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Mit1/Lb9 and *Copg2*, new members of mouse imprinted genes closely linked to *Peg1/Mest*[1]

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Abstract Two mouse genes, *Mit1/Lb9* and *Copg2*, linked to *Peg1/Mest* on mouse chromosome 6, were identified to be imprinted maternally and paternally, respectively. *Mit1/Lb9* encoding untranslated transcripts resides within the intron 20 of *Copg2*. The gene is maternally imprinted in adult mouse brain, partially imprinted in other tissues. *Copg2* consists of 24 exons within the >40 kb genomic region, being expressed ubiquitously in mouse tissues with a partial imprinting pattern in embryos, neonates, and adult brain in contrast to maternally imprinted human *COPG2*. In addition, we identified an antisense transcript of *Copg2*, *Copg2AS*, which overlaps 3′-UTRs of *Copg2* and *Peg1/Mest*. The *Copg2AS* transcript is maternally imprinted in embryos, neonates, and adult tissues.

Key words: Imprinting; *Peg1/Mest*; Coatomer protein complex; Antisense RNA

1. Introduction

Genomic imprinting is an epigenetic mechanism in mammals that directs differential silencing of a gene depending on the parental allelic origin in the offspring's soma [1,2]. More than two dozens of human and mouse genes have been identified to be imprinted through various efforts including genome-wide systematic searches for monoallelically expressed genes [3–9]. The clustering behavior of imprinted genes was exploited in a strategic screening for the identification of new imprinted genes. The increased wealth of genomic and EST database enabled us to ambulate around four genomic regions each of which contains only a single previously identified imprinted gene. We have identified two mouse imprinted genes, *Mit1/Lb9* and *Copg2*, closely linked to *Peg1/Mest*. The expression profiles and imprinting patterns of the genes were determined.

2. Materials and methods

2.1. Determination of imprinting status

Human genes and ESTs closely linked to the known imprinted genes were chosen from Human GeneMap'99 (http://www.ncbi.nlm.nih.gov/genemap). Mouse homologues of the selected human genes were identified from the GenBank database using BLAST programs. Polymorphisms of the candidate genes were used to physically discriminate one of parental alleles from the other in the F1 hybrids crossed between two inbred mouse strains, C57BL/6J (a strain of *Mus musculus domesticus*) and KJR/Msf (a strain of *Mus musculus molossinus*). Restriction fragment length polymorphism (RFLP) analyses of RT-PCR products were carried out to determine the allele-specific expression of the genes in the (KJR/Msf×C57BL/6J)F1 and the (C57BL/6J×KJR/Msf)F1 hybrids. Total RNAs were isolated using Tri reagent (Molecular Research Center). Reverse transcription was performed with SuperScript II Reverse Transcriptase (GibcoBRL) using random hexamer for *Mit1/Lb9* or strand-specific primers for *Copg2* and *Copg2AS*. The PCR primers 4–5 (5′-AACAAAACTAGCTTTACTTGAGAG-3′) and 4–32 (5′-ATCTGTAACTGTAACCCTGGGTCG-3′) were used for amplification of the *Mit1/Lb9* cDNA. The primer 4–32 ends at one nucleotide upstream to the polymorphic site and contains a C residue rather than a native G residue as the 3′-penultimate nucleotide. Therefore, the PCR product of the KJR/Msf allele would be digested by *Taq*I restriction enzyme while that of the C57BL/6J allele would not (see Fig. 4A). The PCR products were digested with *Taq*I restriction enzyme and analyzed on a 5% polyacrylamide gel. PCR amplifications were carried out for 33 cycles in 50 μl of 10 mM Tris–HCl (pH 8.3), 40 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 0.5 μg/ml acetylated BSA, and 200 μM of each dNTP with 50 pmol of each primer and 2.5 units of *Taq* DNA polymerase at 94°C for 30 s, at 55°C for 30 s, and at 72°C for 30 s. The PCR primers G2-F1 (5′-GCTGCTGCCTGGGAAGAGGT-3′) and G2-PF (5′-GAAGAAACTCTAAAGCTCATGCTC-3′) located in exon 20 and exon 24, respectively, were used for amplification of the *Copg2* cDNA. To amplify the *Copg2AS* cDNA, the PCR primers G2-PR (5′-GGCCCTGCGCCAGCAGATCAAACA-3′) and Peg1-3UF (5′-CCTAAGAGCAAATGGTGCTG-3′) located in exon 24 of *Copg2* and exon 12 of *Peg1/Mest*, respectively, were used. The PCR amplifications were performed under the same conditions as above except that the annealing temperature was 62°C. Genomic DNA was amplified using G2-PR and G2-PF primers. The PCR products of *Copg2* and *Copg2AS* were digested with *Hha*I and *Bsm*AI restriction enzymes, respectively and analyzed on a 20% polyacrylamide gel.

