



Genomic Organization of the Murine Miller–Dieker/Lissencephaly Region: Conservation of Linkage with the Human Region

Shinji Hirotsune, Svetlana D. Pack, Samuel S. Chong, et al.

Genome Res. 1997 7: 625-634

Access the most recent version at doi:[10.1101/gr.7.6.625](https://doi.org/10.1101/gr.7.6.625)

References This article cites 27 articles, 8 of which can be accessed free at:
<http://genome.cshlp.org/content/7/6/625.full.html#ref-list-1>

License

Email Alerting Service Receive free email alerts when new articles cite this article - sign up in the box at the top right corner of the article or [click here](#).

To subscribe to *Genome Research* go to:
<https://genome.cshlp.org/subscriptions>

Cold Spring Harbor Laboratory Press

LETTER

Genomic Organization of the Murine Miller–Dieker/Lissencephaly Region: Conservation of Linkage with the Human Region

Shinji Hirotsume,¹ Svetlana D. Pack,² Samuel S. Chong,²
Christiane M. Robbins,³ William J. Pavan,¹ David H. Ledbetter,^{2,4} and
Anthony Wynshaw-Boris^{1,5}

¹Laboratory of Genetic Disease Research, ²Diagnostic Development Branch, and ³Sequencing Core Facility, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD 20892-4470

Several human syndromes are associated with haploinsufficiency of chromosomal regions secondary to microdeletions. Isolated lissencephaly sequence (ILS), a human developmental disease characterized by a smooth cerebral surface (classical lissencephaly) and microscopic evidence of incomplete neuronal migration, is often associated with small deletions or translocations at chromosome 17p13.3. Miller–Dieker syndrome (MDS) is associated with larger deletions of 17p13.3 and consists of classical lissencephaly with additional phenotypes including facial abnormalities. We have isolated the murine homologs of three genes located inside and outside the MDS region: *Lis1*, *Mnt/Rox*, and *14-3-3ε*. These genes are all located on mouse chromosome 11B2, as determined by metaphase FISH, and the relative order and approximate gene distance was determined by interphase FISH analysis. The transcriptional orientation and intergenic distance of *Lis1* and *Mnt/Rox* were ascertained by fragmentation analysis of a mouse yeast artificial chromosome containing both genes. To determine the distance and orientation of *14-3-3ε* with respect to *Lis1* and *Mnt/Rox*, we introduced a super-rare cutter site (*VDE*) that is unique in the mouse genome into *14-3-3ε* by gene targeting. Using the introduced *VDE* site, the orientation of this gene was determined by pulsed field gel electrophoresis and Southern blot analysis. Our results demonstrate that the MDS region is conserved between human and mouse. This conservation of linkage suggests that the mouse can be used to model microdeletions that occur in ILS and MDS.

Contiguous gene syndromes are complex human genetic diseases caused by the deletion of physically contiguous but functionally unrelated genes (Ledbetter and Ballabio 1995). These syndromes are the consequence of de novo deletions of characteristic chromosomal regions, resulting in hemizygoty and haploinsufficiency of genes contained within the deleted regions. Deletions of 17p13.3 result in two well-characterized disorders: isolated lissencephaly sequence (ILS) and Miller–Dieker syndrome (MDS). ILS is a brain malformation disorder characterized by smoothness of the cerebral surface with

disordered organization of the cortical layers (classical lissencephaly), the result of defective neuronal migration at 9–13 weeks of embryonic development (Dobyns 1987). This disease is associated with de novo translocations or submicroscopic deletions within chromosome 17p13.3 in almost 40% of patients (Dobyns et al. 1993). MDS consists of classical lissencephaly, characteristic facial abnormalities, and occasionally other birth defects (Dobyns et al. 1992). In MDS, either visible cytogenetic or submicroscopic deletions are detected in >90% of patients. By mapping the extent of deletions in patients with ILS and MDS, critical regions responsible for each of these disorders have been described (Chong et al. 1997). The deleted regions in patients with these disorders have considerable overlap, although the

⁴Present address: Center for Medical Genetics, The University of Chicago, Chicago, Illinois 60637-1470.

⁵Corresponding author.

E-MAIL tonywb@nhgri.nih.gov; FAX (301) 402-2170.

HIROTSUNE ET AL.

MDS region extends more telomeric on 17p13.3. These results suggest that clinical severity correlates with the extent of deletion of the critical region, resulting in haploinsufficiency.

Recently, a candidate gene for lissencephaly, *LIS1*, was isolated in 17p13.3 (Reiner et al. 1993). *LIS1* was later identified as the human homolog of the 45-kD brain isoform of a subunit of platelet-activating factor acetylhydrolase (Hattori et al. 1994). Point mutations and rearrangements of *LIS1* have been found in several patients with ILS (Chong et al. 1997; LoNigro et al. 1997). Exons of other genes have also been identified within the chromosome region of MDS (S.S. Chong and D.H. Ledbetter, unpubl.). However, the relationships between gene function and haploinsufficiency in ILS and MDS remain to be elucidated. To address these issues, it will be valuable to analyze mice with gene disruptions and defined deletions within the MDS critical region.

As a first step toward creating a mouse model for lissencephaly and MDS, we isolated murine homologs of three genes, *Lis1*, *Mnt/Rox*, and *14-3-3 ϵ* , located within the critical regions for MDS and ILS. We have found that the location and order of these genes is evolutionarily conserved between human and mouse, suggesting that it will be possible to model MDS deletions in the mouse.

RESULTS

Isolation of Mouse Genomic Clone of *Lis1*, *Mnt/Rox*, and *14-3-3 ϵ*

A 129Sv λ phage genomic library was screened using human *LIS1*, *MNT/ROX*, and *14-3-3 ϵ* cDNA. We isolated 11, 2 and 2 positive phage clones for *Lis1*, *Mnt/Rox*, and *14-3-3 ϵ* , respectively. To screen for overlap of these genomic clones, we compared restriction fragment patterns of each clone (fingerprinting analysis) using *EcoRI* and *PstI*. The two independent clones isolated for *Mnt/Rox* showed similar fingerprinting patterns, indicating that the two clones were overlapping or identical (data not shown). Similarly, the two clones of *14-3-3 ϵ* displayed identical fingerprinting patterns (data not shown). The fingerprinting profiles revealed that the 11 *Lis1* clones were comprised of three different overlapping groups of clones (data not shown), consistent with the presence of at least three *Lis* family members in the mouse (Reiner et al. 1995).

The mouse cDNA sequences of *Lis1* (Peterfy et al. 1994) and *14-3-3 ϵ* (McConnell et al. 1995) have been reported previously, and *Mnt/Rox* was se-

quenced recently for both human and mouse (Hurlin et al. 1997; Meroni et al. 1997). To identify exons located within phage clones of all three genes, we subcloned phage inserts into pBluescript after digestion with *AluI* or *Sau3AI*. These subclone libraries were screened using cDNA probes for each gene. Clones containing exons of each gene were sequenced to identify exons and exon-intron junctions. The alignment of genomic sequences with each mouse cDNA sequence indicated that they represent genomic clones for *Lis1*, *Mnt/Rox* and *14-3-3 ϵ* (data not shown).

