

# Identification of a Large Novel Imprinted Gene Cluster on Mouse Proximal Chromosome 6

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Mice with maternal duplication of proximal chromosome 6 die in utero at an early embryonic stage. Recently, two imprinted genes, paternally expressed *Sgce* and maternally expressed *Asb4*, were identified in this region. This report analyzes the imprinting status of genes within a 1-Mb region containing these two genes. *Peg10*, which is next to *Sgce*, shows complete paternal expression, like *Sgce*. Conversely, *Neurabin*, *Pon2*, and *Pon3* show preferential maternal expression at embryonic stages, although they all show biallelic expression in neonatal tissues. These results demonstrate that there is a large novel imprinted gene cluster in this region. 5'-RACE (Rapid Amplification of cDNA Ends) analysis of *Peg10* revealed the existence of a novel first exon separate from the second exon, which encoded two putative ORFs similar to the viral Gag and Pol proteins. A differentially methylated region established in sperm and eggs is located just within the region containing the two first exons of *Peg10* and *Sgce*, and may play an important role in regulating the two paternally expressed genes: *Peg10* and *Sgce*.

[Supplemental material is available online at [www.genome.org](http://www.genome.org). The sequence data from this study have been submitted to DDBJ under accession nos. AB091827–AB091829.]

Maternal uniparental duplication in mouse proximal chromosome 6 causes early embryonic lethality (Beechey 2000; also see <http://www.mgu.har.mrc.ac.uk/imprinting/imprinting.html>). Previously, we identified *Peg1/Mest* as the first imprinted gene in the mouse proximal chromosome 6 (Kaneko-Ishino et al. 1995). However, *Peg1/Mest*-deficient mice show perinatal growth retardation and abnormal maternal behavior, but do not show early embryonic lethality (Lefebvre et al. 1998). Recently, the existence of two imprinted regions has been demonstrated in this region; mice with a maternal duplication proximal to T77H die in utero before 11.5 dpc, whereas those with a duplication distal to T77H show growth retardation (Beechey 2000). *Peg1/Mest* maps to the latter region, and two novel imprinted genes, paternally expressed *Sgce* and maternally expressed *Asb4*, have been identified in the former region (Piras et al. 2000; Mizuno et al. 2002). We have also reported that human retrotransposon-derived *PEG10*, which is adjacent to *SGCE*, is a paternally expressed imprinted gene (Ono et al. 2001).

Because many imprinted genes form clusters in some chromosome regions, it is very possible that there is a large imprinted gene cluster in the *Sgce-Asb4* region. Therefore, we analyzed the imprinting status of six other genes in this region (*Cas1*, *Peg10*, *Neurabin*, *Pon1*, *Pon3*, and *Pon2*) and showed that four genes were imprinted. *Peg10* was paternally expressed, whereas the other three genes showed preferential maternal expression in the embryonic stage. In relation to these imprinted genes, we discuss the phenotypes observed in

mice with maternal duplication of proximal chromosome 6 (early embryonic lethality and perinatal growth retardation) and in human maternal disomy of chromosome 7, which is associated with the growth retardation phenotype known as Silver-Russell syndrome (SRS; OMIM No.180860).

