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The Mouse *Murr1* Gene Is Imprinted in the Adult Brain, Presumably Due to Transcriptional Interference by the Antisense-Oriented *U2af1-rs1* Gene

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The mouse *Murr1* gene contains an imprinted gene, *U2af1-rs1*, in its first intron. *U2af1-rs1* shows paternal allele-specific expression and is transcribed in the direction opposite to that of the *Murr1* gene. In contrast to a previous report of biallelic expression of *Murr1* in neonatal mice, we have found that the maternal allele is expressed predominantly in the adult brain and also preferentially in other adult tissues. This maternal-predominant expression is not observed in embryonic and neonatal brains. In situ hybridization experiments that used the adult brain indicated that *Murr1* gene was maternally expressed in neuronal cells in all regions of the brain. We analyzed the developmental change in the expression levels of both *Murr1 and U2af1-rs1* in the brain and liver, and we propose that the maternal-predominant expression of *Murr1* results from transcriptional interference of the gene by *U2af1-rs1* through the *Murr1* promoter region.

Genomic imprinting is a type of gene regulation wherein one of the two parental alleles is predominantly expressed according to parental origin (5). Nearly 131 imprinted genes have been identified in mammals (http://cancer.otago.ac.nz/IGC/Web/home.html). Many of the genes have essential functions in embryonic development, and thus abnormalities of imprinted genes are often associated with human diseases, including disorders affecting cell growth, development, and behavior (23). However, the mechanism of imprinting is still poorly understood. Some common features have been observed in the genome structure of the imprinted genes that have been reported to date. Most imprinted genes are found in clusters in specific chromosomal domains. Colocalization of the imprinted genes implies that some mechanisms related to genomic imprinting may function over a long chromosomal region. For example, approximately 10 of the known mouse imprinted genes map to a distal region of mouse chromosome 7 (3, 31; http://www.mgu.har.mrc.ac.uk/imprinting/imprinting-.html). This region consists of two imprinted domains in terms of imprinting control. The imprinted genes in each domain are under the control of a region termed the imprinting control center (11, 39). Epigenetic modification of chromatin is considered to be a fundamental and important mechanism involved in the generation and maintenance of the imprinted states of imprinted genes (8). Epigenetic modifications, such as DNA methylation, modifications of histones, and the organization of nucleosomes, have been regarded as important because they have essential and general functions, not only for imprinted genes but also in the regulation of all genes (19, 26, 28, 45).

U2af1-rs1 is a mouse imprinted gene and is transcribed exclusively from the paternal allele in embryonic and neonatal mice (16, 17). This gene is intronless and located within the first intron of *Murr1*. It is transcribed in the direction opposite of that of the host gene in the proximal region A3.2 of mouse chromosome 11 (27). The presence of two maternal copies of this region resulted in prenatal growth retardation, while two paternal copies resulted in fetal overgrowth (6). The *U2af1-rs1* gene was supposedly created by retrotransposition during mouse evolution after the divergence of mice and humans, as the human *MURR1* gene does not contain the homologue of the *U2af1-rs1* gene within its introns (reference 27 and unpublished data).

Imprinted genes are a suitable model system for the study of epigenetic control of gene expression because their allelic expressions are essentially controlled by this mechanism. We initiated the analysis of the *Murr1* gene in order to investigate the imprinting mechanisms of the *U2af1-rs1* locus. The *U2af1-rs1* gene was the only gene reported to be imprinted in this chromosomal region at that time. It was necessary to know if there were other imprinted genes around the *U2af1-rs1* locus because clustered imprinted genes are thought to be controlled by a common regulatory mechanism rather than being independently regulated (10, 30). Although *Murr1* has been reported to be expressed biallelically in the neonatal mouse (27), we have analyzed *Murr1* expression in the tissues of both adult and embryonic mice because allelic expression of some imprinted genes is tissue- and developmental stage specific (7, 15, 24, 38). In this study we have found that the expression of the *Murr1* gene shows a moderate bias of expression toward the maternal allele in the adult tissues analyzed so far. Moreover, this gene showed a predominant expression of the maternal

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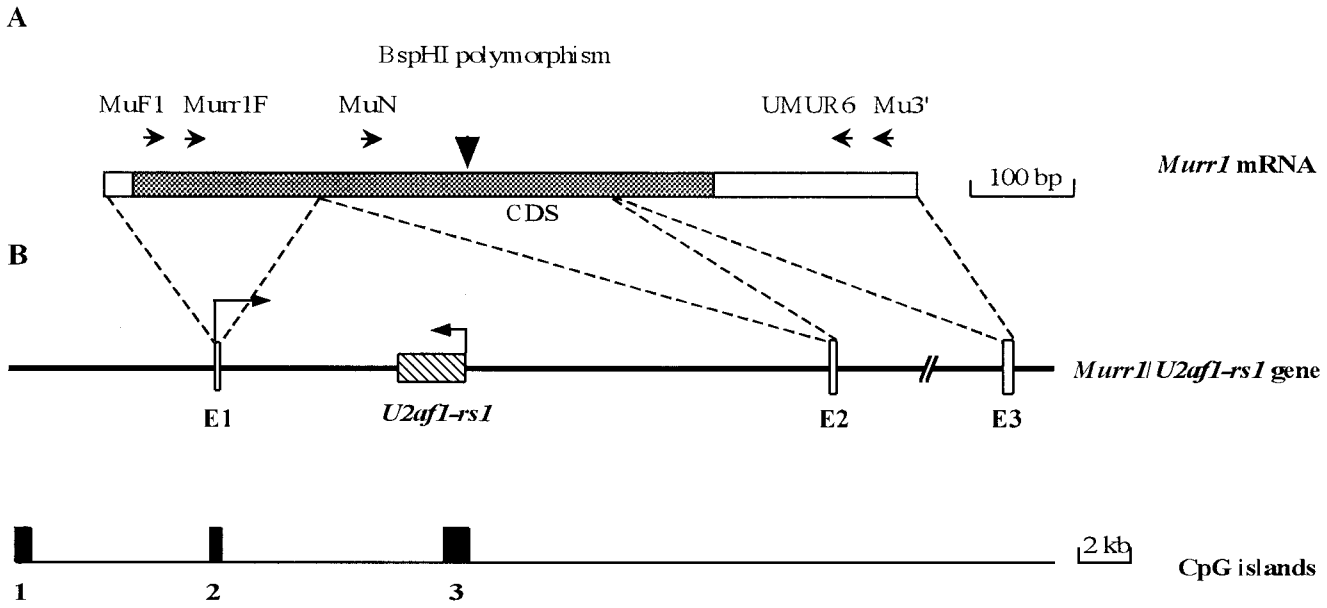

FIG. 1. Structure of the mouse *Murr1* gene. (A) The mRNA and coding sequence (CDS, shown as gray box) of *Murr1*. The arrows indicate the primers employed in the allelic expression analysis (MuF1 and UMUR6) and probe syntheses for Northern blot (MuN and UMUR6) and in situ hybridization (Murr1F and Mu3′). The *Bsp*HI site, which is polymorphic between the C57BL/6 and PWK strains, is shown above the mRNA. The site exists only in the C57BL/6 sequence. (B) The *Murr1* gene consists of three exons and two introns with a length of about 83 kb. An imprinted gene, *U2af1-rs1*, is located within the first intron of the *Murr1* gene. Three CGIs were detected in the region ranging from 10 kb upstream to 10 kb downstream of *Murr1*.

allele specifically in adult brain but not in brains of neonatal or embryonic mice. We proposed that this mechanism might come from transcriptional interference by antisense-oriented *U2af1-rs1*.