2.2. Characterization of cDNA and genomic DNA clones

Six cDNA clones of *Mit1/Lb9* and three cDNA clones of *Copg2* were isolated from mouse brain cDNA library and testis cDNA library, respectively. Six genomic clones for *Mit1/Lb9* and *Copg2* were isolated from mouse 129 genomic DNA library. Library screening was carried out according to the standard protocol. The nucleotide sequences were determined using a *Taq*Trak Sequencing System (Promega) and a Bigdye Terminator cycle sequencing kit (Perkin-Elmer).

2.3. Southern blot and Northern blot analyses

Fifteen micrograms of mouse genomic DNA were digested with *Stu*I, *Bgl*II, *Pvu*II, *Hin*dIII, *Eco*RI, *Xba*I, or *Dra*I, electrophoresed on a 0.8% agarose gel, and transferred to a Hybond-N^+ membrane

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[1] The nucleotide sequences reported in this paper have been submitted to the GenBank with accession numbers: mouse *Mit1/Lb9*, AF217545; mouse *Copg2*, AF205065.

Abbreviations: *Mit1*, *Mest*-linked imprinted transcript 1; *Copg*, coatomer protein complex subunit γ

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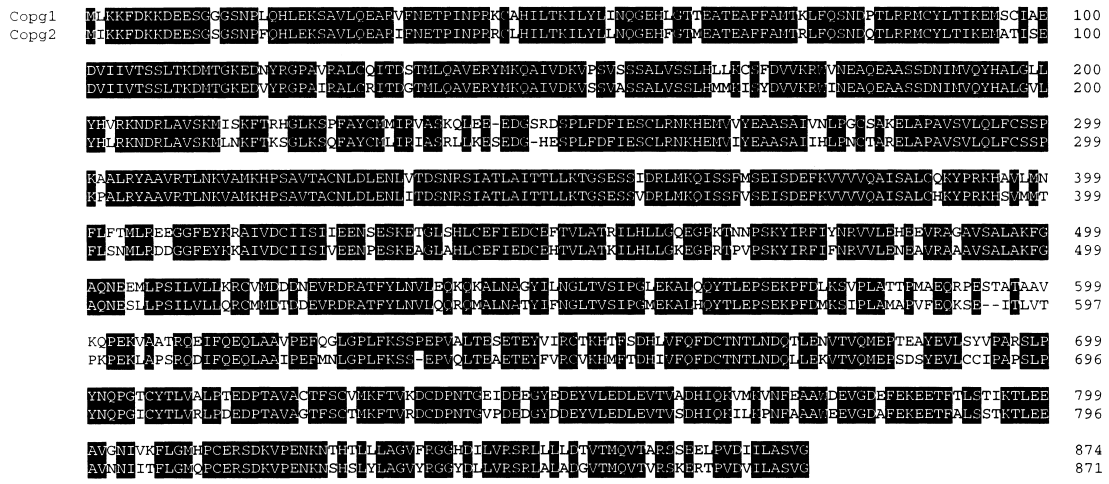

Fig. 1. Comparison of deduced amino acid sequences between mouse *Copg1* and mouse *Copg2*. Identical amino acids are shaded.

(Amersham) in the Southern hybridization experiment. Thirty micrograms of total RNAs were electrophoresed on a 1% agarose gel and transferred to a Hybond-N^+ membrane for the Northern hybridization. The [α-^{32}P]dCTP random primed *Mit1*/*Lb9* and *Copg2* cDNAs were used as the probes. Hybridization was carried out at 65°C using the QuickHyb solution (Stratagene).