## RESULTS

### Gene Alignment and Analysis of CpG Islands of the *Colla2-Asb4* Region in the Mouse

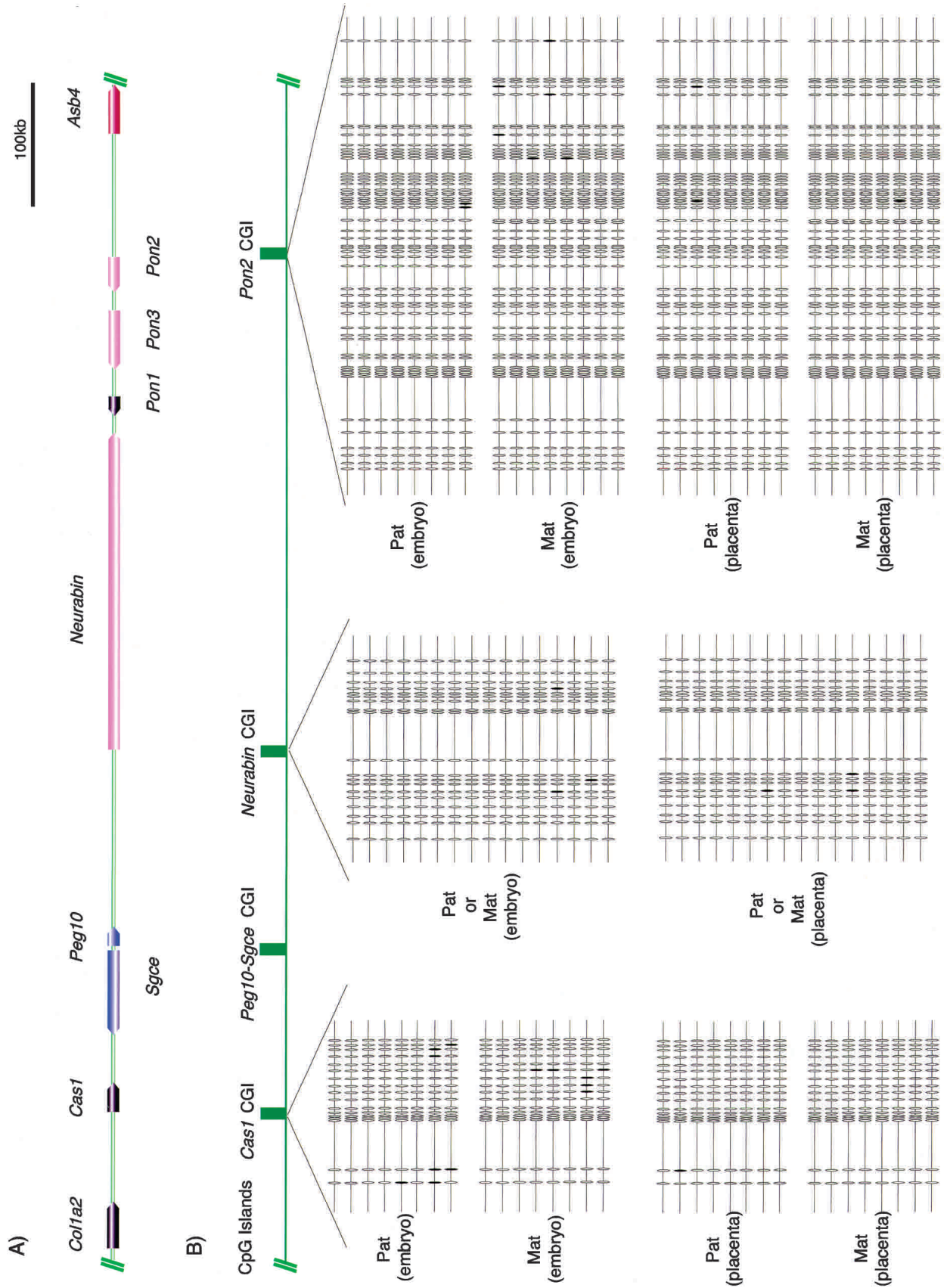
Nine genes in mouse proximal chromosome 6 map to a 1-Mb region between 1.2 and 2.2 Mb of the *Mus musculus* Whole-Genome Shotgun (WGS) supercontig Mm6\_WIFeb01\_97 (GenBank accession no. NW\_000272): *Colla2*, *Cas1*, *Sgce*, *Peg10*, *Neurabin*, *Pon1*, *Pon3*, *Pon2*, and *Asb4* (from proximal to distal, Fig. 1A). This gene alignment is conserved in the human syntenic 7q21 region (data not shown). In this report, we characterize the imprinting status of six of these genes (*Cas1*, *Peg10*, *Neurabin*, *Pon1*, *Pon3*, and *Pon2*), because *Sgce* and *Asb4* are already known to be imprinted and *Colla2* is reported to show biallelic expression (Piras et al. 2000; Mizuno et al. 2002). This region contained four CpG islands (CGIs) corresponding to the promoter regions of the *Cas1*, *Peg10-Sgce*, *Neurabin*, and *Pon2* genes. Bisulfite sequencing analyses of both day 10 embryo and placenta showed that three regions were nonmethylated (Fig. 1B), and that of *Peg10-Sgce* was differentially methylated (Fig. 2A).

We previously reported that human *PEG10* was derived from a Sushi-ichi retrotransposon encoding two ORFs (ORF1 and ORF2) showing similarity to retroviral Gag and Pol proteins, respectively (Fig. 2A; Poulter and Butler 1998; Ono et al. 2001). *Peg10* exists in all five mammals that we have exam-

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**Figure 1** (Legend on next page)

ined (human, cat, dog, olive baboon, and chimpanzee), in the same location as in the mouse (Fig. 1A; GenBank accession no. AC069292, AC108197, AC113572, AC092529, and AC094111, respectively). Expressed sequence tags (ESTs) corresponding to *Peg10* are also registered for the cow, rat, mink, and pig (GenBank accession no. AV608102, AI599367, MVU00594, and BF191703, respectively). Therefore, retrotransposon-derived *Peg10* is highly conserved in mammals.

### Genomic Structure and Verification of Imprinting of Mouse *Peg10*

We determined the full-length sequence of *Peg10*, which consists of 6407 bp, by 5'-RACE (Rapid Amplification of cDNA Ends; GenBank accession no. AB091827). The first exon of *Peg10* was identified 6.5 kb upstream of the second exon and was within 250 bp of the first exon of *Sgce*, which is oriented in a head-to-head manner (Fig. 2A). The genomic structure of these two genes is also conserved in the human genome (data not shown). Therefore, it is not an intron-less gene, as we previously reported in humans (Ono et al. 2001).

A CGI overlaps the first two exons of *Sgce* and *Peg10* (Fig. 2A), and there are 12 direct repeats of a 29-bp GC-rich sequence in the *Peg10* intron 1, just downstream of the first *Peg10* exon (Fig. 2B). Bisulfite sequencing analyses showed that the whole CGI was differentially methylated in paternal and maternal alleles in both day 10 embryo (Fig. 2A, lower part) and placenta (data not shown). Furthermore, it was revealed that differential methylation was already established in oocytes and sperm. Therefore, this region is the primary differentially methylated region (DMR), which indicates that it has an important function in regulating the paternal expression of both *Peg10* and *Sgce* (see following).

To verify tissue-specific expression, we carried out Northern blot analyses using a *Peg10* 3'untranslated region (UTR) fragment as a probe. As shown in Figure 2C, a high level of *Peg10* expression was observed only in placentas. The major transcript was estimated to be ~6.5 kb, which is consistent with the full-length cDNA that we identified. Strong expression of human *SGCE* and *PEG10* in placenta has been reported previously (McNally et al. 1998; Ono et al. 2001).