FISH Analysis of *Lis1*, *Mnt/Rox*, and *14-3-3 ϵ* Phage Clones

Multicolor fluorescence in situ hybridization (FISH) analysis of the phage clones for *Lis1*, *Mnt/Rox*, and *14-3-3 ϵ* on metaphase and interphase chromosomes was performed to determine the chromosome location and establish the order of these three genes. With metaphase FISH, all three genes were located within the same region of mouse chromosome 11B2 when examined individually (Fig. 1A-C) or simultaneously (Fig. 1D). Chromosome 11 was identified using *Brca1* as a marker (Schröck et al. 1996). The other two groups of the genomic clones that were isolated by *LIS1* cDNA were mapped to different chromosomes by metaphase FISH (data not shown). Interphase FISH was performed with the three phage clones to determine relative gene order. As in the human, the gene order was *Lis1*, *Mnt/Rox*, and *14-3-3 ϵ* (Fig. 1E). In addition, the relative distance between these genes was estimated by comparing the separation between hybridization signals in several nuclei using FISH on halo preparations (Haaf and Ward 1994a,b) from interphase nuclei (fiber-FISH). The distance between *Lis1* and *Mnt/Rox* was about one-third of the distance between *Mnt/Rox* and *14-3-3 ϵ* (Fig. 1F). Thus, these three genes colocalized to the same region of the mouse chromosome syntenic to human 17p13.3, and gene order and relative distance were conserved between mouse and human.

Determination of the Transcriptional Orientation of *Lis1* and *Mnt/Rox* by YAC Fragmentation

To determine the relative transcriptional directions of *Lis1* and *Mnt/Rox*, yeast fragmentation (Pavan et al. 1990) of a yeast artificial chromosome (YAC) clone containing *Lis1* and *Mnt/Rox* was performed. The Princeton mouse YAC library was screened as

MURINE MDS/ILS REGION

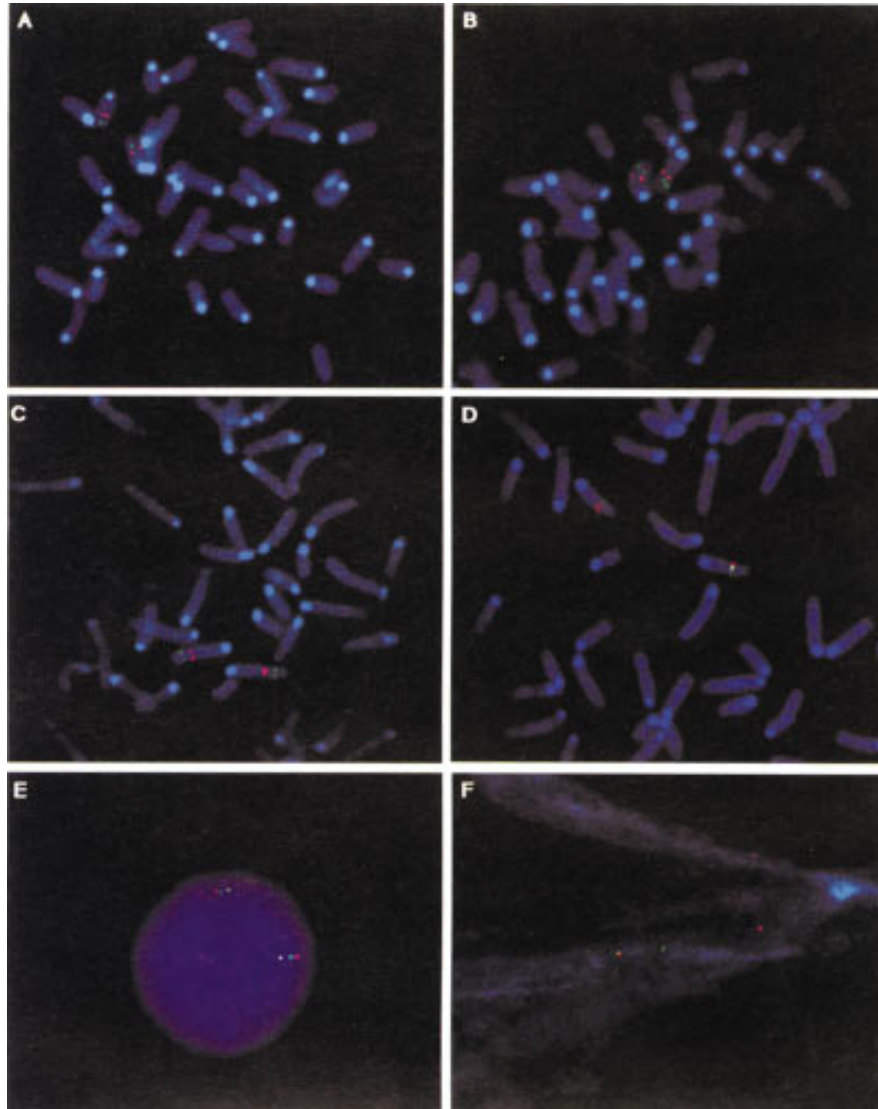


Figure 1 FISH on normal mouse metaphase chromosomes, interphase nuclei, and extended chromatin. *Brca1* (FITC, green fluorescence) was used as a marker for chromosome 11, and colocalized on metaphase spreads to chromosome 11 with: (A) *Lis1*; (B) *Mnt/Rox*; (C) *14-3-4 ϵ* ; (D) *Lis1*, *Mnt/Rox*, and *14-3-3 ϵ* (rhodamine, red fluorescence). (E) Gene order was established by three-color FISH on mouse interphase nuclei: *Lis1* (red); *Mnt/Rox* (green); *14-3-3 ϵ* (orange, mix of biotin- and digoxigenin-labeled DNA, 1:1 ratio). (F) FiberFISH was used for evaluation of relative distances between *Lis1* (orange), *Mnt/Rox* (green), and *14-3-3 ϵ* (red).

pools, and one positive YAC clone was isolated that contained both genes. The YAC containing *Lis1* and *Mnt/Rox* and the fragmentation strategy are represented in Figure 2A. Fragmentation vectors for each gene were created by inserting genomic clones of *Lis1* and *Mnt/Rox* into the yeast fragmentation vector pB1R (Lewis et al. 1992) in both orientations. Homologous recombination between the YAC and

the fragmentation vector will result in deletion of all sequences distal to the integration site, including *TRP1*, and integration of a new auxotrophic marker *LYS2*. The viability of the recombinant yeast depends on the ability of the fragmentation to result in a functional YAC, which in turn is dependent upon the orientation of the DNA fragment inserted into the yeast fragmentation vector. If the gene is inserted in the correct orientation in the fragmentation vector, recombination with the YAC will result in a deleted YAC with a centromere and *LYS2*. In contrast, after recombination with the fragmentation vector containing the gene in the opposite orientation, the deleted YAC will contain *LYS2*, but will lose the centromere. This will cause loss of the deleted YAC during mitosis, and result in inefficient growth on plates lacking lysine. Gene orientation can be inferred by comparison of the frequency of the fragmentation vectors in both directions, and confirmed by Southern analysis of the resulting deleted YACs.