MATERIALS AND METHODS

Mice. C57BL/6 (B6), PWK mouse strains, and the F_1 progeny obtained by reciprocal crosses between them were used as the sources of total RNAs and genomic DNAs. BPF1 progeny resulted from the cross of B6 females and PWK males. PBF1 progeny resulted from crosses of PWK females and B6 males.

Mice with two paternal copies of proximal chromosome 11, including the *Murr1* locus (paternal duplication [PatDp] prox11), were produced by a standard protocol using the mouse reciprocal translocation T(7;11)40Ad and identified by using genetic markers (4).

Allelic expression of the *Murr1* and *U2af1-rs1* genes. Total RNAs were isolated with ISOGEN (Nippon Gene) from various tissues of adult F_1 progeny obtained by reciprocal crosses between C57BL/6 and PWK mice. Contaminating chromosomal DNA was removed by DNase I treatment. Reverse transcription was conducted with oligo(dT) primers according to the manufacturer's protocol of the RNA PCR kit (Takara). To exclude the problem of heteroduplex formation skewing the results of restriction endonuclease digestion, hot-stop reverse transcription-PCR (RT-PCR) was employed (40). Thirty cycles of PCR with primers MuF1 (5′-TGGAGGGTGGCAAGTCCCTG-3′) and UMUR6 (5′-GGTAACA CCAGTGGGCAAAG-3′) (Fig. 1) were followed by one cycle of amplification with primer UMUR6 labeled with α-^{32}P. The resulting 686-bp fragment was digested with *Bsp*HI and electrophoresed. The intensity of electrophoretic bands was measured by using BAS2000 (Fujifilm). For the analysis of *U2af1-rs1* gene, a regular PCR with primers 5′-U2af1rs1 (5′-AGATAACCACGGATACCTGG-3′) and 3′-U2af1rs1 (5′-AGTACATAGGCCTGCCCATG-3′) was performed. The resulting 236-bp PCR product was digested with *Msp*I and electrophoresed.

Quantification of mRNAs by real-time RT-PCR. A real-time kinetic PCR method using the SYBR Green and Light-Cycler technique (Roche) was employed to quantify the amount of the *Murr1* and *U2af1-rs1* mRNAs in the brains and livers of different developmental stages (13.5-days-postcoitum [dpc] embryonic, newborn, 2-week-old, and 12-week-old mice). Total RNAs were isolated from the tissues of BPF1 and PBF1 mice by using ISOGEN (Nippon Gene). The RNAs were reverse transcribed by using oligo(dT) primer and then were used as templates in each real-time kinetic PCR containing a primer pair for *Murr1, U2af1-rs1*, or *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase) mRNA. The primer pairs used were as follows. For *Murr1*, MU109 (5′-ACTCTATCCGGA AGTGCCAC-3′) and UMUR5 (5′-AGGCTTGCCATCGACTCTCC-3′) were used; for *U2af1-rs1*, U2F1 (5′-AGACCTACCAGCAGTTCTTG-3′) and U2R (5′-GGTCACTGGACAGAATTCAC-3′) were used; and for *Gapdh*, GAP DHR1 (5′-CAGTCTTCTGGGTGGCAGTG-3′) and GAPDHF (5′-GCCAAA CGGGTCATCATCTC-3′) were used.

The kinetics of PCR amplification was monitored as the kinetics of enhancement of the fluorescence of SYBR Green (a dye specific for double-stranded DNA) in the reactions. The relative amounts of three mRNAs were obtained by determining the numbers of cycles required to obtain the same levels of fluorescence in each PCR. In order to evaluate the quantification of this assay and to condition the PCRs, pilot experiments were performed by using serially diluted cDNAs of these mRNAs as the templates according to the manufacturer's protocol.

In situ hybridization of *Murr1* mRNAs in mouse brain. The fresh cuts of brains were fixed immediately in paraformaldehyde after removal and sectioned after being embedded in paraffin. The product of RT-PCR with primers Murr1F (5′-TAGCGCAGAACGCCTTTCAC-3′) and Mu3′ (5′-ATCCTTGAAGACTT TCATGC-3′) (Fig. 1) was cloned into the pT7Blue T-vector (Takara) and used as the template for in vitro transcription of antisense and sense single-stranded RNA probes for *Murr1*. The antisense and sense RNA probes for *U2af1-rs1* were prepared in the same manner from the RT-PCR product with primers U2F (5′-GGAAACACGACTTTCCCACG-3′) and U2AluI-R (5′-GCTGTCCTCTT CTCTTCCTC-3′). The digoxigenin-labeled antisense and sense probes were synthesized with T7 RNA polymerase and digoxigenin-UTP according to the manufacturer's protocol (RNA labeling and detection kit; Roche). The transcripts were used as probes at a concentration of 10 ng/μl in hybridization buffer. The hybridized probe was detected with alkaline phosphatase-coupled antibodies to digoxigenin according to the manufacturer's protocol (RNA labeling and detection kit; Roche).

Methylation analyses. The DNA methylation status of the CpG islands (CGIs) was assessed by sodium bisulfite PCR and sequencing assay as described previously (46). Briefly, genomic DNA from the brain of a C57BL/6 mouse was extracted by standard phenol-chloroform extraction methods and treated with sodium bisulfite prior to PCR with each primer set. PCR products were cloned into pT7Blue T-vector, and each cloned PCR product was amplified by colony

PCR using a Takara Ex *Taq* Kit (Takara Shuzo). The amplified products were subjected to sequencing reaction using BigDye (Applied Biosystems) and DYEnamic ET terminator kits (Amersham Pharmacia Biotech) after treatment with exonuclease I and shrimp alkaline phosphatase (both from Amersham Pharmacia Biotech).

Detection and quantification of the primary transcript of the *Murr1* and *U2af1-rs1* by strand-specific RT-PCR and semiquantitative RT-PCR. DNase I-treated total RNAs (0.25 μg) isolated from the brain, liver, heart, or lung of adult mice using ISOGEN (Nippon Gene) were mixed with 5 pmol of either forward primers 1, 3, 4, and 5 or reverse primer 2 (see Fig. 6) in a final volume of 10 μl; the RNAs were then reverse transcribed for 30 min at 55°C, heat inactivated at 99°C for 5 min, and cooled on ice. Following the reverse transcription and denaturation, 33 PCR cycles with four different primer sets corresponding to the primers for reverse transcription were performed, respectively.

Semiquantitative RT-PCR was employed to detect the downstream primary transcript of the *Murr1* and *U2af1-rs1*. The product of reverse transcription with either primer 1 or 3 as described above was subjected to 33 and 36 PCR cycles, respectively, the products of which were verified as linear ranges of the PCR with corresponding primers. After agarose electrophoresis and staining with ethidium bromide, the quantity of the PCR products was analyzed by National Institutes of Health Image software (version 1.62).