To verify the imprinting status of *Peg10*, we examined DNA polymorphisms in the *Peg10* 3'UTR between JF1 and C57BL/6 (the G and A residues indicated by arrows in Fig. 2D, respectively) by direct sequencing. In day 10 F<sub>1</sub> embryos of the crosses (B6 × JF1 and JF1 × B6), only paternal G residues were detected in the placentas, yolk sacs, and neonatal brains in the former, and only paternal A residues were detected in the latter samples. Therefore, paternal expression of *Peg10* was confirmed in mice, as occurs with human *PEG10*.

### Genes Showing Preferential Maternal Expression

To test the imprinting status of the remaining five genes (*Cas1*, *Neurabin*, *Pon1*, *Pon3*, and *Pon2*), we examined DNA polymorphisms between JF1 and C57BL/6. Allele-specific expression analyses were carried out using a restriction fragment length polymorphism (RFLP) method combined with the Hot-stop RT-PCR method (Uejima et al. 2000). To exclude the possible misinterpretation of imprinting status because of the existence of overlapping RNAs, we confirmed that no other cDNA bands of different sizes or genomic DNA bands of the same size were detected in RT-PCR experiments for each gene, when the two primers were located in different exons. In addition, the amplified bands were directly sequenced to confirm that they were derived from the genes in question.

*Cas1*, which encodes a putative glycosyltransferase, is a conserved gene found in humans, *Drosophila*, plants, and bacteria (Janbon et al. 2001). In the mouse, RT-PCR experiments with RNA from a range of adult tissues showed that *Cas1* was expressed ubiquitously (Fig. 3A). In the RFLP analysis, *Cas1* showed complete or equal biallelic expression in neonatal brain. It was also biallelically expressed in day 10 and 13 embryo, placenta, and yolk sac samples, but there seemed to be weak maternal biases, especially in extraembryonic tissues (data not shown). Therefore, an RFLP analysis combined with Hot-stop PCR was performed to quantify the expression of each allele precisely. The levels of JF1 and B6 expression went up and down reciprocally in two reciprocal F<sub>1</sub> samples (Fig. 3B), indicating its imprinting. However, the expression ratios of maternal/paternal alleles (M/P values) in these samples were small (no more than twofold) and were not conclusive.

*Neurabin* was first isolated as an actin filament (F-actin)-binding protein in the rat. It regulates synapse formation in vitro and is specifically expressed in neural tissues (Nakanishi et al. 1997). As shown in Figure 3C, *Neurabin* was strongly expressed in the brain. Allelic expression analysis using RFLP with Hot-stop PCR showed biallelic expression of *Neurabin* in the neonatal brain, and preferential maternal expression was observed in placenta (day 10) and yolk sac (day 10 and 13) samples (Fig. 3D). In these tissues, the ratio of expression of a 240-bp band derived from the B6 allele to that of a 100-bp band from the JF1 allele was constantly higher in (B6 × JF1) F<sub>1</sub> samples and lower in reciprocal (JF1 × B6) F<sub>1</sub> samples. The M/P values of placentas were 3.5 and 2.6 (day 10) in (B6 × JF1) F<sub>1</sub> and (JF1 × B6) F<sub>1</sub>, respectively, and these values for yolk sac samples were 2.2 and 4.9 (day 10) and 2.0 and 4.6 (day 13). Because it is apparent that placenta samples from day 13 contain significant amounts of maternal tissue, we did not analyze these samples. We also cannot exclude the possibility of maternal tissue contamination from placenta samples completely, even when isolated from day 10 conceptus, whereas yolk sac samples can be recovered free from maternal contamination. Therefore, we conclude that *Neurabin* is

**Figure 1** Genomic structure of the *Col1a2-Asb4* region in the mouse. (A) Physical map of the genes identified in a 1-Mb region in the *Mus musculus* WGS supercontig Mm6\_WIFeb01\_97 (GenBank accession no. NW\_000272). The arrows show the direction of each transcription unit, and imprinted genes are indicated by color: red and pink indicate strong and weak preferential maternal expression, respectively, and blue indicates complete paternal expression. Genes showing no expression biases between parental alleles are shown in black. (B) CpG islands (CGIs) and their DNA methylation states in the domain. There are four CGIs (length over 300 bp) shown in green boxes in the *Col1a2-Asb4* region, and the three nonmethylated CGIs determined by bisulfite sequencing of genomic DNA isolated from (B6 × JF1) F<sub>1</sub> embryo and placenta (day 10) are shown. DNA polymorphisms were used to determine paternal and maternal alleles of *Cas1* CGI and *Pon2* CGI, and showed that both alleles were nonmethylated in this region. No available DNA polymorphisms were found in *Neurabin* CGI, but no methylated CpGs were observed in this region either. Each horizontal line indicates the sequence from a single clone. Each CpG dinucleotide is represented by an oval. White and black ovals indicate nonmethylated and methylated CpGs, respectively. Details of the differentially methylated region of the *Peg10-Sgce* CGI are shown in Fig. 2A.