Four fragmentation vectors were constructed that contained *Lis1* or *Mnt/Rox* in both orientations. Vectors were linearized, transformed singly with each YAC, and selected for *LYS* prototrophy. For the *Lis1* vectors, there were 7.8-fold more stable *LYS*⁺ transformants produced with the R fragmentation vector than with the L vector. For *Mnt/Rox*, 2.1-fold more clones were produced with the L vector than with the R vector (Fig. 2A). These results suggest that *Lis1* and *Mnt/Rox* are transcribed divergently. The sizes of the fragmented YAC clones were compared with the original YAC, which was 600 kb (data not shown), by pulsed field gel electrophoresis (PFGE) and Southern analysis us-

HIROTSUNE ET AL.

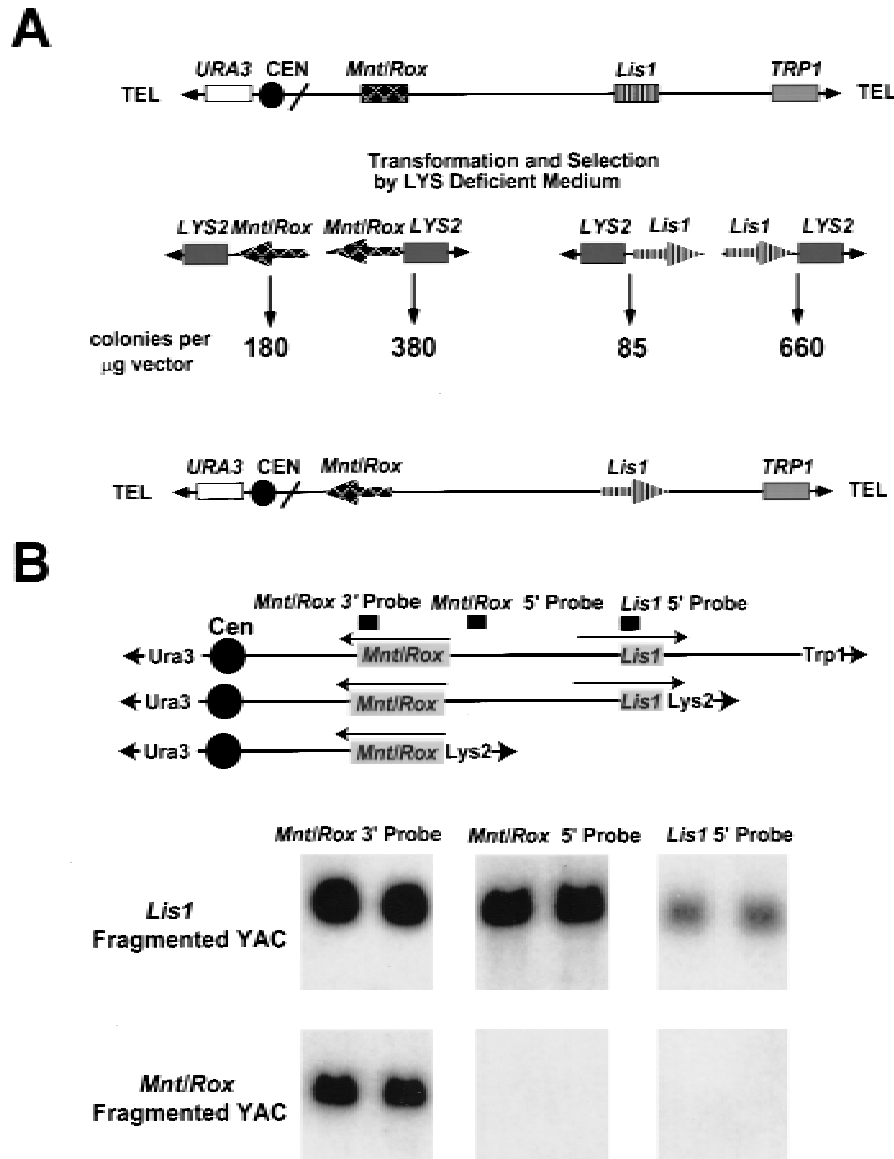


Figure 2 *Lis1-Mnt/Rox* YAC fragmentation for transcriptional orientation. (A) Schematic representation of YAC fragmentation. (Top) The *Lis1-Mnt/Rox* YAC clone is shown, with the location of the two genes, *URA3* and *TRP1* selectable markers, and the position of the centromere and telomeres. (Middle) The fragmentation vectors with *LYS2* as the selectable marker near the telomere, and the *Mnt/Rox* (left) or *Lis1* (right) cloned in both orientations relative to the telomere. Underneath the fragmentation vectors, the number of colonies per microgram of vector are indicated. (Bottom) The gene orientation on the YAC relative to centromere was inferred by comparison of the recombinant frequency of each fragmentation vector. The arrows indicate the 5' → 3' direction of each gene. (B) Southern analysis of the fragmented YAC. (Top) The original YAC is shown on the first line, followed by the organization of the *Lis1*- and *Mnt/Rox*-deleted YACs. The locations and transcriptional directions of the two genes, as determined by colony number, and the locations of three hybridization probes are shown. The arrows indicate the 5' → 3' direction of each gene. (Bottom) DNA from two *LYS2*⁺ transformants from the *Lis1* fragmentation, and two from the *Mnt/Rox* fragmentation, were digested with *EcoRI*, transferred to nylon membranes, and probed with three different probes: a *Mnt/Rox* 3' probe, a *Mnt/Rox* 5' probe, and a *Lis1* 5' probe.

ing pBR322 as probe. The fragmented YACs with *Lis1* and *Mnt/Rox* were 400 kb and 200 kb, respectively, indicating that the distance between these genes is ~200 kb (data not shown) and demonstrating that fragmentation had occurred. To further support this relative gene orientation, YAC DNAs were isolated from fragmented clones recovered from *Lis1* or *Mnt/Rox* vectors, digested with *EcoRI*, and probed with three independent probes: a *Mnt/Rox* 3' probe, a *Mnt/Rox* 5' probe, and a *Lis1* 5' probe (Fig. 2B). Yeast DNA of *LYS2*⁺ clones from the *Lis1* fragmentation hybridized to all three probes, whereas DNA of clones from the *Mnt/Rox* fragmentation hybridized only with the *Mnt/Rox* 3' probe, as expected (Fig. 2B). Thus, the orientation estimated by the recombination frequency was completely consistent with the Southern hybridization data, demonstrating that *Lis1* and *Mnt/Rox* are transcribed divergently.