3. Results

3.1. Isolation and characterization of Mit1/Lb9 and Copg2

We investigated parent of origin-specific expression patterns of 12 mouse genes of which human homologues are closely linked to *Peg1*/*Mest* [10], *PEG3* [11], *PEG5*/*NNAT* [12], and *MEG1*/*GRB10* [13]. It has not been investigated whether the genomic regions of those imprinted genes harbor additional imprinted genes yet. The allelic expression patterns of the 12 candidates were determined in the F1 hybrids of C57BL/6J (a strain of *Mus musculus domesticus*) and KJR/Msf (a strain of *Mus musculus molossinus*). Mouse homologues of *UBE2H*, *IRF5*, *KIAA0265*, Cda15a02, and *CALU* linked to *Peg1*/*Mest*, WI-8030, *EMAP2*, AAD23609.1, and CGI-146 linked to *PEG3*, *RBL1* linked to *PEG5*/*NNAT*, and *TTC4* linked to *MEG1*/*GRB10* were ascertained to be expressed biallelically in adult mouse brain, heart, lung, and kidney (data not shown). However, a gene closely linked to *Peg1*/*Mest*, a maternally imprinted gene on mouse chromosome 6 [3], was identified to be imprinted. It was found that the mouse cDNA sequence (GenBank accession no. AA611551), a homologue of a human clone 23582 (GenBank accession no. AF038190), was maternally imprinted in mouse brain (see below). The gene was designated as *Mit1* (*Mest*-linked imprinted transcript 1; GenBank accession no. AF217545). It shares 67.3% nucleotide sequence identity with human clone 23582. Subsequently, cDNA and genomic DNA clones of *Mit1* were isolated and characterized. Sequencing analyses showed that *Mit1* is located within the intron 20 of an adjacent gene, *Copg2* (Fig. 1), a homologue of coatomer protein complex subunit γ (γ-COP) [21] (Fig. 2A). *Copg2* shares 80.8% amino acid sequence identity with mouse *Copg1* (GenBank accession no. AF187079) (Fig. 1) and 97.6% amino acid sequence identity with human *COPG2* [9]. A CpG island was found in the 5′-region of *Copg2* (Fig. 2A). The 3′-UTR of *Copg2* overlapped at its terminal 52 nucleotides with the 3′-UTR of *Peg1*/*Mest* (Fig. 2A). The overlapping is conserved in mouse, human [9], and rat (GenBank accession nos. AI408151 and AA851833). The exon–intron boundaries of mouse *Copg2* and human *COPG2* are conserved. Database analyses of the cDNA and genomic DNA sequences of *Mit1* revealed that the previously identified *Lb9* cDNA (GenBank accession no. U20263) was located at the upstream of *Mit1* (Fig. 2A). RT-PCR experiments using the PCR primers, one of which annealed to the

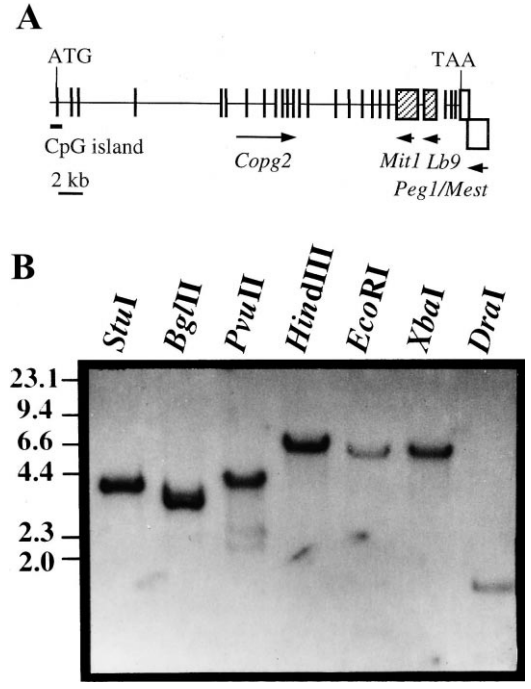

Fig. 2. Genomic organization of *Mit1*/*Lb9* and *Copg2*. A: Genomic structure of *Mit1*/*Lb9* and *Copg2*. The protein coding regions are indicated by solid boxes. Open boxes represent 3′-UTRs of *Copg2* and *Peg1*/*Mest*. *Mit1* cDNA and *Lb9* cDNA are indicated by hatched boxes. Introns of which the sizes were determined are represented by horizontal lines. Transcriptional orientations of *Mit1*/*Lb9*, *Copg2*, and *Peg1*/*Mest* are indicated by arrows. Translation start codon (ATG), stop codon (TAA), and CpG island of *Copg2* are indicated. B: Southern blot analysis of *Mit1*/*Lb9*. The size markers are indicated in kb.