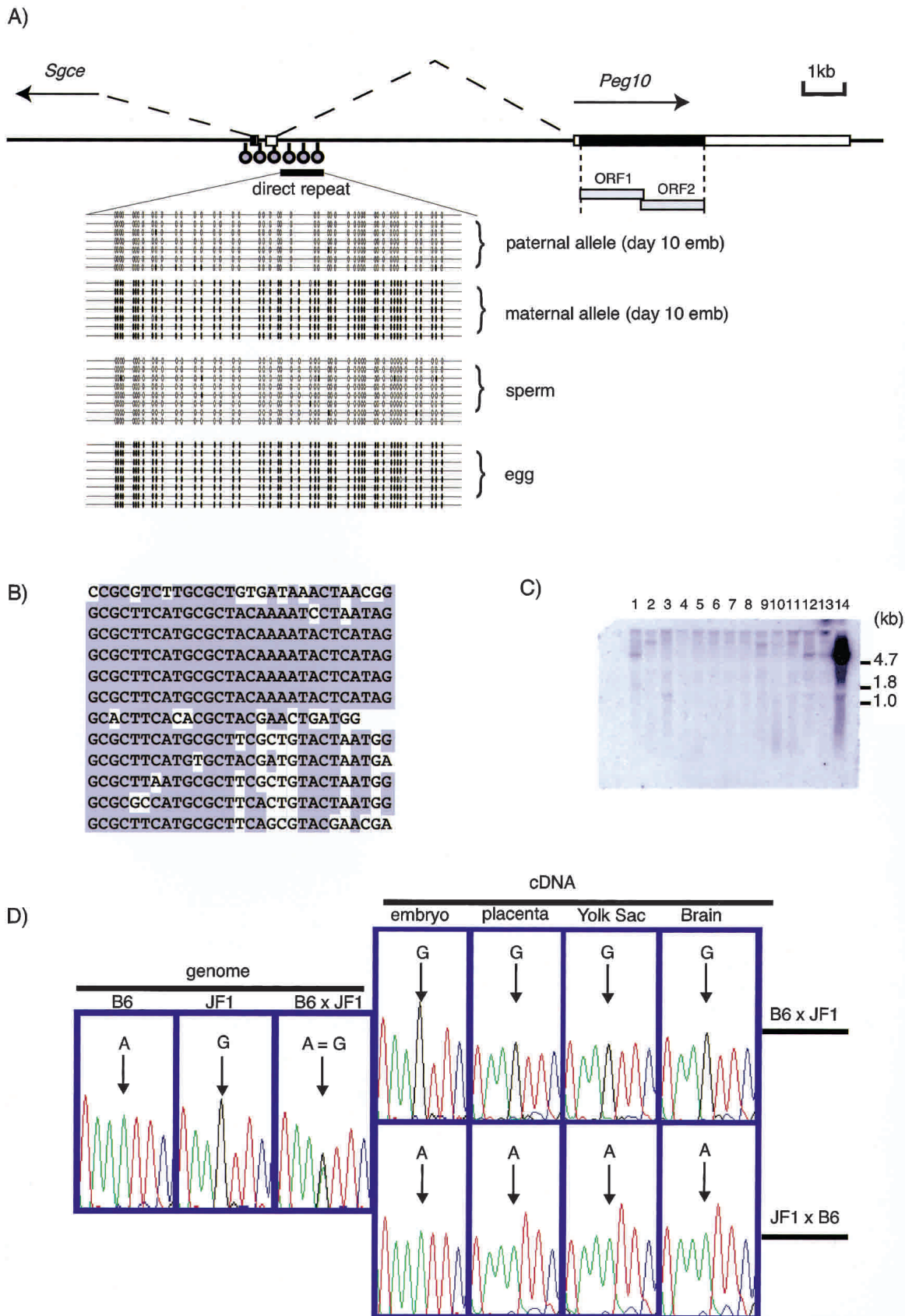


Figure 2 (Legend on next page)

also a maternally expressed imprinted gene at the embryonic stage, especially in extraembryonic tissues.

The three *Pon* genes are located adjacent to each other on mouse chromosome 6 and human chromosome 7 (Fig. 1A; Primo-Parmo et al. 1996). Human PON1, PON2, and PON3 share 65% similarity at the amino acid level. PON1 is an enzyme associated with high-density lipoprotein (HDL) that is believed to protect against the early events of atherogenesis via its ability to hydrolyze oxidized phospholipids. It is also involved in the detoxification of organophosphate insecticides, such as parathion and chlorpyrifos. In contrast, PON2 and PON3 lack paraoxonase activity, although they have similar antioxidant properties. PON3 is also found in HDL, whereas PON2 is not associated with HDL (Davies et al. 1996; Shih et al. 1998; Draganov et al. 2000; Ng et al. 2001).

As shown in Figure 4A, *Pon1* expression was detected mainly in liver and lung. Therefore, we tested the imprinting status of *Pon1* in liver and lung from neonates. As shown in Figure 4B, the same biallelic expression pattern of *Pon1* was observed between reciprocal  $F_1$  crosses, although it is apparent that the expression of the JF1 allele was constantly higher than that of the B6 allele in both tissues. It has been reported that DNA polymorphisms in human promoter regions affect the levels of *IL-4* and *TNF- $\alpha$*  expression (Song et al. 1996; Wilson et al. 1997). Therefore, it is highly possible that genes showing strain-specific (or strain-biased) expression exist in the mouse. In this experiment, apparent allele-biased expression was also observed in *Pon2* and *Pon3* (see following). To distinguish imprinted parental allele-specific expression from strain-specific expression, it is very important to analyze the imprinting status in the  $F_1$  of both crosses. It was difficult to obtain reliable results from embryos, placentas, and yolk sacs because of low expression of *Pon1* in these tissues (data not shown). Mouse *Pon2* is ubiquitously expressed (Fig. 4A) and biallelic expression was observed in neonatal brain (Fig. 4C, right-hand lanes) and other neonatal tissues (skeletal muscle, spleen, kidney, liver, and lung; data not shown). However, significant maternally biased expression was observed in day 10 placenta (3.5 and 4.0) and to a lesser degree in day 13 yolk sac samples (1.9 and 2.0) in (B6  $\times$  JF1)  $F_1$  and (JF1  $\times$  B6)  $F_1$ , respectively (Fig. 4C). Therefore, *Pon2* is imprinted, at least, in these restricted tissues at the embryonic stages.