Insertion of Super-Rare Cutter *VDE* at *14-3-3ε* Locus by Homologous Recombination

A single YAC clone containing both *Mnt/Rox* and *14-3-3ε* could not be isolated by repeated screening. Therefore, we could not use YAC fragmentation to determine gene distance and transcriptional orientation. Because the physical distance between *Lis1* and *Mnt/Rox* is ~200 kb (see above), using relative distances in fiberFISH (Fig. 1F), the distance between *Mnt/Rox* and *14-3-3ε* was estimated to be 600 kb (Fig. 1F), consistent with the human physical map.

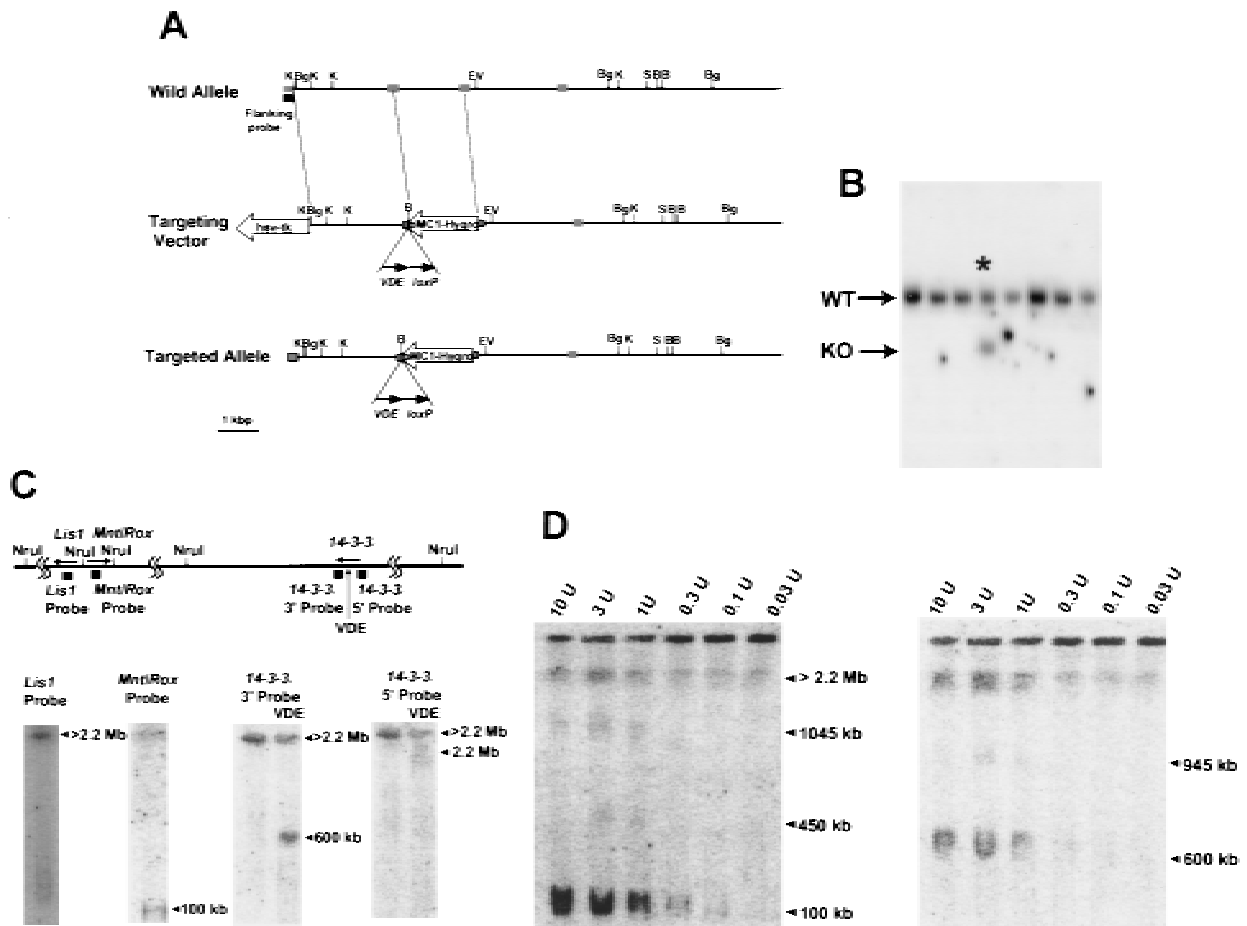


Figure 3 *14-3-3 ϵ* targeting of VDE site for determination of transcriptional orientation. (A) The restriction map and the targeting vector are shown. pMC1hyg and PGK-*tk* indicate the genes used for positive and negative selection with hygromycin and FIAU; arrow directions indicate transcriptional orientation. *VDE* and *loxP* sites are indicated. The dotted boxes indicate *14-3-3 ϵ* exons. Restriction sites are as follows: (B) *Bam*HI; (Bg) *Bgl*II; (EV) *Eco*RV; (H) *Hind*III; (K) *Kpn*I; (S) *Sal*I; (VDE) the super-rare cutter. (B) The result of the screening of the transfected ES cell by Southern hybridization using a flanking probe and *Bam*HI digestion. (Asterisk) The positive clone; (WT) wild-type allele; (KO) knock-out allele. (C, top) The *Nru*I restriction map of the MDS region in the mouse, with the location of the three genes (the direction of transcription indicated by arrows), and the location of probes used for hybridization. (Bottom) PFGE and Southern hybridization of DNA from the targeted ES clone with a *VDE* site inserted into the *14-3-3 ϵ* locus. DNA was digested with *Nru*I alone (nothing above the lane), or *Nru*I and *VDE* (indicated by *VDE* above the lane), and hybridized with the indicated probes. The arrows indicate the sizes of the bands detected with each probe. (D) PFGE Southern hybridization for complete *VDE* digestion and complete or partial *Nru*I digestion. The enzyme concentration per microliter is above each lane. The arrowheads indicated the band detected by the *14-3-3 ϵ* 3' probe.

To determine the relative gene orientation spanning this long distance, we inserted the super-rare cutter site *VDE* at the *14-3-3 ϵ* locus by homologous recombination in embryonic stem (ES) cells. *VDE* is a DNA endonuclease derived from the *VMA1* gene product of *Saccharomyces cerevisiae* (Gimble and Thorner 1992) and is related to other nucleases involved in rearrangement (Bremer et al. 1992). Considering its long recognition sequence (34 bp), it

seemed reasonable to assume that this inserted site would be unique or rare within the mouse genome. To confirm this, mouse genomic DNA was embedded into agarose blocks and digested with *VDE*, followed by PFGE analysis. The ethidium bromide staining profile displayed no significant difference with or without *VDE* digestion (data not shown).