RESULTS

***Murr1* contains *U2af1-rs1* in its first intron.** *Murr1* spans 83 kb and consists of three exons and two introns. An imprinted gene, *U2af1-rs1*, which shows paternal allele-specific expression, is located within the first intron of *Murr1* and transcribed in a direction opposite to that of the host gene (Fig. 1) (27). The genomic sequence, including that of the *Murr1* gene, is available from DDBJ under the accession numbers AB089806 and NT_039515. This gene encodes a mRNA of approximately 1 kb and is expressed ubiquitously in adult tissues as shown by Northern blotting in Fig. 2. Cloning and sequencing of the cDNA revealed that *Murr1* mRNA is 793 bases long, excluding the polyA tail, and encodes a protein of 188 amino acids. The transcriptional initiation site was determined by 5′ rapid amplification of cDNA ends analysis. Several of the largest clones had the same 5′ ends (data not shown). The cDNA sequence has been submitted to the DDBJ database under accession numbers D85430 (27) and AB104816. Although the function of the protein has not yet been analyzed in mice, the homologue in the dog was reported to be involved in copper metabolism (41, 42).

The maternal allele is expressed preferentially in all tissues but predominantly in adult brain. The existence of an imprinted gene within the *Murr1* gene has led us to analyze the allelic expression of *Murr1* because many imprinted genes are found to be organized as clusters (3, 10). The gene has been reported to show biallelic expression in the whole body of neonatal mice specimens (27). Tissues of adult mice were examined by using the F_1 progeny of reciprocal crosses between C57BL/6 and PWK mice. We performed hot-stop RT-PCR followed by digestion with *Bsp*HI that could distinguish between the *Murr1* alleles of each parental strain (Fig. 1). As seen in Fig. 3A, *Murr1* showed preferential expression of the maternal allele in all the adult tissues examined. In the brain, predominant expression of the maternal allele was the most significant. In order to know whether this allelic expression also occurs in early developmental stages, we analyzed embryonic and neonatal brains (Fig. 3B). These brains express the *Murr1* gene biallelically with moderate preference for the maternal allele, as seen in adult tissues other than the brain. The brain of a 2-week-old mouse showed predominant expression of the maternal allele to the same extent as that found in the brains of 6- and 12-week-old mice.

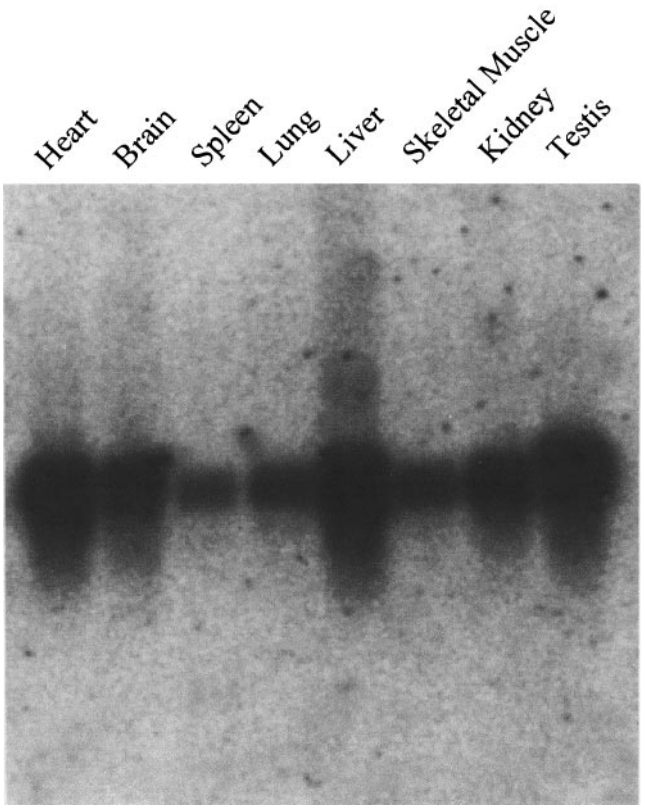

FIG. 2. Expression of the *Murr1* gene in mouse adult tissues. A premade mouse multiple tissue Northern blot (Clontech Co.) was probed with the PCR fragment synthesized with primers MuN and UMUR6 (Fig. 1). The *Murr1* gene is expressed ubiquitously but is most abundant in the heart, liver, and testis.

Because there is a possibility that the paternal expression of *U2af1-rs1* affects the allelic expression of its host gene, *Murr1*, its allelic expression was also examined in the same tissues. In previous reports, *U2af1-rs1* was shown to be expressed exclusively from the paternal allele in the early embryo and whole neonate (16, 17). Our analysis showed that the *U2af1-rs1* gene was expressed exclusively from the paternal allele in all the adult tissues and in the brains of mice in all the developmental stages so far examined (Fig. 3C and D).

Predominant maternal expression was observed in all regions of adult brain. Maternal allele-predominant expression of the *Murr1* gene in the adult brain may suggest a function of the gene in the development of the brain after birth. In order to gain insight into this function, we determined the regions expressing the gene by RNA in situ hybridization on the adult brains of C57BL/6 mice (Fig. 4a, b, c, f, i, and l). The expression of *Murr1* was observed in all the regions of the cerebrum, cerebellum, and medulla oblongata. The hippocampus region of the cerebrum had the highest levels of expression. The expression was seen mainly in neuronal cells.

Maternal allele-predominant expression of *Murr1* raises two possibilities for the allelic expression of this gene in adult brain. First, *Murr1* may be expressed predominantly from the maternal allele in all the regions of adult brain. Alternatively,