As shown in Figure 4A, mouse *Pon3* is expressed mainly in liver and lung in the adult. There was no evidence of its imprinting in neonatal liver and lung (Fig. 4D, right-hand lanes). However, *Pon3* showed maternally biased expression in day 10 placenta and day 13 yolk sac samples, because the B6 (534 bp) and JF1 (727 bp) alleles went up and down recip-

rocallly in these tissues of two different  $F_1$ s (Fig. 4D). Similar maternal biases were also observed in day 10 embryo to a lesser degree. It was also evident that the expression of B6 alleles in embryo, placenta, and yolk sac was much higher than that of JF1 alleles in both reciprocal  $F_1$ s. Therefore, M/P values are not a good indicator of imprinting in this case.

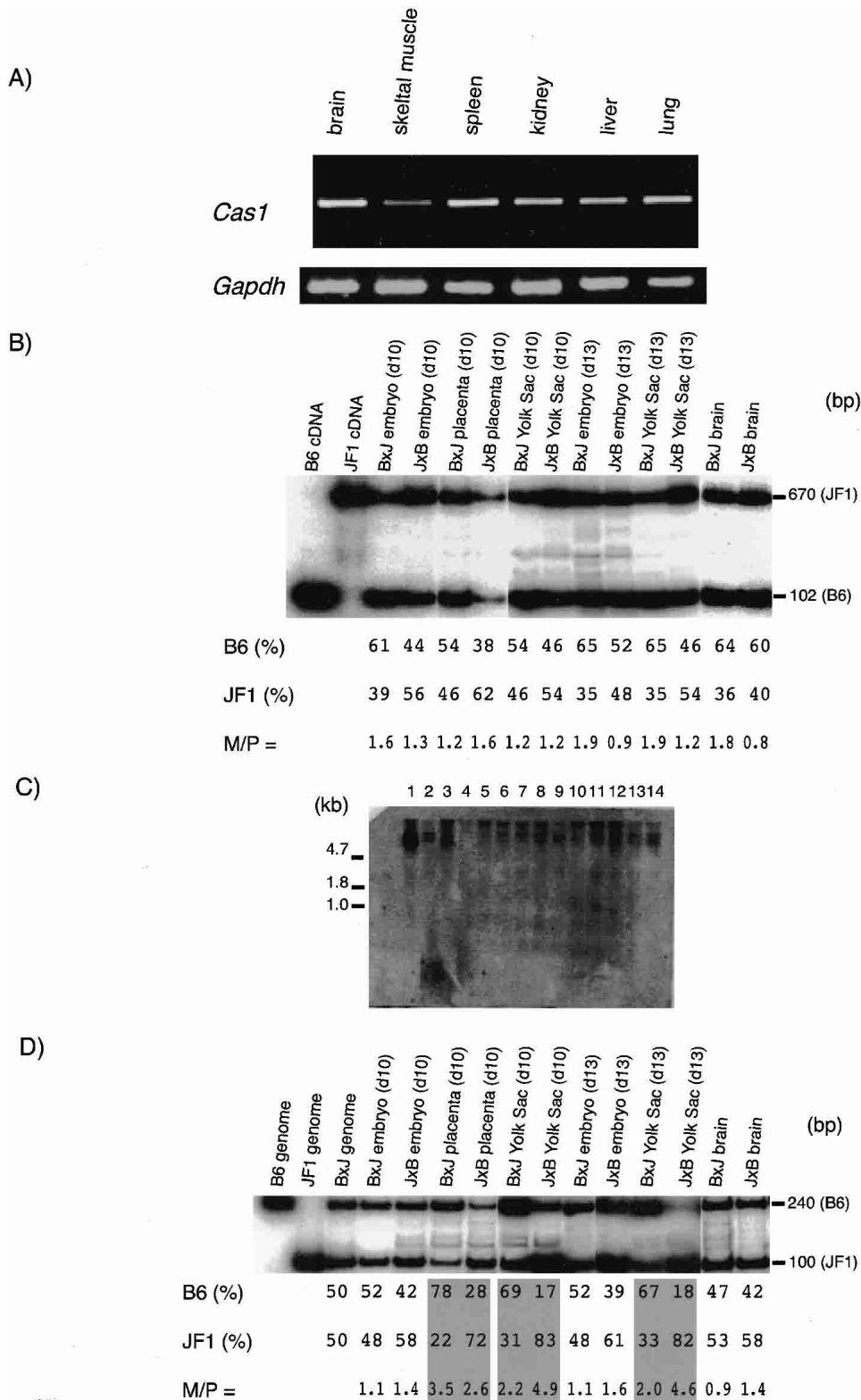
## DISCUSSION

In this study, we demonstrated that at least two paternally expressed genes and four maternally expressed genes are located in a 1-Mb region between *Col1a2* and *Asb4* in the mouse proximal chromosome 6 and form a large cluster of imprinted genes. Of these, *Sgce* and *Peg10* show paternal expression in an all-or-none fashion, and *Asb4* shows mainly maternal expression with a lesser paternal contribution (Piras et al. 2000; Mizuno et al. 2002). One characteristic of this cluster is that three genes (*Neurabin*, *Pon2*, and *Pon3*) show preferential maternal expression in the embryonic stage. They show biallelic expression in neonatal tissues, but their expression in extra-embryonic tissues appears maternally biased. Although most imprinted genes identified so far show clear all-or-none monoallelic expression patterns, some imprinted genes showing weak parental preferences were recently reported, including *Tnfrif1* on mouse distal chromosome 7 and *Dio3* on mouse distal chromosome 12 (Clark et al. 2002; Tsai et al. 2002). The former shows a weak maternal bias in several organs and the latter shows preferential paternal expression in embryos and placentas to differing degrees. It is rational to expect that genes showing differential expression of paternal and maternal alleles that are always associated with the same parental biases be classified as imprinted genes.