Lb9 cDNA and the other to the *Mit1* cDNA, showed both sequences were within the same gene transcript (data not shown).

Mit1/Lb9 is a single copy gene as shown by Southern blot analysis (Fig. 2B). The transcription products of *Mit1/Lb9* seem to be highly heterogeneous since multiple transcripts were detected in Northern blot analysis (Fig. 3A). In addition, we could find six different forms of the *Lb9* cDNA (GenBank accession nos. U20262–U20267) from the GenBank database. It is likely that the observed multiple *Mit1/Lb9* transcripts are generated by alternative splicing and/or differential usage of promoters and polyadenylation sites. It is of interest that no considerable open reading frame could be predicted from the sequences of *Mit1/Lb9* cDNAs. It seems that *Mit1/Lb9* functions at the RNA level as *H19* [14], *Igf2rAS* [15], *IPW* [16], or *LIT1* [17] does.

3.2. Expression of Mit1/Lb9 and Copg2

The expression patterns of *Mit1/Lb9* and *Copg2* were determined by Northern blot analyses with total RNAs isolated from various tissues (Fig. 3). The transcription level of *Mit1/Lb9* varied among different tissues (Fig. 3A). The expression of *Mit1/Lb9* was highest in brain. The major transcript, about 7 kb in length, was also expressed in heart, lung, and muscle, while the expression in other tissues was negligible. On the other hand, *Copg2* was ubiquitously expressed in most mouse tissues with the highest expression in testis where the *Mit1/Lb9* was hardly expressed (Fig. 3B). The major transcript of *Copg2* was about 3 kb in length.

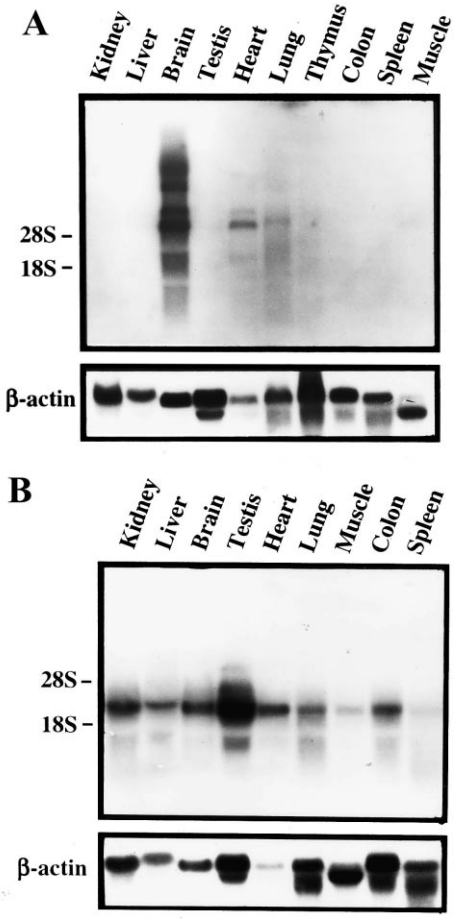

Fig. 3. Expression analyses of *Mit1/Lb9* and *Copg2*. Total RNAs from different tissues of mouse were blotted and hybridized with *Mit1/Lb9* (A) or *Copg2* (B) cDNA as a probe. Reprobing of the same blot with the β-actin probe was used for the control.