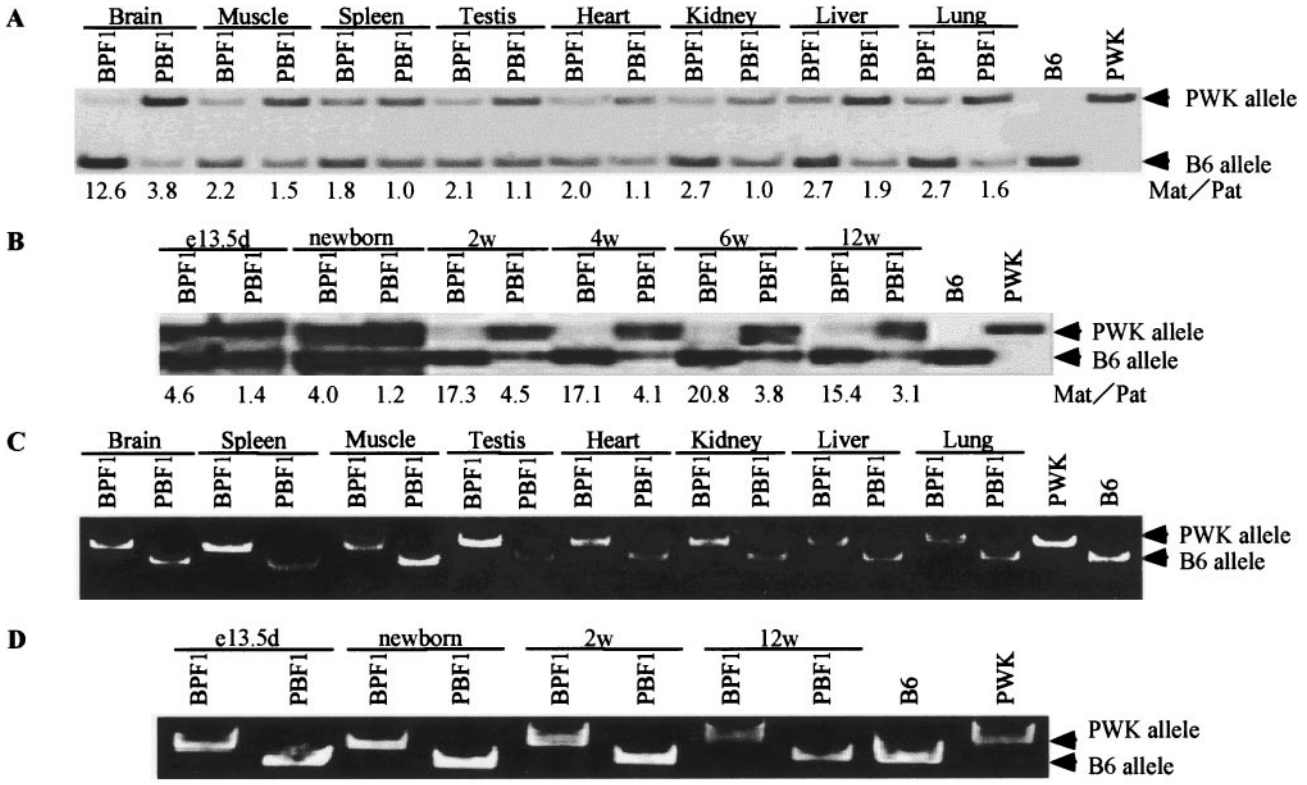

FIG. 3. Analysis of allelic expression of *Murr1* and *U2af1-rs1*. Total RNAs were prepared from various tissues of adult, newborn, and embryonic F_1 progeny obtained from crosses between C57BL/6 and PWK mice. Allelic expression analyses of *Murr1* in the tissues of adult mice (A) or in the brains of 13.5-dpc embryonic, newborn, 2-, 4-, 6-, and 12-week-old mice (B). The products of hot-stop RT-PCR were digested with *Bsp*HI, which digests only the product from the B6 allele (Fig. 1). The ratio of the intensity of maternal product to paternal product is indicated beneath each lane. Allelic expression analyses of *U2af1-rs1* in the tissues of adult mice (C) or in the brains of 13.5-dpc embryonic, newborn, 2-week-old, and 12-week-old mice (D). The products of RT-PCR were digested with *Msp*I, which digests only the product from the B6 allele.

the gene may be expressed exclusively from the maternal allele in some regions but biallelically in other regions. *Ube3a* is an imprinted gene with such a regional-imprinted expression (1). In situ hybridization was performed using adult mouse brain cells with two paternal copies of proximal chromosome 11, including the *Murr1* locus [PatDp(prox11)]. Weak but detectable signals for *Murr1* mRNA were observed in all the regions and were essentially similar to those of wild-type C57BL/6 brains (Fig. 4d, g, j, and m). There was no region lacking hybridization signal, and the expression pattern of *Murr1* appeared to be normal in the PatDp(prox11) brain. This result indicated that the gene was expressed maternal allele predominantly in all the regions expressing the gene.

The CGIs associated with the *Murr1* promoter region are not methylated. Three CGIs have been identified in the region spanning from 10 kb upstream to 10 kb downstream of the *Murr1* gene (Fig. 1). CGI1 is located approximately 8 kb upstream from the *Murr1* gene, and CGI2 spans from the promoter region to the 5′ part of exon 1. CGI3 is the region spanning from the promoter to the body of the *U2af1-rs1* gene. CGI3 has been reported to be methylated only on the maternal allele and has been thought to be involved in the paternal expression of *U2af1-rs1* (17). These three CGIs were analyzed for methylation in the brain genome by a sodium bisulfite sequencing method (data not shown). One can expect that the CGIs 1 and/or 2 may be paternally methylated if differential methylation is involved in the maternal expression of *Murr1*. The CGI3 was found to be differentially methylated in the brain as reported previously. On the other hand, no methylation was found in the other two CGIs, 1 and 2. Nonmethylation of these CGIs suggests that differential methylation within the genome is not involved in the allelic expression of *Murr1*, in contrast to many of the imprinted genes.

The expression levels of *Murr1* and *U2af1-rs1* change inversely during brain development. In the adult brain, *U2af1-rs1* and *Murr1* show reciprocal allelic expression. The *U2af1-rs1* gene is expressed exclusively from the paternal allele, and the *Murr1* gene is expressed predominantly from the maternal allele. This fact suggests that there may be some kinds of interaction, such as transcriptional interference, between allelic expressions of these genes. In order to investigate this possibility, the mRNA levels of these genes were quantified in the brain and liver during development by quantitative real-time RT-PCR (Fig. 5).

In the brain (Fig. 5A), *Murr1* expression occurs at a relatively high level during the embryonic and neonatal periods. The expression levels decrease considerably between the neonatal stage and 2 weeks after birth, the period during which maternal allele-predominant expression of *Murr1* becomes remarkable (Fig. 3B). This overall decrease from the embryo to adult