Therefore, we used gene targeting to insert a *VDE* site, along with a pMC1hyg gene, into the *14-*

HIROTSUNE ET AL.

3-3 ϵ locus using a replacement-type targeting construct (Fig. 3A). Correct targeting should also result in the disruption of this gene. ES cells were transfected and screened for the presence of targeted events at the 14-3-3 ϵ locus. DNA from each clone was digested with *Bam*HI followed by agarose gel electrophoresis. After Southern blotting and hybridization with an external probe, one positive clone out of 121 was identified (Fig. 3B), as demonstrated by the presence of a 7-kb polymorphic targeted band. Southern analysis with several restriction enzymes and probes confirmed that this clone was correctly targeted at the 14-3-3 ϵ locus (data not shown).

Determination of the Transcription Orientation of 14-3-3 ϵ

The 14-3-3 ϵ targeted ES clone, with insertion of the *VDE* site, was used to determine the gene orientation and the distance between *Mnt/Rox* and 14-3-3 ϵ . Using wild-type DNA, PFGE and Southern analysis demonstrated that *Mnt/Rox* is located within a 100-kb *Nru*I fragment (Fig. 3C). On the other hand, both *Lis1* and 14-3-3 ϵ are located within very large (>2.2 Mb) *Nru*I fragments (Fig. 3C). To determine the relative orientation and distance of 14-3-3 ϵ from *Mnt/Rox*, DNA from the 14-3-3 ϵ -targeted ES clone was digested with *Nru*I and *VDE*, followed by PFGE, Southern analysis, and hybridization with 5' or 3' probes of 14-3-3 ϵ on either side of the *VDE* site (Fig. 3D). The size of the bands detected by 5' and 3' probes were used to determine the transcriptional orientation of 14-3-3 ϵ relative to *Mnt/Rox*. The physical map of the human and the mouse interphase and fiberFISH data estimated that *Mnt/Rox* and 14-3-3 ϵ are located within 600 kb (see above). The 14-3-3 ϵ 5' probe detected a very large fragment (>2.2 Mb), and the 14-3-3 ϵ 3' probe detected a 600-kb fragment. These results suggest that 14-3-3 ϵ is transcribed in a direction toward *Mnt/Rox*, because this is the only orientation compatible with the detection of these sized bands. This interpretation was supported by partial digestion of DNA from the targeted clone with

*Nru*I (Fig. 3D). The targeted 14-3-3 ϵ ES cell DNA was digested completely with *VDE*, followed by partial digestion with *Nru*I using various enzyme concentrations. After PFGE and Southern analysis with a 3' probe of 14-3-3 ϵ , 945- and 600-kb fragments were detected, whereas a *Mnt/Rox* probe on the same filter detected bands of 100, 450, and 1045 kb (Fig. 3D). Because 14-3-3 ϵ and *Mnt/Rox* are located within 600 kb by fiberFISH, and *Lis1* and 14-3-3 ϵ are located on large (>2.2-Mb fragments), the Southern data is consistent only with the interpretation that 14-3-3 ϵ is transcribed toward *Mnt/Rox*. The gene distance and orientation estimated from the *Nru*I restriction map was also supported by partial *Not*I digestion and Southern analysis (data not shown).

The gene organization of the human MDS/ILS region and murine syntenic region is summarized in Fig. 4. The organization of three genes in this region is similar between the mouse and human. In the mouse, *Lis1* and *Mnt/Rox* are transcribed divergently, and are located 170 kb apart. 14-3-3 ϵ is transcribed toward *Mnt/Rox*, and these genes are located within 1 Mb of each other.

DISCUSSION

Several human syndromes are associated with the hemizygous deletion of a specific chromosomal re-

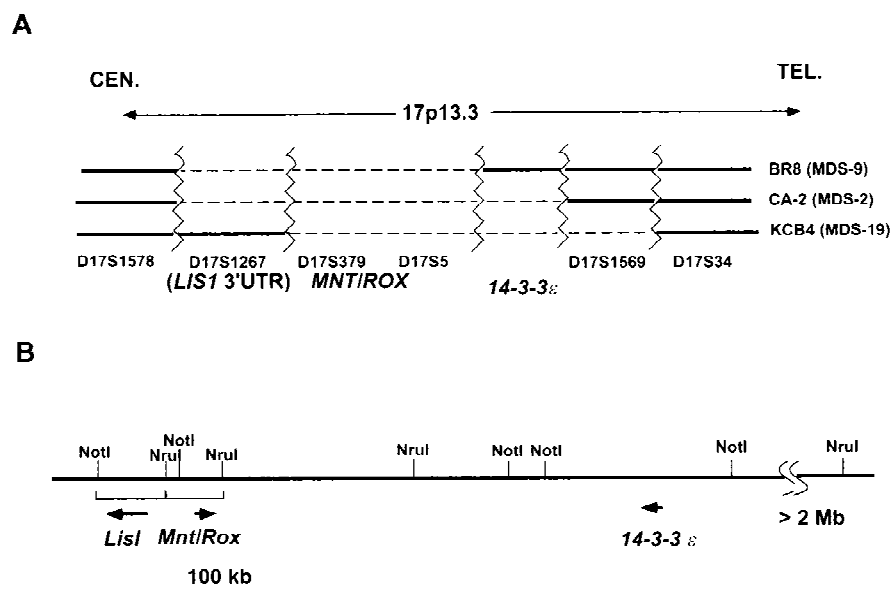


Figure 4 Comparison of human and mouse genomic organization in the MDS/ILS critical region. (A) Gene organization in the human MDS/ILS region on human 17p13.3 (after Chong et al. 1997). The broken line indicates the extent of deletion in each patient. The locations of genes and polymorphic markers are indicated at the bottom. (B) Murine syntenic region of 11B2.

gion (Ledbetter and Ballabio 1995). A subset of these appear to be classical contiguous gene syndromes, in which the inactivation of several unrelated genes is involved in a complex phenotype, and the severity of the phenotype is determined by the genes that are deleted. MDS appears to be a typical example of a contiguous gene syndrome. Certain deletions in this region result in ILS, whereas overlapping and larger deletions result in MDS. The relationships between gene function and phenotype in human contiguous gene syndromes are difficult to study in patients, so the creation of a mouse model would be a valuable tool to examine these relationships. A detailed determination of genomic organization of the MDS critical region is a prerequisite to the creation of a mouse model of ILS/MDS.