What is the mechanism of this preferential expression? There are three possibilities: (1) the existence of overlapping transcripts from other gene(s) showing paternal or biallelic expression; (2) the existence of two promoters, one for maternal expression and the other for biallelic expression; and (3) the relaxation of genomic imprinting: the change from an imprinted state to a nonimprinted state occurs in certain situations.

The first possibility, overlapping RNA, such as readthrough products of *Peg10*, can be excluded, because no other cDNA bands of different sizes or genomic DNA bands of the same size were detected in RT-PCR experiments for *Pon1* and *Pon2*, when the two primers were located in different exons. The amplified bands were directly sequenced to confirm that they were derived from the genes in question, as mentioned. Moreover, as far as the expression profile of *Peg10*

**Figure 2** *Peg10* imprinted expression associated with primary DMR. (A) The genomic structure of *Peg10* and bisulfite sequencing analyses of the *Peg10* repeated sequence region. The full-length *Peg10* sequence was determined by 5'-RACE analysis. The white boxes are untranslated regions and putative open reading frames are shown with black boxes. Two putative ORFs of *Peg10* are shown in gray boxes below; ORF1 shares 30% amino acid identity with the gag protein, and ORF2 predicted from a -1 frameshift of ORF1 shares 25% identity with the pol protein of Sushi-ichi retrotransposon, respectively. The translational frameshifting of ORF1-ORF2 is commonly observed in retroviruses and gypsy-type LTR retrotransposons. The arrows indicate the 5'-3' direction of *Peg10* and *Sgce*. The DNA methylation status of *Peg10* repeats in day 10 embryo, eggs, and sperm are shown. Differential methylation was observed in the day 10 embryo as well as the day 10 placenta (data not shown). DNA polymorphisms between JF1 and B6 were used to distinguish parental alleles. The entire *Peg10-Sgce* CpG island indicated by gray lollipops shows a differentially methylated status similar to that in the *Peg10* repeats (data not shown). (B) Twelve *Peg10* intronic 29-bp repeat sequences. Shaded boxes indicate homology with the most frequent sequence, GCGCTTCATGCGCTACAAAATACTCATAG (four times). (C) Northern blot profiles of *Peg10* in mouse adult tissues. Total RNA from mouse adult brain (lane 1), heart (lane 2), lung (lane 3), liver (lane 4), spleen (lane 5), kidney (lane 6), stomach (lane 7), small intestine (lane 8), skeletal muscle (lane 9), skin (lane 10), thymus (lane 11), testis (lane 12), uterus (lane 13), and placenta (lane 14) was analyzed. Ribosomal RNA detected by ethidium bromide (EtBr) staining was used as a marker. Strong *Peg10* expression was observed only in placenta, and the major transcript was estimated to be ~6.5 kb long. (D) Paternal expression of *Peg10*. Paternal expression of *Peg10* in day 10 embryo, placenta, yolk sac, and neonatal brain is demonstrated by direct sequencing of the RT-PCR products, by comparing the sequence profiles of genomic DNA containing B6 and JF1 alleles equally.



**Figure 3** Characterization of *Cas1* and *Neurabin*. (A) Expression profiles of *Cas1*. The RT-PCR products (30 cycles for *Cas1* and 25 cycles for the control *Gapdh*) using total RNA from a range of adult tissues (brain, skeletal muscle, spleen, kidney, liver, and lung) are shown. (B) *Cas1* expression in the embryonic stage. In the *Ddel* RFLP experiment combined with Hot-stop PCR using a radioisotope-labeled primer, the B6 and JF1 alleles yielded 102- and 670-bp fragments, respectively. Three experiments were carried out in the same samples and the average ratios between the B6 and JF1 alleles, and also between maternal and paternal alleles (M/P values), are shown below. Similar results were obtained in the tissues from different individuals (data not shown). (C) Northern blot profiles of *Neurabin* in mouse adult tissues. Strong expression is observed in the brain sample, whereas weak ubiquitous expression is observed in all other tissues. (D) Maternal expression of *Neurabin*. In the *Acil* RFLP analysis with Hot-stop PCR, the B6 and JF1 alleles yielded 240- and 100-bp fragments, respectively. Preferential maternal expression was observed in placenta (day 10) and yolk sac (day 10 and 13) samples. The gray boxes indicate clear maternal biases in reciprocal crosses.

is concerned, this hypothesis is unlikely, because *Peg10* is strongly expressed only in placentas, where the maternal preferential expression of these genes was observed; the genes showed completely biallelic expression in neonatal tissues, where *Peg10* expression is very low.

The second possibility is more likely, because several EST clones possessing different transcription start sites for *Neurabin* have been registered in GenBank (accession no. AW048064, BB617696, and BB639924). There are also several examples in which different promoters show different expression profiles depending on tissue and developmental stage. These include *Igf2* and human *PEG1/MEST* (paternal and biallelic), *Gnas*, *Nesp*, and *GnasXL* (paternal, maternal, and biallelic) and mouse *Meg1/Grb10* (maternal and paternal) and human *GRB10* (biallelic and paternal; Vu and Hoffman 1994; Peters et al. 1999; Kosaki et al. 2000; Li et al., 2000; Kobayashi et al., 2001; Hikichi et al. 2003). In such cases, intermediate types of expression should be observed where two different transcripts overlap. Further analyses to identify the precise promoters of these genes will be necessary to test this possibility.