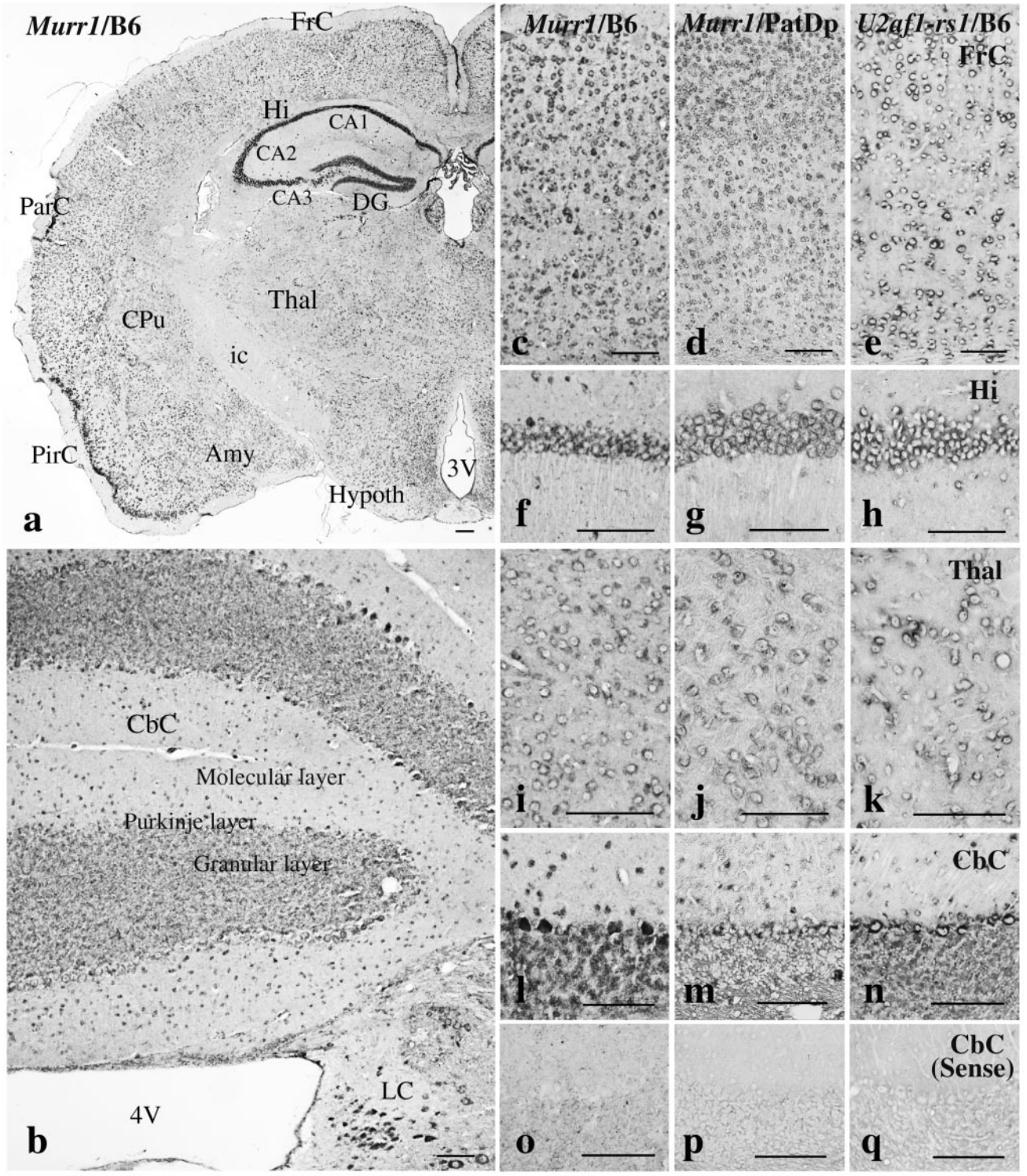

FIG. 4. In situ hybridization of *Murr1* and *U2af1-rs1* mRNAs in mouse brain. RNA probes of *Murr1* and *U2af1-rs1* were hybridized on brains of C57BL/6 (B6) and PatDp(prox11) mice. Antisense probes (panels a through n) and sense probes as negative control (panels o through q) were used. Shown are coronal sections through the forebrain (a) and the cerebellum (b) of C57BL/6 mice hybridized with *Murr1* probe; also shown are magnified images of the frontal cortex (c through e), hippocampus (f through h), thalamus (i through k), and cerebellar cortex (l through q) hybridized with the *Murr1* probe on C57BL/6 brain (panels c, f, i, l, and o), *Murr1* probe on PatDp(prox11) brain (panels d, g, j, m, and p), and *U2af1-rs1* probe on C57BL/6 brain (panels e, h, k, n, and q) are shown. FrC, frontal cortex; Hi, hippocampus; Thal, thalamus; Hypoth, hypothalamus; CA1 to -3, fields CA1 to CA3 of Ammon's horn; DG, dentate gyrus; ParC, parietal cortex; PirC, piriform cortex; CPu, caudate putamen; Amy, amygdala; ic, internal capsule; CbC, cerebellar cortex; 3V, third ventricle. Scale bars, 100 μm.

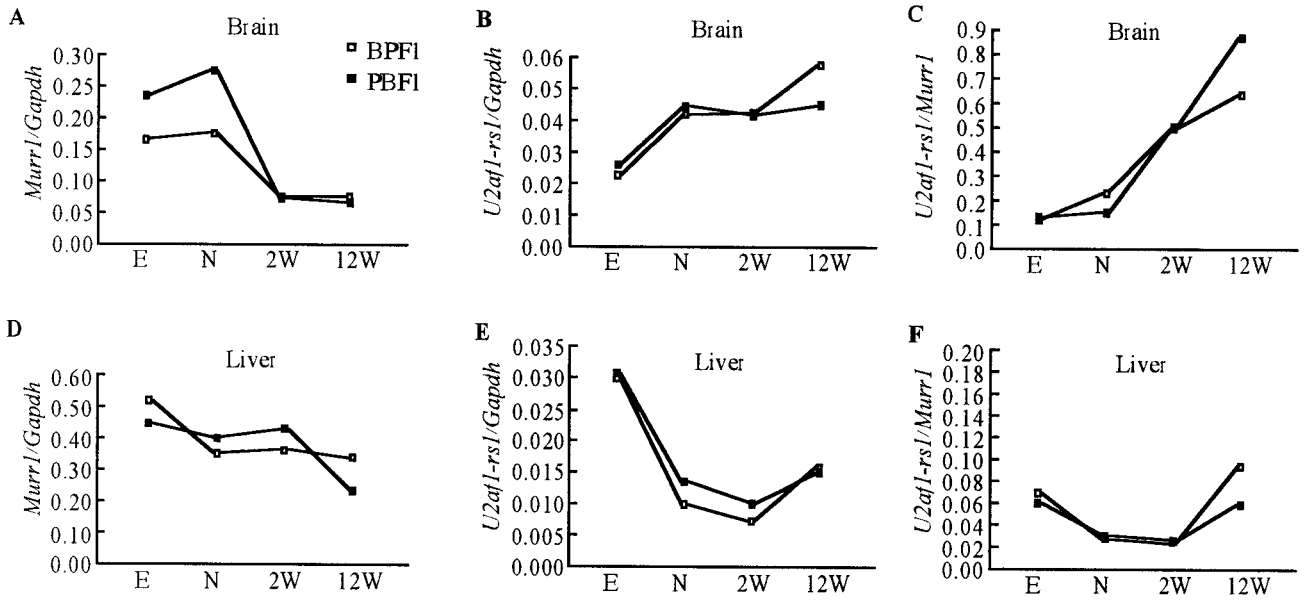

FIG. 5. Change of the expression of *Murr1* and *U2af1-rs1* during development in the brain and liver. The expression of *Murr1* and *U2af1-rs1* was analyzed in the brains and the livers of BPF1 and PBF1 mice during development by quantitative real-time PCR. All the analyses were performed twice for each of the RNA samples. The relative amounts of *Murr1* and *U2af1-rs1* mRNAs were calculated by normalizing their values with the housekeeping gene *Gapdh*. Shown are expression levels of *Murr1*/*Gapdh* (A), *U2af1-rs1*/*Gapdh* (B), and *Murr1*/*U2afi-rs1* (C) in the brains; also shown are expression levels of *Murr1*/*Gapdh* (D), *U2af1-rs1*/*Gapdh* (E), and *Murr1*/*U2afi-rs1* (F) in the livers. E, 13.5-dpc embryo; N, newborn; 2W, 2-week-old mice; 12W, 12-week-old mice; □, BPF1; ■, PBF1.

stages is approximately fourfold. In contrast, the expression of *U2af1-rs1* continues to increase during development (Fig. 5B). Overall, this increase is approximately two- to threefold. As a result, the ratio of expression of *U2af1-rs1* versus that of *Murr1* increased about 10-fold during development (Fig. 5C). The final level of *U2af1-rs1* expression (0.05) is almost the same as that of *Murr1* expression (0.06) in the adult brain.

On the other hand, the expression of both genes decreased simultaneously during development in the liver, in which the maternal allele-predominant expression of *Murr1* does not occur (Fig. 5D and E). Relative levels of expression of *U2af1-rs1* and *Murr1* do not change significantly during development (Fig. 5F). The expression of *Murr1* is more than 10-fold higher than that of *U2af1-rs1* in both embryonic and adult periods.