In this paper, we describe the genomic organization of the MDS critical region in the mouse. The mouse homologs of three human genes present in the MDS region were cloned and sequenced. Comparison of this sequence with the cDNA sequence of murine *Lis1* (Peterfy et al. 1994) and *14-3-3 ϵ* (McConnell et al. 1995) and human and murine *Mnt/Rox* (Hurlin et al. 1997; Meroni et al. 1997) allowed us to identify exons and exon-intron junctions of these three genomic clones. All three genes were mapped to the region of mouse chromosome 11 that is syntenic to the MDS region on human 17p13.3 by FISH. Using interphase FISH, we were able to determine relative gene orientation and distance of these murine genes, and they were similar to the gene organization in the human genes (Chong et al. 1996). We determined the relative transcriptional directions of these three murine genes by using two novel approaches. The transcription direction of *Lis1* relative to *Mnt/Rox* was determined using YAC fragmentation (Pavan et al. 1990), demonstrating that these genes are divergently transcribed. The relative direction of *14-3-3 ϵ* was determined by the introduction of a super-rare cutter site for *VDE* digestion into the locus through homologous recombination in ES cells. PFGE and Southern analysis then allowed us to determine that *14-3-3 ϵ* is transcribed the same direction as *Lis1*.

The murine homologs of *Lis1*, *Mnt/Rox*, and *14-3-3 ϵ* each encode known proteins. *Lis1* was isolated as a gene that showed partial deletion in two patients with lissencephaly (Reiner et al. 1993), and was later identified as the homolog of one of the subunits of platelet-activating factor acetylhydrolase (Hattori et al. 1994). *Lis1* is expressed in the cortical plate in the developing mouse (Reiner et al. 1995), consistent with its presumed role in neuronal migration. *Mnt/Rox* is deleted in all patients with

MDS. *Mnt/Rox* is a basic helix-loop-helix domain that interacts with Max and Sin3 to regulate the activity of c-Myc (Hurlin et al. 1997; Meroni et al. 1997). *14-3-3 ϵ* is located in a region with frequent loss of heterozygosity in several cancers (Phillips et al. 1993; McDonald et al. 1994), raising the possibility that this gene may act as a tumor suppressor. Additionally, an interaction between *14-3-3 ϵ* and *cdc25B* has been described (Conklin et al. 1995), suggesting a role in cell cycle regulation. Thus, all three genes that were analyzed have potential function in development, and may play a role in the ILS and/or MDS phenotypes.

Conservation of synteny is a feature of mammalian chromosomes (DeBry and Seldin 1996). Within these regions, the gene order appears to be similar, although detailed examination of distances and orientation have been performed for few of those regions. We have examined the conservation of synteny between human chromosome 17p13.3 and mouse 11B2 in detail. Our data demonstrate that the gene order and distance of these genes have been conserved between the mouse and human. The evolutionary conservation suggests either that there is some functional importance to the organization of this genomic region or that there is not sufficient evolutionary distance between these species for divergence to have occurred. Because we have not identified all genes in this region in either mouse or human, we cannot rule out the possibility that small rearrangements may be present. However, the overall organization of these regions is conserved between these two species. Because there is conservation of linkage, it will be possible to create deletions in the mouse that mimic those of ILS and MDS. Such models will likely be effective reagents for the molecular dissection of the genes involved in the pathogenesis of these disorders. The detailed organization of the murine homologous region described here provides an important framework for the creation of a mouse model of ILS and MDS.

METHODS

Isolation of Mouse Genomic Clones of *Lis1*, *Mnt/Rox*, and *14-3-3 ϵ*

A λ phage genomic library (strain 129/Sv, Stratagene) was screened with labeled cDNAs for human *LIS1*, *MNT/ROX*, and *14-3-3 ϵ* . As probes, the 1.5-kb *HindIII* fragment of human *LIS1* cDNA (Reiner et al. 1993), the 0.8-kb *HindIII*-*PvuII* fragment of human *MNT/ROX* cDNA (Meroni et al. 1997), and the 1.7-kb PCR-amplified product from a human *14-3-3 ϵ* cDNA clone (Chong et al. 1996) were used. The phage DNAs were purified

HIROTSUNE ET AL.

by standard procedures (Sambrook et al. 1989). Phage inserts were cloned into the *NotI* site of the pBluescript (Stratagene) and digested with *NotI* and *EcoRI* or *PstI* for fingerprinting.

To determine exons and exon-intron junctions, the plasmid subclones were digested with *AluI* or *Sau3AI* and subcloned into the *EcoRV* or *BamHI* sites of pBluescript II (Stratagene), respectively. These subcloned libraries were screened with cDNA probes as described above. The plasmid DNAs of the positive colonies were extracted by the Qiagen plasmid kit, and sequenced by automated cycle methods (model 377, Perkin-Elmer Applied Biosystem Inc.; Robbins et al. 1996). GenBank accession nos. of *Lis1*, *Mnt/Rox*, and *14-3-3ε* are U34277, U77536, and Z19599, respectively.

FISH Analysis Using Phage Clones

Slides with metaphase spreads, interphase nuclei, and extended chromatin were prepared from normal mouse fibroblasts. Interphase and metaphase FISH were performed as described (Pinkel et al. 1986; Haaf and Ward 1994 a,b), using phage and cosmid genomic probes labeled with digoxigenin-11-dUTP. Hybridization signals were scored using a Zeiss Axioptot fluorescence microscope and three-color images were captured on a Photometrics cooled-CCD camera (Photometrics, Ltd., Tucson, AZ) using IP Lab image software (Signal Analytics Corporation, Vienna, VA). Genomic clones of *Lis1*, *Mnt/Rox*, and *14-3-3ε*, as well as *Brca1* (Schröck et al. 1996) as a marker for chromosome 11, were used as FISH hybridization probes. At least 25 metaphase spreads, 100 interphase nuclei, and 15 fiberFISH preparations were examined with identical results.

Isolation of YACs Containing *Lis1* and *Mnt/Rox*

The YAC clone containing *Lis1* and *Mnt/Rox* was obtained by screening the Princeton mouse YAC library by PCR. Pools of YAC DNA prepared from the library were screened in two steps using a pair of *Mnt/Rox* specific primers (5'-CTTCTTCAGCCGGTGCCAACTTCA-3' and 5'-CCCTC-CCTGCGCCCATCCTG-3'). We identified two positive YAC clones containing *Mnt/Rox*. YAC DNA was prepared according to standard procedures (Smith et al. 1988). These two DNAs were amplified by PCR using a pair of *Lis1*-specific primers (5'-TACCGTGCATGGTTCTGATGC-3' and 5'-TCTCAA-GGGCCATACAGACTCTG-3') with the same conditions described above. One positive YAC clone containing both *Lis1* and *Mnt/Rox* was identified.

YAC Fragmentation for *Lis1* and *Mnt/Rox* Transcriptional Orientation

To make fragmentation vectors for *Lis1* and *Mnt/Rox*, the 6-kb *SalI-SalI* (Fig. 2A) fragment of the *Lis1* genomic clone and the 10-kb *EcoRI-EcoRI* fragment (Fig. 2B) of the *Mnt/Rox* genomic clones were inserted into *EcoRI* site of the yeast fragmentation vector, pB1R (Lewis et al. 1992), in both orientations. The orientation of the inserts was determined by restriction site analysis.

