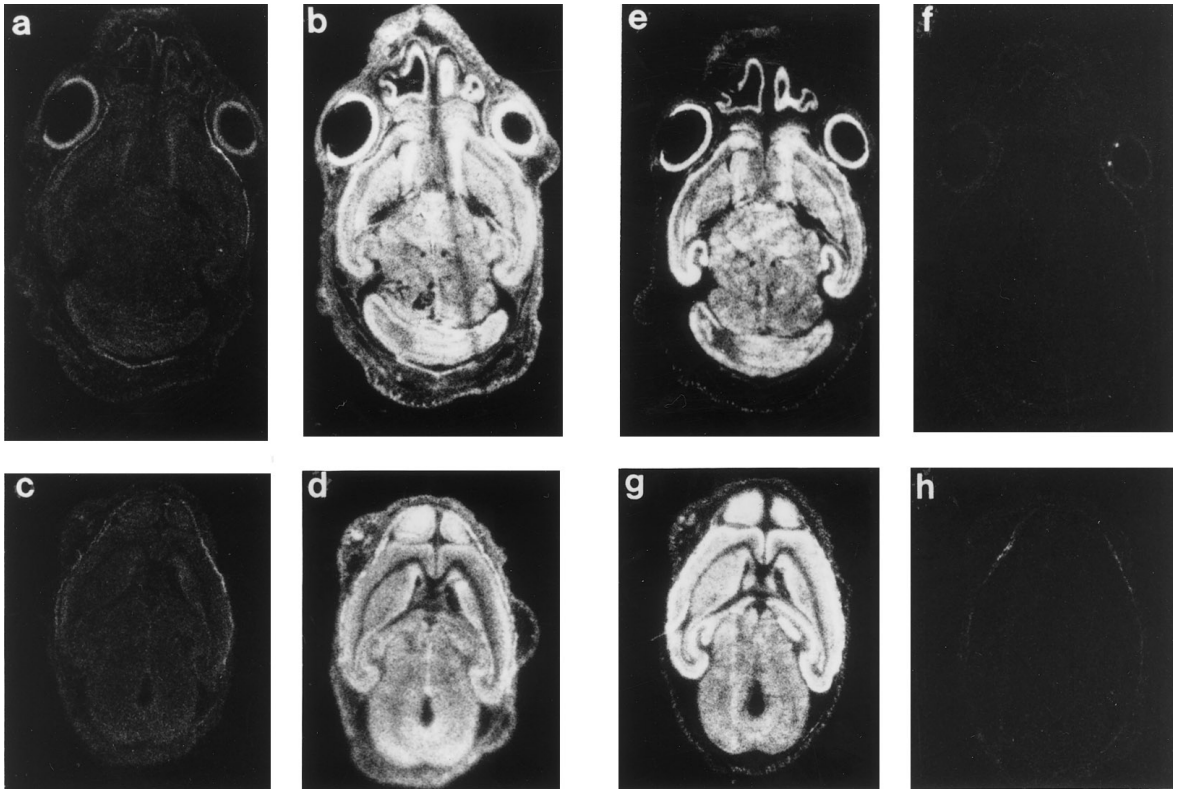

FIG. 6. *In situ* hybridization histochemistry of newborn mouse brain. (**A**) MARCKS mRNA expression. Brain sections were hybridized with sense (**a, c**) or antisense (**b, d**) MARCKS RNA probes. (**B**) MLP mRNA expression. Brain slices were hybridized with sense (**f, h**) or antisense (**e, g**) MLP RNA probes.

microsatellite repeat, *MLP1,* within *MLP,* with a heterozygosity value of 0.52. Subsequent screening of DNA from multiple families affected with a lumbosacral myelomeningocele and analysis by the transmission equilibrium test (Spielman *et al.*, 1993) showed no evidence for linkage disequilibrium with alleles at these loci and the spina bifida phenotype, suggesting that *MLP* is not a major gene for the spina bifida phenotype in these families. Similarly, evaluation of these pedigrees for linkage disequilibrium with markers surrounding the *MACS* locus on chromosome 6 also revealed no evidence for disequilibrium. The transmission equilibrium test is well suited to the investigation of candidate genes in complex disorders such as neural tube defects (Spielman *et al.*, 1993). The primary advantage of this approach is that it requires the availability of only nuclear pedigrees with just one affected individual. Although these initial studies did not implicate *MLP* or *MACS* as major genes for the spina bifida phenotype, it is important to keep in mind the differing phenotypes that were reported in the MLP-deficient mice, the presence of exencephaly rather than spina bifida in the MARCKS-deficient mice, and the fact that our investigations were performed in families with one affected member. Testing of multiplex pedigrees and investigation of other types of neural tube defects, such as anen-

```
1    GTATACCTGT AATCCCAGCT ACTTCGGAGG CTGAGGCAGG AGAATCGCTT
51   GAACCCGGAA GGCAGAGGTT GCAGTCAGCT GAGATCACGC CACTGTACTC
                →
101  CAGCCTGGTG ACAGAGGGAG ATTCTGTCTC AAAAAACAAA ACAAAACATA
151  CAGACACACA CACACACACA CACACACACA CACACACACA CACCAAAAAA
                                                ←
201  CCCGTGCATT TGAGGTGCAG ACCACACTGG AGGATTTCAA TAATGTTCCT
251  CAGGGCTGTT ACATAAAATT CCGGGGCAGG TGCCAGACAC AAAACTTGGT
301  GCTCAACAGT TTCCCATTCT TGTCCTCTCT CTCTCTCCTC TCTTTCTGCC
351  CTGGGGAGGG GAGCTGGGAG CCTAGGATTG CCCTGAGTCA
```

FIG. 7. Sequence surrounding the novel, *MLP*-linked marker *MLP1.* Nucleotide sequence surrounding the *MLP1* CA repeat marker. The CA repeat is boxed and starts at base 155 and ends at base 192. The PCR primers used to type this locus are underlined with the forward primer represented by → and the reverse primer represented by ←.

TABLE 2

Allele Sizes and Frequencies at *MLP1*

Allele	Size (nt)	Frequency
A1	130	0.08
A2	132	0.11
A3	134	0.67
A4	136	0.09
A5	138	0.01
A6	140	0.01
A7	142	0.03

Note. DNA from a series of 53 unrelated white Caucasians was analyzed by PCR as described under Materials and Methods.

cephaly or myelomeningoceles at levels higher than lumbosacral, may yield different results and is now practical given the identification of polymorphic markers for both *MACS* and *MLP*.

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