Transcription from *U2af1-rs1* may act through the promoter region of *Murr1*. Antisense orientation of the *U2af1-rs1* gene relative to *Murr1* suggests the possibility that transcription originating from *U2af1-rs1* promoter overlaps the promoter region of the *Murr1* gene and interferes with *Murr1* transcription. The 3′ end of a mature mRNA is created by the cleavage of the primary transcript. A primary transcript is synthesized beyond the end of the gene, sometimes far more than a distance of 1 kb (29). If this is because of the paternal expression of *U2af1-rs1*, this interference should occur only on the paternal allele and may cause the reduction of the paternal expression of *Murr1*. We employed strand-specific and semiquantitative RT-PCR to detect and quantify the transcripts in the region downstream of *U2af1-rs1* up to the promoter region of *Murr1* (Fig. 6).

In the analysis performed using adult brain RNA, the transcript was detected in the promoter region when primer 1 with sense orientation was used for cDNA synthesis. In contrast, no transcript was detected in this region when primer 2 with antisense orientation was used as the primer for cDNA synthesis (Fig. 6B). This result indicated that the *Murr1* promoter region was transcribed in the antisense direction relative to the *Murr1* gene. The sequencing of the PCR product revealed that the transcript was expressed exclusively from the paternal allele (Fig. 6D). On the other hand, when liver RNA was used for this analysis, no transcript was detected with either of the primers for cDNA synthesis using PCR with same number of amplification cycles (33 cycles) (Fig. 6B). An antisense transcript was, however, detected in the liver when PCR was done with 36 amplification cycles (data not shown). In the brain, transcripts with antisense orientation relative to *Murr1* were also detected at two sites in the first intron downstream of *U2af1-rs1*. Only the transcript at the site close to *U2af1-rs1* was detected in the liver, however. No transcript with antisense orientation was detected at the promoter region of *U2af1-rs1* in either the brain or liver (Fig. 6C). These results strongly suggest that transcription from the *U2af1-rs1* promoter to the upstream region of the *Murr1* gene on the paternal chromosome occurs more abundantly in the brain than in the liver, although we have no direct evidence that these antisense transcripts are parts of one continuous transcript. This finding implies that the transcription of *U2af1-rs1* interferes with the expression of the paternal allele of *Murr1* in the adult brain. If this is the case, *U2af1-rs1* should be expressed in the regions where *Murr1* is expressed in the brain. This has been shown to be true by in situ hybridization, in which the expression of *U2af1-rs1* observed in the regions was essentially the same as that of *Murr1* expression in C57BL/6 brain (Fig. 4e, h, k, and n). We also detected the same antisense transcripts at the *Murr1* promoter in lung and kidney cells. Semiquantitative

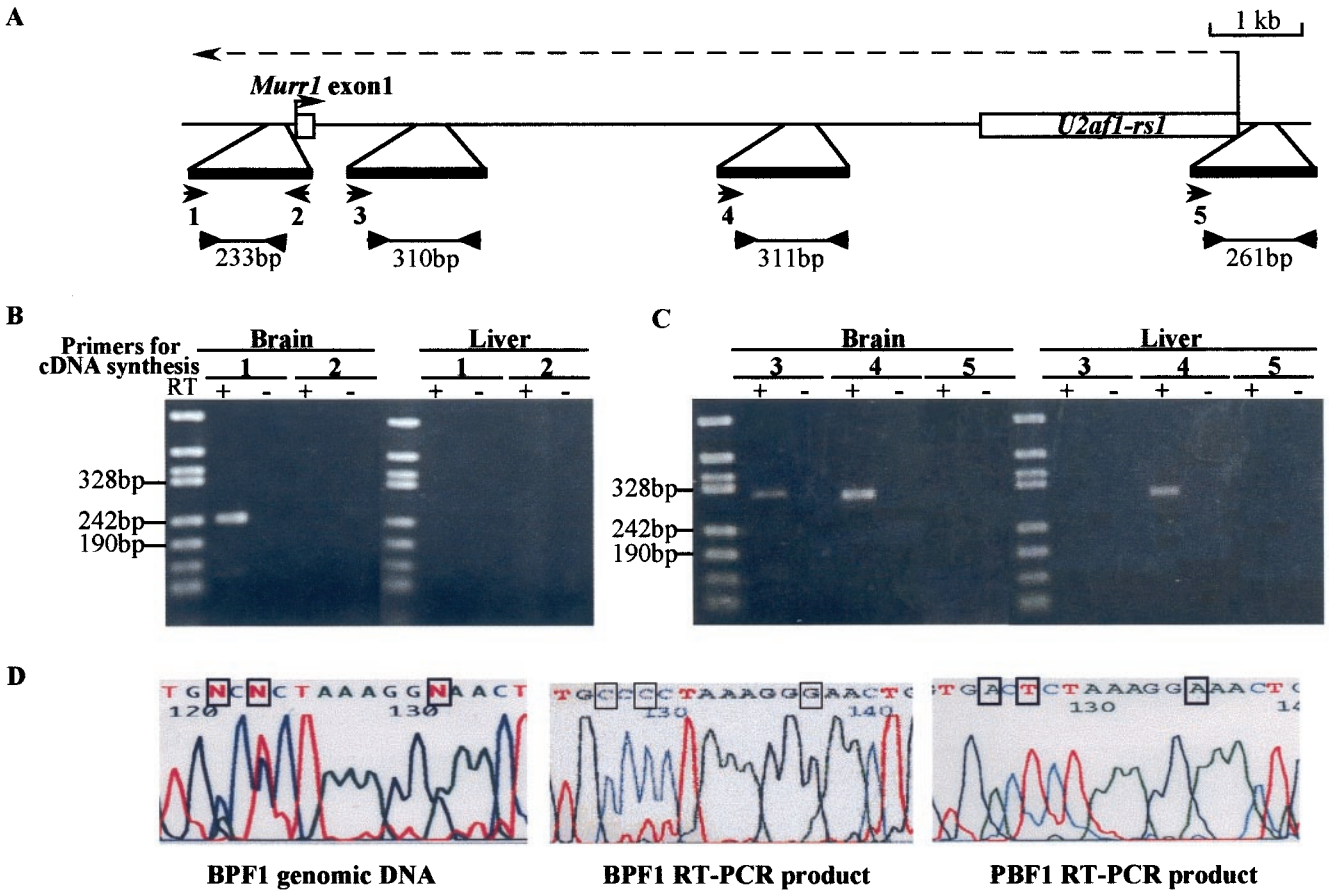

FIG. 6. Strand-specific RT-PCR of the *Murr1* gene. (A) Indicated are the locations of the primers for RT (1, 2, 3, 4 and 5) and the regions amplified by PCR in the *Murr1*/*U2af1-rs1* region. (B) RT-PCR in the 300-bp region upstream of *Murr1* with the total RNAs of adult brain and liver of an adult C57BL/6 mouse. A transcript with antisense orientation relative to *Murr1* was detected in the brain but not in the liver. (C) Transcripts with antisense orientation were also investigated in the intronic region downstream and upstream of *U2af1-rs1* with the total RNAs of adult brain and liver. A transcript near the exon 1 of *Murr1* was detected in the brain but not in the liver, while the transcript near *U2af1-rs1* was detected in both the brain and the liver. The antisense primary transcript was not detected in the region upstream of the *U2af1-rs1* gene. All PCRs were performed with 33 amplification cycles. (D) The sequence analysis of the PCR products in the 300-bp region upstream of *Murr1*. The genomic DNA and the total RNAs were obtained from the brains of the adult F_1 progeny from the crosses of B6 and PWK mice. The polymorphic bases are boxed in the sequences.