Yeast cells (5×10^7) were transformed with 2 μ g of the fragmentation vectors digested with *NotI* and *SalI*. Transformation was accomplished with lithium acetate using the Alkali-Cation Yeast Kit (BIO 101). Transformants were selected

on plates lacking lysine but supplemented with uracil. *LYS+* transformants were further tested for the ability to grow in the absence of lysine and uracil or tryptophan and uracil. For PFGE analysis, high-molecular-weight yeast DNA of *URA+*, *TRP-*, and *LYS+* transformants were prepared (Smith et al. 1988), and electrophoretic karyotypes were assessed using CHEF DR11 (Bio-Rad). In addition, yeast DNA from transformants was digested with *EcoRI*, and separated by agarose gel electrophoresis, and DNAs were subsequently transferred to Hybond N+ (Amersham). Probes used for hybridization included a 0.5-kb *SalI-BamHI* fragment of the *Lis1* genomic clone, a 0.7-kb *EcoRI-SalI* fragment, a 0.5-kb *BamHI-BamHI* fragment of the *Mnt/Rox* genomic clone, and pBR322.

Construction of Targeting Vector for Insertion of *VDE* into *14-3-3ε*

Genomic fragments of *14-3-3ε* were amplified from 129Sv mouse genomic DNA directly using long PCR. The primers were synthesized based on the reported mouse *14-3-3ε* sequence (McConnell et al. 1995). The primer pair 5'-ATGGACGTGGAGCTGACAGTTGAAGA-3' and 5'-TGTTAGCTGCTGGAATGA GGTGTTG-3' was used for amplification of a 3.5-kb fragment located at the 5' site of the *14-3-3ε*. The primer pair, 5'-ACTTCTCCACGCAC-CCCATT-3' and 5'-TCACTGATTCATCTCACATCCT-3' was used for amplification of a 9.0-kb fragment located at 3' site of the *14-3-3ε*. A shuttle PCR program was performed: 5 min at 95°C, 30 cycles each of 1 min at 95°C and 15 min at 68°C, followed by additional extension of 15 min at 72°C using a LA PCR Kit (Takara, Oncor). The amplified fragments were inserted into pCRII using the TA Cloning Kit (Invitrogen).

These fragments were cloned into a modified version of the targeting vector pPNT (Tybulewicz et al. 1991), where the PGK-*neo* gene was replaced with pMC1-*hyg*. In this vector, pPNT-*hyg loxP VDE*, a cassette was inserted at unique cloning sites between the 3' end of the pMC1-*hyg* and PGK-*tk*. The cassette contains several novel restriction sites, a *loxP* site, and a *VDE* restriction site. To create the targeting construct for *14-3-3ε*, the 3.3-kb *BglII-BamHI* fragment of the 5' region was inserted at a unique *BamHI* site of pPNT-*loxPVDE*. The 9.0-kb *XhoI-SacI* fragment of the 3' region was made blunt with T4 DNA polymerase (NEB) followed by insertion at a unique *XhoI* site of pPNT-*loxPVDE* (Fig. 3A).

Insertion of Super-Rare Cutter *VDE* to *14-3-3ε* locus of ES Cell by Homologous Recombination

TC1 ES cells (Deng et al. 1996) were transfected with *NotI*-linearized *14-3-3ε* targeting vector and selected with hygromycin and FIAU. The culture, electroporation, and selection of TC1 cells were carried out as described (Deng et al. 1994). ES cell colonies that were resistant to both hygromycin and FIAU were analyzed by Southern blotting for homologous recombination events within the *14-3-3ε* locus by a novel method that used 96-well plates and low-melting-temperature agarose (S. Hirotune and A. Wynshaw-Boris, unpubl.). Genomic DNAs from drug-resistant ES clones were digested with *BamHI* and transferred to Hybond N+ after separation by electrophoresis in 0.8% agarose gel in $0.5 \times$ TBE. The membrane was probed with a 0.2-kb *EcoRI-BglII* fragment derived from the 5' genomic fragment of *14-3-3ε* (Fig. 3A).

Determination of the Orientation and Distance of 14-3-3 ϵ

We used the inserted *VDE* site for determination of the orientation and distance of 14-3-3 ϵ relative to *Lis1* and *Mnt/Rox*. The 14-3-3 ϵ targeted ES cells were embedded in low melting agarose (0.5×10^7 cells/300 μ l), and DNA was prepared as described above. DNA of the targeted clone was digested completely with *VDE* and partially with *NruI*, separated by PFGE, and transferred and hybridized with a 5' or 3' probe of 14-3-3 ϵ as described above. The PCR-amplified product used for screening ES clones for targeting was used for the 5' probe. As the 3' probe, a PCR-amplified product using two primers (5'-TCACTCTTGCCTTAAGAAGTTCTGAAAA-3', 5'-GCTGCAGGATGTGGAAGATGAGAAT-3') was used. To construct a restriction map with *NruI*, the agarose block was digested with *VDE* completely followed by partial *NruI* digestion with various enzyme concentrations (1–0.003 unit/ μ l).

ACKNOWLEDGMENTS

We thank Denise Larson and Lisa Garrett for excellent technical support; Robert Eisenman and Romeo Carrozzo for communicating data prior to publication; and Robert Nussbaum for his generous support.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