It is also worth considering the third possibility, the relaxation of imprinting, because there is evidence for a change in the expression of imprinted genes associated with a change in DNA methylation in DMR regions in some diseases and cancers as a result of mutation or deletions. However, we do not know of a mechanism that relaxes imprinting functions in normal development without changes in DNA sequences.

In conclusion, the maternal bias in expression should be reflected by some imprinting-related mechanism, overlapping transcripts from different promoters showing different parental expression profiles, or the relaxation of imprinting.

There are two conserved imprinted regions in the mouse proximal chromosome 6 and the human syntenic region of the long arm of chromosome 7. In the mouse, the *Peg1/Mest* region is responsible for perinatal growth retardation, and the region examined in this work is responsible for early embryonic lethality when maternal duplication occurs. However, human maternal disomy of the chromosome 7 containing both imprinted regions is associated with the perinatal growth retardation known as Silver-Russell syndrome, but there is no evidence of early embryonic lethality (Preece et al. 1997). Therefore, two questions arise concerning the latter imprinted region: what gene(s) is responsible for early embryonic lethality in the mouse and why is no severe lethal phenotype observed in humans?

One simple possibility is the existence of a mouse-specific imprinted gene(s) that causes early embryonic lethality. We have confirmed that both human *PEG10* and mouse *Peg10* are paternally expressed in an all-or-none manner. It has been reported that a mutation in human *SGCE* on chromosome 7q21 causes myoclonic dystonia (OMIM no.159900), which is characterized by bilateral, alcohol-sensitive myoclonic jerks involving mainly the arms and axial muscles (Zimprich et al. 2001). The family trees of patients show paternal inheritance of this disease, indicating that human *SGCE* is also a paternally expressed gene. However, it is not known whether it shows completely monoallelic expression, as with mouse *Sgce*, or paternal expression with a maternal contribution. The imprinting status of the other four human homologs corresponding to the mouse maternally expressed genes, *Neurabin*, *Pon2*, *Pon3*, and *Asb4*, is not known. Therefore, analyses of these human homologs are required to elucidate phenotype differences between the human and mouse. In this study, we analyzed genes in a 1-Mb region near

*Sgce* and *Asb4*. It is also probable that there are other imprinted genes responsible for the mouse lethal phenotype downstream from *Asb4*.

Another explanation for the human–mouse phenotype discrepancy is the difference in the mechanisms producing human uniparental disomies and mouse uniparental duplications. It has been reported that some human chromosomal disomies arise from trisomies, because congenital chromosome mosaicism showing trisomy in placentas containing both paternal and maternal alleles is sometimes observed in human uniparental disomy patients. In contrast, mice with maternal duplication have been constructed by mating mice with Robertsonian translocations or reciprocal translocations at the same locus (see <http://www.mgu.har.mrc.ac.uk/imprinting/imprinting.html>). Consequently, all the tissues in the embryos and placentas possess two maternal alleles for the duplicated regions. Therefore, it is possible that the mouse early embryonic phenotype results from a placental abnormality, which is not apparent in the human case. In this case, *Sgce* and *Peg10* are most likely involved, because they show complete paternal expression and are strongly expressed in placentas. Gene targeting approaches to these genes are necessary to test this possibility.

In this report, we identified a primary DMR in the region where the first exons of *Sgce* and *Peg10* overlap. Twelve repeat sequences of a 29-bp fragment are located within a CGI just downstream from the *Peg10* first exon. It would be very interesting to determine whether this DMR regulates only the two paternally expressed genes or all the genes in the cluster, including the maternally expressed genes, because there are no apparent DMRs in this region. A gene-targeting study of the DMR will be very important for elucidating the mechanism regulating this imprinted region in mouse proximal chromosome 6.