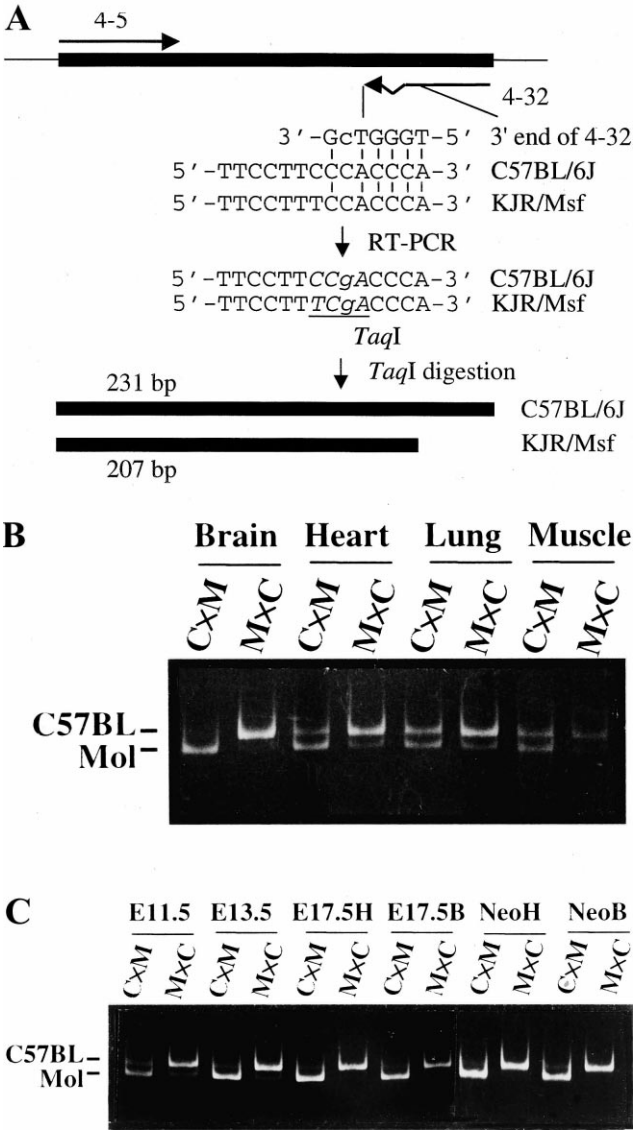

Fig. 4. Determination of imprinting status of *Mit1/Lb9*. A: Expected restriction DNA fragments using mismatch PCR-mediated site-directed mutagenesis. The polymorphic site between C57BL/6J and KJR/Msf is indicated in boldface. The mismatched base in the primer 4–32 is shown in lowercase. Polymorphic *Taq*I site created in the KJR/Msf allele is underlined. B: Imprinting status of *Mit1/Lb9* in adult tissues. RNAs derived from the (C57BL/6J×KJR/Msf)F1 hybrid (C×M) and the (KJR/Msf×C57BL/6J)F1 hybrid (M×C) were used for RT-PCR amplification. PCR products were digested with *Taq*I and separated on a 5% polyacrylamide gel. C: Imprinting status of *Mit1/Lb9* in mouse embryos and neonates. C57BL, C57BL/6J allele; Mol, KJR/Msf allele; E17.5H, head of E17.5; E17.5B, body of E17.5; NeoH, head of neonate; NeoB, body of neonate.

3.3. Imprinting status of Mit1/Lb9 and Copg2

The imprinting status of *Mit1/Lb9* was determined using a single base polymorphism between mouse strains, C57BL/6J and KJR/Msf. Since the polymorphism did not provide any available RFLP site, mismatch PCR-mediated site-directed