RT-PCR indicated that the primary antisense transcript in the *Murr1* promoter region was much more abundant in the adult brain than in the heart, lung, and liver (data not shown).

DISCUSSION

We have shown that the mouse *Murr1* gene is imprinted in the adult brain. The gene is expressed predominantly from the maternal allele in the neurons of all regions of the adult brain but shows only a preferential maternal expression in the brain at earlier developmental stages. Some imprinted genes have been reported to show a change in allelic expression during development (7, 15, 24, 38). These are, however, genes that are imprinted only in the early stages of development. *Murr1*, therefore, is the first imprinted gene which shows allelic expression in later stages of development. *Murr1* also has a moderate bias toward expression of the maternal allele in all other adult tissues analyzed so far.

Our preliminary analysis indicates that *MURR1*, the human homologue of mouse *Murr1*, is expressed biallelically in the adult brain and contains no imprinted gene in the first intron (Zhang et al., unpublished data). This strongly suggests that the antisense gene, *U2af1-rs1*, causes the maternal expression of *Murr1* in the mouse. The expressions of *Murr1* and *U2af1-rs1* show a reverse change during the development of the brain (Fig. 5A through C). *Murr1* expression decreases during brain development, whereas *U2af1-rs1* expression increases. Given the assumption that the mRNA levels reflect the transcriptional activities of these genes, these results indicate that relative transcriptional activity of *U2af1-rs1* is increasing compared to that of *Murr1* during brain development. On the other hand, analyses by strand-specific RT-PCR suggested that the transcription from the *U2af1-rs1* promoter reaches to the promoter region of *Murr1* on the paternal allele in adult brain, liver, kidney, and lung to a different extent (Fig. 6B and C and data not shown). A possible hypothesis is that the transcriptional interference of the *U2af1-rs1* gene against *Murr1* causes maternal allele-preferential or -predominant expression of the

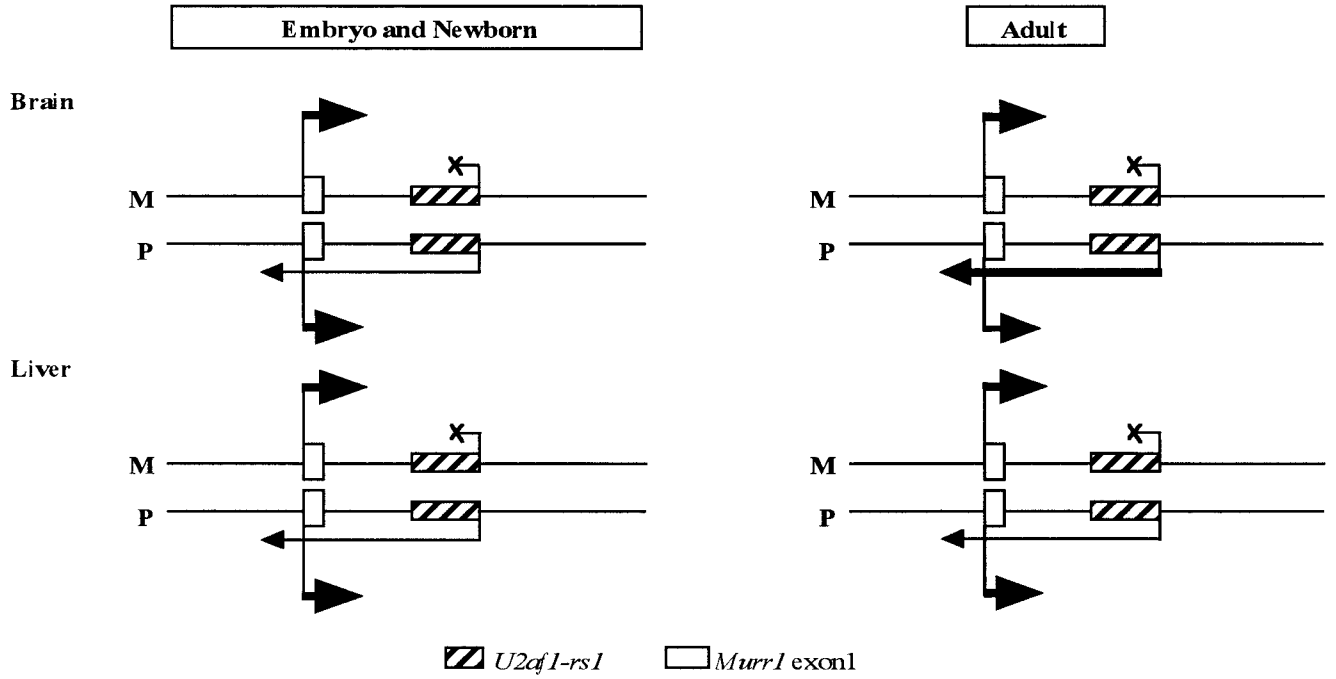

FIG. 7. The expression-interference model between the *Murr1* and *U2af1-rs1* genes. The antisense transcription from the *U2af1-rs1* gene (starting from the slashed square) is hypothesized to interfere with the *Murr1* transcription (starting from the blank square) on the paternal allele and to cause the maternal allele-preferential or -predominant expression in the adult brain.

Murr1 gene (Fig. 7). In this hypothesis, we assume that the transcription from *U2af1-rs1* gene may interfere with *Murr1* transcription, possibly by its initiation on the paternal allele, and cause a reduction in *Murr1* expression dependent on the balance of the transcriptional activities of both genes. Transcriptional interference has been reported in yeast, where two genes are closely spaced and transcribed in the same direction. The transcription of the downstream gene is affected when the upstream gene is expressed (13). A similar phenomenon has been reported in mammalian cells (14).

In the embryonic and neonatal brain, *U2af1-rs1* expression is relatively low compared to *Murr1* expression. We propose that during these developmental stages this low expression of *U2af1-rs1* would moderately interfere with the transcription of *Murr1* on the paternal chromosome, resulting in the maternal allele-preferential expression of *Murr1*. The expression of *Murr1* decreases in the period between the neonatal and 2-week-old stages, a time when the maternal expression of *Murr1* becomes noticeable (Fig. 3B). The expression of *U2af1-rs1*, on the other hand, continues to increase during development. This could enhance the interference with *Murr1* transcription and result in maternal allele-predominant expression in the adult brain. During the development of the liver, in contrast to that of the brain, the relative level of expression of *U2af1-rs1* compared with that of *Murr1* does not change significantly (Fig. 5D through F). This would cause only a slight reduction of the paternal transcription of *Murr1* and lead to the maternal allele-preferential expression as observed in most of the adult tissues and in the immature brain. Such a transcriptional interference should be reciprocal; that is, the *Murr1* transcription may also interfere with the *U2af1-rs1* transcription. Even if this is the case, the allelic expression of *U2af1-rs1* should not be affected. Since *U2af1-rs1* is expressed exclusively from the paternal allele, the gene still shows the exclusive paternal expression even when the transcriptional interference by *Murr1* decreases the paternal expression of *U2af1-rs1*. In order to observe the interference with *U2af1-rs1*, the transcription of *Murr1* should be stopped on the paternal chromosome by targeted deletion of the *Murr1* promoter region.