REFERENCES

- Bremer, M.C.D., F.S. Gimble, J. Thorner, and C.L. Smith. 1992. *VDE* endonuclease cleaves *Saccharomyces cerevisiae* genomic DNA at a single site: Physical mapping of the *VMA1* gene. *Nucleic Acids Res.* 20: 5484.
- Chong, S.S., A. Tanigami, A.V. Roschke, and D.H. Ledbetter. 1996. 14-3-3 ϵ has no homology to *LIS1* and lies telomeric to it on chromosome 17p13.3 outside the Miller-Dieker syndrome chromosome region. *Genome Res.* 6: 735–741.
- Chong, S.S., S.D. Pack, A.V. Roschke, A. Tanigami, R. Carrozzo, A.C.M. Smith, W.B. Dobyns, and D.H. Ledbetter. 1997. A revision of the lissencephaly and Miller-Dieker syndrome critical region in chromosome 17p13.3. *Hum. Mol. Genet.* 6: 147–155.
- Conklin, D.S., K. Galaktionov, and D. Beach. 1995. 14-3-3 proteins associate with *cdc25* phosphatases. *Proc. Natl. Acad. Sci.* 92: 7892–7896.
- DeBry, R.W. and M.F. Seldin. 1996. Human/mouse homology relationships. *Genomics* 33: 337–351.
- Deng, C., A. Wynshaw-Boris, M. Shen, C. Daugherty, D.M. Ornitz, and P. Leder. 1994. Murine FGFR-1 is required for early post-implantation growth and axial organization. *Genes & Dev.* 8: 3045–3058.
- Deng, C., A. Wynshaw-Boris, A. Kuo, F. Zhou, and P. Leder. 1996. Fibroblast growth factor-3 is a negative regulator of bone growth and development. *Cell* 84: 911–921.
- Dobyns, W.B. 1987. Developmental aspects of lissencephaly and the lissencephaly syndromes. In *Genetic aspects of developmental pathology*. (ed. E.F. Gilbert and J.M. Opitz), pp. 225–241. Alan R. Liss, New York, NY.
- Dobyns, W.B., E.R. Elias, A.C. Newlin, R.A. Pagon, and D.H. Ledbetter. 1992. Casual heterogeneity in isolated lissencephaly. *Neurology* 42: 1375–1388.
- Dobyns, W.B., O. Reiner, R. Carrozzo, and D.H. Ledbetter. 1993. Lissencephaly, a human brain malformation associated with deletion of the *LIS1* gene located at chromosome 17p13. *J. Am. Med. Assoc.* 270: 2838–2842.
- Gimble, F.S. and J. Thorner. 1992. Homing of DNA endonuclease gene by meiotic gene conversion in *Saccharomyces cerevisiae*. *Nature* 357: 301–306.
- Haaf, T. and D.C. Ward. 1994a. High resolution ordering of YAC contigs using extended chromatin and chromosomes. *Hum. Mol. Genet.* 3: 629–633.
- . 1994b. Structural analysis of alpha-satellite DNA and centromere proteins using extended chromatin and chromosomes. *Hum. Mol. Genet.* 3: 697–709.
- Hattori, M., H. Adachi, M. Tsujimoto, H. Arai, and K. Inoue. 1994. Miller-Dieker lissencephaly gene encodes a subunit of brain platelet-activating factor. *Nature* 370: 216–218.
- Hurlin, P., C. Queva, and R.N. Eisenman. 1997. *Mnt*, a novel Max-interacting protein is coexpressed with *Myc* in proliferating cells and mediates repression at *Myc* binding sites. *Genes & Dev.* 11: 44–58.
- Ledbetter, D.H. and A. Ballabio. 1995. Molecular cytogenetics of contiguous gene syndrome: Mechanisms and consequences of gene dosage imbalance. In *The metabolic basis of inherited disease* (ed. C. Scriver, A.L. Beudet, W.S. Sly, and D. Valle) pp. 881–839. McGraw-Hill, New York, NY.
- Lewis, B.C., N.P. Shah, B.S. Braun, and C.T. Denny. 1992. Creation of yeast artificial chromosome fragmentation vector based on lysine-2. *Genet. Anal. Tech. Appl.* 9: 86–90.
- LoNigro, C., S.S. Chong, A.M.C. Smith, W.B. Dobyns, R. Carrozzo, and D.H. Ledbetter. 1997. Point mutation and intragenic deletion in *LIS1*, the lissencephaly causative gene in isolated lissencephaly sequence and Miller-Dieker syndrome. *Hum. Mol. Genet.* 6: 157–164.
- McConnell, J.E., J.F. Armstrong, P.E. Hodges, and J.B. Bard. 1995. The mouse 14-3-3 *epsilon* isoform, a kinase regulator whose expression pattern is modulated in mesenchyme and neural differentiation. *Dev. Biol.* 168: 218–228.
- McDonald, J.D., L. Daneshvar, J.R. Willert, K. Matsumura, F. Waldman, and P.H. Cogen. 1994. Physical mapping of chromosome 17p13.3 in the region of a putative tumor suppressor gene important in medulloblastoma. *Genomics* 23: 229–232.
- Meroni, G., A. Reymond, M. Alcalay, G. Borsani, A.

HIROTSUNE ET AL.

Tanigami, R. Tonlorenzi, C. Lo Nigro, S. Messali, M. Zollo, D.H. Ledbetter, R. Brent, A. Ballabio, and R. Carrozzo. 1997. Rox: A novel bHLHZip protein expressed in quiescent cells that heterodimerizes with Max, binds a non-canonical "E-box," and acts as a transcriptional repressor. *EMBO J.* (in press).

Pavan, W.J., P. Hieter, and R.H. Reeves. 1990. Generation of deletion derivatives by targeted transformation of human-derived yeast artificial chromosomes. *Proc. Natl. Acad. Sci.* 87: 1300-1304.

Peterfy, M., T. Gyuris, R. Basu, and L. Takacs. 1994. Lissencephaly-1 is one of the most conserved proteins between mouse and human: A single amino-acid difference in 410 residues. *Gene* 150: 415-416.

Phillips, N., M. Ziegler, B. Saha, and F. Xynos. 1993. Allelic loss on chromosome 17 in human ovarian cancer. *Int. J. Cancer* 54: 85-91.

Pinkel, D., T. Straune, and J.W. Gray. 1986. Cytogenetic analysis using quantitative, high sensitivity, fluorescence hybridization. *Proc. Natl. Acad. Sci.* 83: 2934-2938.

Reiner, O., R. Carrozzo, Y. Shen, M. Wehnert, F. Faustinella, W.B. Dobyns, T. Caskey, and D.H. Ledbetter. 1993. Isolation of a Miller-Dieker lissencephaly gene containing G protein β -subunit-like repeats. *Nature* 364: 717-721.

Reiner, O., U. Albrecht, M. Gordon, K.A. Chianoso, C. Wong, O. Gai-Gorber, T. Sapir, L.D. Siracusa, A.M. Buchberg, C.T. Caskey, and G. Eichele. 1995. Lissencephaly gene (LIS1) expression in the CNS suggests a role in neuronal migration. *J. Neurosci.* 15: 3730-3738.

Robbins, C.M., E. Hsu, and P. Gillevet. 1996. Sequencing homopolymer tracts and repetitive elements. *BioTechniques* 20: 862-868.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: A laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schröck, E., P. Badger, D. Larson, M. Erdos, A. Wynshaw-Boris, T. Ried, and L. Brody. 1996. The murine homolog of the human breast and ovary cancer susceptibility gene Brca1 maps to mouse chromosome 11D. *Hum. Genet.* 97: 256-259.

Smith, C.L., S.R. Klco, and C.R. Cantor. 1988. Pulsed-field gel electrophoresis and the technology of large DNA molecules. In *Genome analysis: A practical approach*. (ed. K.E. Davies), pp. 41-72. IRL Press, Oxford, UK.

Tybulewicz, V.L., C.E. Crawford, P.K. Jackson, R.T. Bronson, and R.C. Mulligan. 1991. Neonatal lethality and lymphopenia in mice with a homozygous disruption of the *c-abl* proto-oncogene. *Cell* 65: 1153-1163.

Received February 11, 1997; accepted in revised form April 1, 1997.