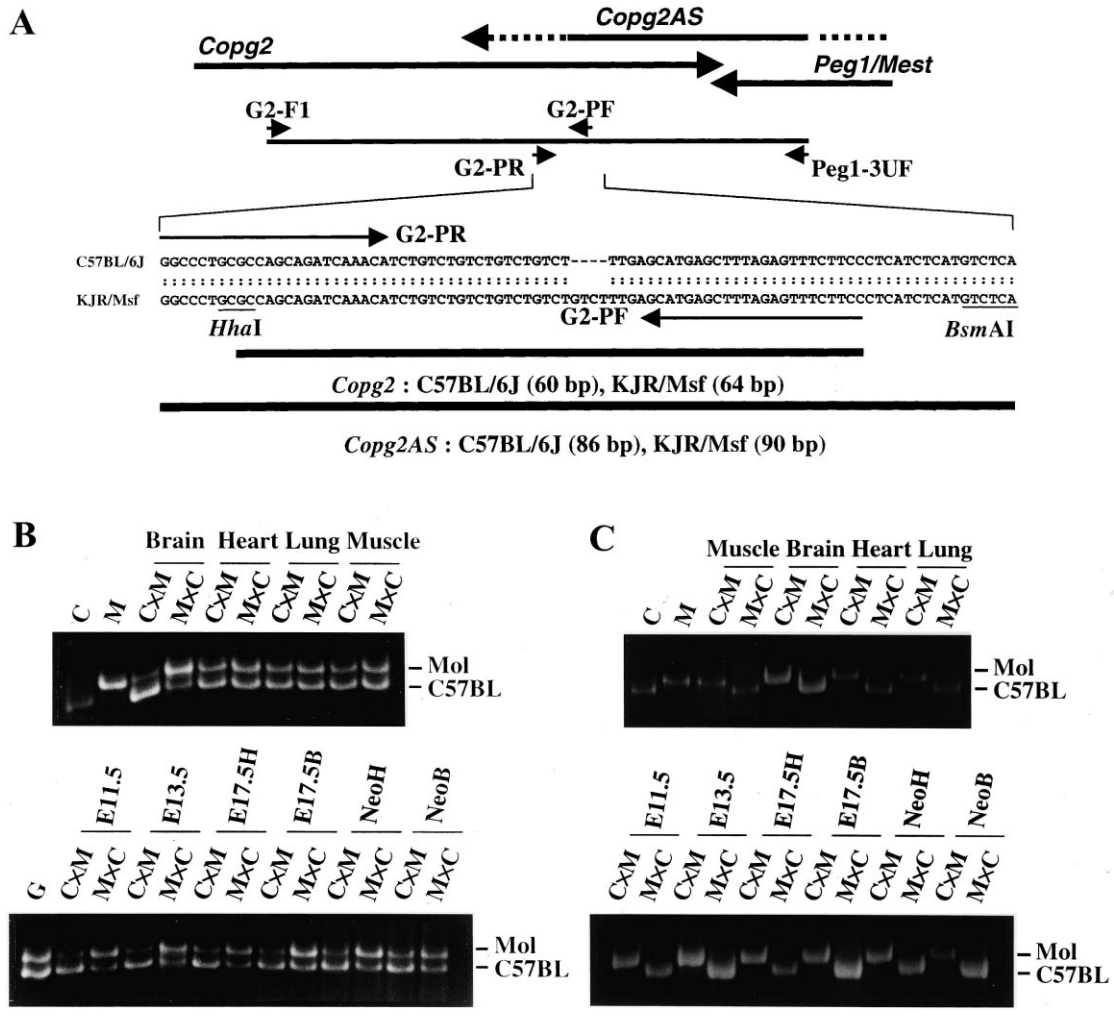

Fig. 5. Determination of imprinting status of *Copg2* and *Copg2AS*. A: Nucleotide sequences of the region containing a length polymorphism between C57BL/6J and KJR/Msf. Annealing sites of primers G2-F1, G2-PF, G2-PR, and Peg1-3UF are indicated. Strand-specific reverse transcription was performed using primer G2-PF for *Copg2* or G2-PR for *Copg2AS*. The RT-PCR product of *Copg2* transcript was amplified with G2-F1 and G2-PF and digested with *Hha*I. The reverse-transcribed product of *Copg2AS* transcript was amplified with Peg1-3UF and G2-PR and digested with *Bsm*AI. Digested DNA fragments were separated on a 20% polyacrylamide gel. Expected restriction DNA fragments are represented. Transcripts of *Copg2*, *Copg2AS*, and *Peg1/Mest* are indicated by arrows. B: Imprinting status of *Copg2* in adult tissues and in mouse embryos and neonates. C: Imprinting status of *Copg2AS* in adult tissues and in mouse embryos and neonates. Imprinting status was determined in the (C57BL/6J×KJR/Msf)F1 hybrid (C×M) and the (KJR/Msf×C57BL/6J)F1 hybrid (M×C). C57BL, C57BL/6J allele; Mol, KJR/Msf allele; C, C57BL/6J; M, KJR/Msf; G, genomic DNA of the (C57BL/6J×KJR/Msf)F1 hybrid; E17.5H, head of E17.5; E17.5B, body of E17.5; NeoH, head of neonate; NeoB, body of neonate.