Another possible explanation for the developmental stage-specific and tissue-specific imprinting is the usage of distinct promoters, such as those reported on the human imprinted gene *IGF2*. Among four promoters of this gene, the expression of the P1 promoter is biallelic, whereas those of the P2 to P4 promoters are paternal allele specific. In human liver, the P1 promoter and the P3 and P4 promoters are used primarily in the adult and fetal stages, respectively. This promoter switch causes fetal stage-specific imprinting of the gene in the human liver (43). We have obtained evidence for a single promoter of the *Murr1* gene by 5′ rapid amplification of cDNA ends. However, performing other assays, such as S1 mapping or RNase protection, would be needed in order to confirm a single promoter of this gene and to exclude the alternative explanation.

Antisense RNAs have been found previously to be associated with imprinted genes (33). The representative pairs of imprinted gene with antisense RNA are *Igf2r*/*Air*, *Ube3A*/*Ube3A-ATS*, *Kvlqt1*/*Lit1*, and *Nesp*/*Nespas*. These antisense RNAs are very long noncoding RNAs (more than 100 kb), and their genes are imprinted oppositely to the partner gene (21, 25, 32, 44). They are suggested to have a role in establishing and/or maintaining imprinting of the partner genes. For example, the antisense RNA, *Air*, has been shown to function in *cis*

to repress not only the partner, *Igf2r*, but also two other imprinted genes located upstream of *Air* (35). Premature disruption of *Air* transcription resulted in the loss of imprinting of all three genes. The imprinting of the upstream genes cannot be due to the sort of transcriptional interference discussed here. One hypothesis is that the *Air* RNA functions in *cis* to cause a repressed chromatin state in the *Igf2r*-imprinted cluster in a way similar to that of *Xist* RNA in X-chromosome inactivation (2). Although we cannot rule out the possibility that *U2af1-rs1* RNA also harbors such a *cis*-acting function to form a repressed chromatin state in this locus, the transcriptional interference is more plausible because of the following reasons. First, *U2af1-rs1* is expressed paternally in all the tissues, whereas adult brain is the only tissue in which *Murr1* is imprinted. In contrast, the pairing of *Igf2r* and *Air* shows reciprocal imprinting in many adult tissues (18). Second, we have searched imprinted genes in 500-kb genomic region around *Murr1*, and no neighboring genes were imprinted (Zhang et al., unpublished data). Third, *U2af1-rs1* RNA is a protein-coding mRNA and should diffuse out to the cytoplasm. Harboring *cis*-acting functions may be difficult in this kind of RNA.

In contrast to the clustering of many imprinted genes, the *Murr1*/*U2af1-rs1* locus seems to be an isolated imprinted locus. *U2af1-rs1* is thought to be a neomorphic gene caused by retrotransposition (27). This gene shows complete imprinted expression, whereas *Murr1*, containing *U2af1-rs1*, shows incomplete imprinting, that is, maternal-predominant or -preferential expression. The mouse *Bc10*/*Nnat* locus shares some features with the *Murr1*/*U2af1-rs1* locus. *Nnat* is expressed exclusively from the paternal allele and is associated with a maternally and differentially methylated region, and it resides within the intron of a gene, *Bc10*, which is transcribed in the opposite direction (20). They have some differences, however. The imprinted gene *Nnat* contains introns, unlike *U2af1-rs1*. The host gene, *Bc10*, is expressed biallelically, although the analysis was done only in embryos. Moreover, a similar arrangement described as "a microimprinted domain" was reported recently for the imprinted genes *Nap115* (chromosome 6) and *Peg13* (chromosome 15) (36). As is true for the *Nnat* gene, the *Nap115* and *Peg13* genes are also expressed exclusively from the paternal allele and located within the introns of other biallelically expressed genes. In order to know whether these loci are isolated imprinted loci, one should analyze neighboring genes on their allelic expression. We are performing this line of analyses with the *Murr1*/*U2af1-rs1* locus, and all the neighboring genes are not imprinted in 500-kb region around *Murr1* as mentioned above. The studies on these isolated loci may be valuable for studies on imprinting mechanisms because of their simplicity and their imprinting mechanisms, which possibly differ from those of large imprinted domains.

DNA methylation can serve as a signal to mark the parental alleles. Allele-specific methylation patterns are a common feature of most imprinted genes (9, 12, 37). Such differentially methylated regions (DMRs) are usually located within CGIs, in contrast to the general observation that CGIs are unmethylated. Many of the DMRs have important functions in the allele-specific expression of the imprinted genes (22, 34). Contrary to this general association of DMRs with imprinted genes, the CGIs found in the *Murr1* locus are not methylated on both alleles in brain cells, except for the maternally methylated CGI3 spanning from the promoter to the body of the*U2af1-rs1* gene. The DMR in CGI3 is considered to be involved in the paternal expression of *U2af1-rs1*. This observation suggests that the allelic expression of *Murr1* does not depend on differential DNA methylation of the genome. Similar results were observed for the Kip2/Lit1 subdomain of mouse chromosome 7F5 (46). There are seven imprinted genes in this region, and no DMR was found in three out of six genes carrying CGIs in the promoter. There was an imprinting control region (ICR) in this subdomain, and results suggested that this ICR governs the imprinted expression of those seven imprinted genes (11). Similarly, an ICR would also probably exist in the *Murr1*/*U2af1-rs1* region. CGI3 is one such candidate. To clarify if this is the case, it would be necessary first to determine the span of the imprinting domain, including *Murr1*/*U2af1-rs1*, and then to search for an ICR in this domain.

Moderate preferential expression of the maternal allele is the general expression pattern of the *Murr1* gene (Fig. 3A and B). This maternal preference is higher in tissues of BPF1 mice than in those of PBF1 mice. This difference between these F_1 mice may be due to the disparity in promoter activities of *Murr1* alleles of the mouse strains C57BL/6 and PWK. It can be assumed that the promoter of the C57BL/6 allele may have higher activity than that of the PWK allele. In the PBF1 mouse, in spite of a mechanism to express the maternal allele preferentially, the greater activity of the paternal allele makes the difference between the two alleles small. In BPF1, the C57BL/6 allele is maternal, and this situation enhances the allelic difference. We have, in fact, found some polymorphisms in the promoter region of the *Murr1* gene between these two strains, although we have not analyzed whether these sequence variations affect the promoter activity.

The dog *Murr1* homologue has recently been reported (42). Exon 2 of the canine *MURR1* gene was found to be deleted in both alleles of a certain purebred dog population that suffered from copper toxicosis. Copper toxicosis is characterized by accumulation of copper in the liver and subsequently in the brain and other organs as a result of inefficient biliary excretion of copper. The presence of the mutation suggests that *MURR1* is the disease-causing gene in copper toxicosis in dogs. Therefore, it is possible that the mouse *Murr1* gene is also involved in copper metabolism. In order to analyze the function of the gene, it would be necessary to produce mice with a deletion of the gene.

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