mutagenesis [18] was used for the discrimination of the expressed alleles (see Section 2 and Fig. 4A). The single base polymorphism and the mismatch PCR primer introduced a *Taq*I restriction enzyme site in the RT-PCR product of the KJR/Msf allele, but not in that of the C57BL/6J allele. The KJR/Msf allele was exclusively expressed in brain of the (C57BL/6J×KJR/Msf)F1 hybrid while the C57BL/6J allele was expressed in the reciprocal (KJR/Msf×C57BL/6J)F1 hybrid (Fig. 4B). However, relaxed imprinting patterns were observed in heart, lung and muscle. The expression level of the C57BL/6J allele was higher than that of the KJR/Msf allele in these tissues of the (KJR/Msf×C57BL/6J)F1 hybrid. In the reciprocal (C57BL/6J×KJR/Msf)F1 hybrid, both alleles were expressed at comparable levels in the same tissues (Fig. 4B). The imprinting status of *Mit1/Lb9* in mouse embryos and neonates was investigated to determine the developmental stage at which the imprinting of *Mit1/Lb9* is established (Fig. 4C). *Mit1/Lb9* was apparently imprinted in E13.5 embryos, E17.5 embryos, and in neonates. However, E11.5 embryos exhibited a relaxed imprinting pattern. The result suggests that the imprinted expression pattern of *Mit1/Lb9* is established at around E13.5.

The four-nucleotide length polymorphism between C57BL/6J and KJR/Msf was used to determine the imprinting status of *Copg2* (Fig. 5A). We initially obtained inconsistent imprinting patterns with different primer sets. Such an inconsistency raised the possibility for the existence of the antisense transcript of *Copg2*. We prepared strand-specific RT products as PCR templates to distinguish *Copg2* from its antisense transcript, *Copg2AS* (Fig. 5A). In contrast to human *COPG2* reported to be maternally imprinted in several human fetal tissues [9], mouse *Copg2* is paternally imprinted. Majority of the *Copg2* transcripts was expressed from the maternal allele in brain (Fig. 5B). Similar imprinting patterns of *Copg2* were

also observed in mouse embryos and neonates (Fig. 5B). *Copg2AS* was maternally imprinted in tested adult tissues, embryos, and neonates (Fig. 5C). *Copg2AS* spans 3′-UTR of *Peg1*/*Mest* and 3′-UTR of *Copg2*. It is unclear that *Copg2AS* is a part of the heterogeneous *Mit1*/*Lb9* transcripts or an uncharacterized *Peg1*/*Mest* transcript.

4. Discussion

We have demonstrated that *Copg2* is parternally imprinted in mouse brain and the whole embryo in a relaxed manner. The relaxed imprinting pattern of *Copg2* shown in this study can be ascribed to the partial imprinting in whole tissues or the tissue-specific imprinting in the whole embryo since we used total RNA of the whole embryos to determine the imprinting status of *Copg2*. The imprinting strengths of *Mit1*/*Lb9* in heart, lung, and muscle are different between (C57BL/6J×KJR/Msf)F1 hybrid and (KJR/Msf×C57BL/6J)F1 hybrid. The inconsistency can be explained by enhanced lability of imprinted expression pattern under some epigenetic environments including strain-specific genetic background as demonstrated in the imprinting pattern of mouse K_v*lqt1* [19].

It is striking that mouse *Copg2* is paternally imprinted in contrast to human orthologue *COPG2* which was reported to be maternally imprinted in several human fetal tissues [9]. Alternatively, putative antisense transcripts of *COPG2* might have obscured the imprinting status of human *COPG2* since Blagitko et al. used random hexamer primers for reverse transcription to determine the allelic expression of *COPG2* [9].

The allele-specific expression is correlated with dynamic change in the allele-specific methylation at CpG island(s) [20]. The CpG island of *Copg2* is a good candidate target for the allele-specific differential methylation. The methylation status of the CpG island is currently being investigated.

Maternal uniparental disomy (UPD) of proximal region of mouse chromosome 6 is associated with embryonic lethality [20]. The overexpression of *Copg2* or the absence of *Mit1*/*Lb9* or *Copg2AS* transcript in the maternal UPD embryo might have caused the embryonic lethality. The sub-proximal end of mouse chromosome 6 shows a conserved synteny with human chromosomal region 7q31–35 [3,10]. The maternal UPD of human chromosome 7 has been reported in approximately 10% of Silver-Russell syndrome (SRS) cases [21,22], suggesting that imprinted gene(s) on human chromosome 7 is responsible for SRS phenotype in the UPD patients [23]. The imprinted *COPG2* and/or its antisense transcripts including human orthologue of mouse *Mit1*/*Lb9* can be candidate genes for the SRS.

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