## METHODS

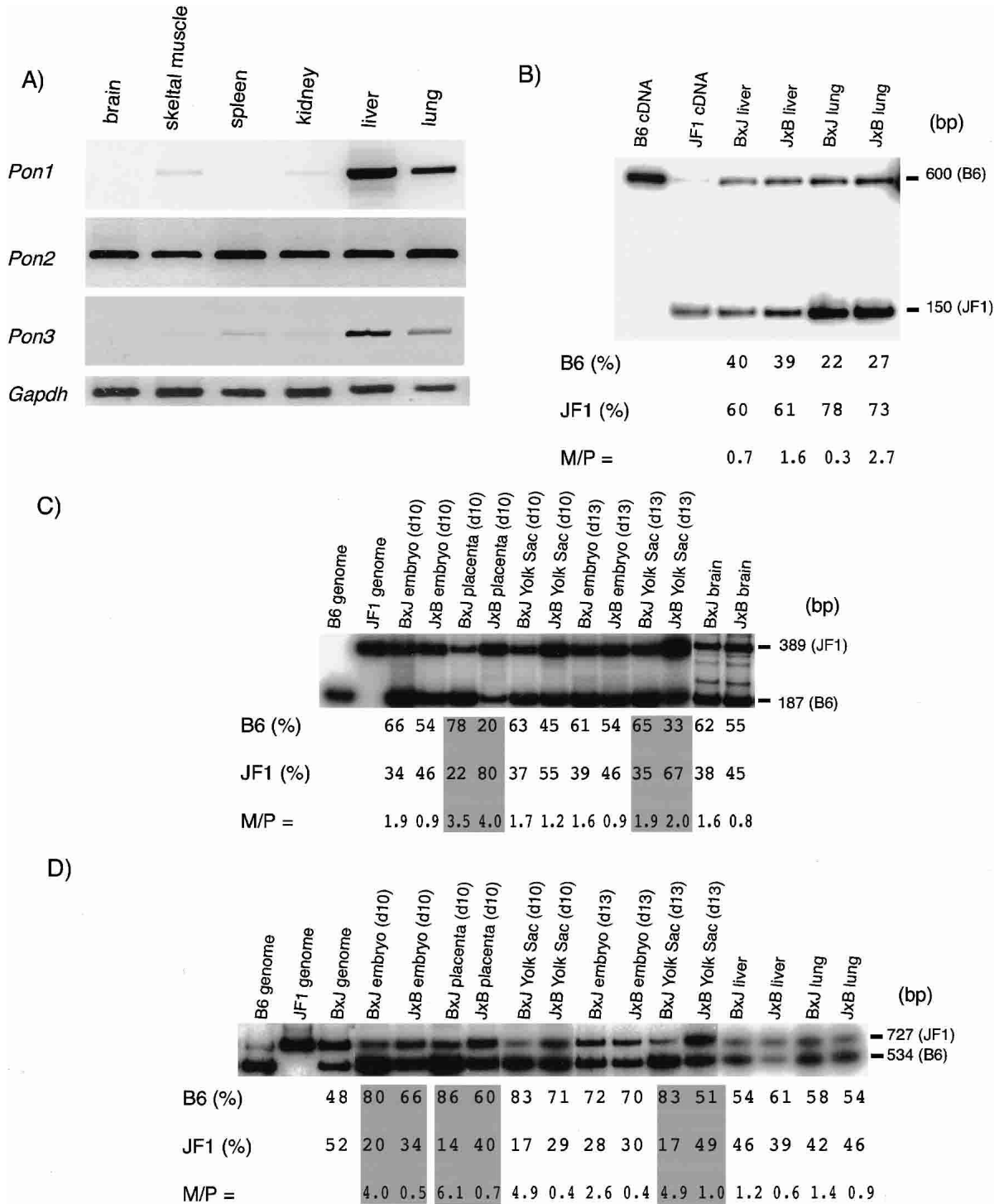
### Identification of Genes and CGIs From a 1-Mb Region Between *Colla2* and *Asb4*

The genomic sequence in the 1-Mb region between Mb 1.2 and 2.2 of *Mus musculus* WGS supercontig Mm6\_WIFeb01\_97 (GenBank accession no. NW\_000272) containing *Sgce* and *Asb4* was repeat-masked using Repeat Masker, and then used in a BLASTN search of dbESTs to identify transcripts. Five of the identified EST clusters were identical to mouse *Colla2* (GenBank accession no. NM\_007743), *Sgce* (NM\_011360), *Pon1* (NM\_011134), *Pon2* (NM\_008896), *Pon3* (NM\_008897), and *Asb4* (NM\_023048), and the rest were orthologs of human *Cas1* (NM\_022900), *PEG10* (NM\_015068), and rat *Neurabin* (NM\_053473). The mouse gene sequences (registered as *Peg10*: AB091827, *Neurabin*: AB091828, and *Cas1*: AB091829) were confirmed using RT-PCR and nucleotide sequencing. To identify CGIs, we ran the program Cpplot (<http://www.ebi.ac.uk/emboss/cpplot/>) with the following parameters: Observed/Expected ratio > 0.60, Percent C + Percent G > 50.00, Length > 300. Four CGIs were identified: *Cas1* CGI (1304815bp–1305668bp; NW\_000272), *Peg10-Sgce* CGI (1453104bp–1453415bp; NW\_000272), *Neurabin* CGI (1609519bp–1610544bp; NW\_000272), and *Pon2* CGI (2009459bp–2010036bp; NW\_000272) corresponding to the promoter regions of these genes.

### RT-PCR and 5'-RACE

Genomic DNA and total RNA were prepared from several tissues from the F<sub>1</sub> of (B6 × JF1), (JF1 × B6), and (B6 × C3H)





**Figure 4** Characterization of *Pon1*, *Pon2*, and *Pon3*. (A) Expression profiles of *Pon1*, *Pon2*, and *Pon3*. RT-PCR products (30 cycles for *Pon1*, *Pon2*, and *Pon3*, and 25 cycles for *Gapdh*) from the same adult tissues used in Fig. 3A are shown. *Pon2* was expressed ubiquitously, whereas *Pon1* and *Pon3* expression were observed mainly in liver and lung. (B) Biallelic expression of *Pon1*. In the *MspI* RFLP experiment with Hot-stop PCR, the B6 and JF1 alleles yielded 600- and 150-bp fragments, respectively. The biallelic expression patterns were unchanged between reciprocal crosses, although JF1 alleles were always expressed more strongly than B6 alleles. (C) Maternal expression of *Pon2*. In the *HaellI* RFLP experiment with Hot-stop PCR, the B6 and JF1 alleles yielded 187-bp and 389-bp fragments, respectively. Preferential maternal expression of *Pon2* was observed in placenta and to a lesser degree in day 13 yolk sac. (D) Maternal expression of *Pon3*. In the *PvuII* RFLP experiment with Hot-stop PCR, the B6 and JF1 alleles yielded 534-bp and 727-bp fragments, respectively. Maternal biases of both the B6 and JF1 alleles were observed in day 10 placenta and day 13 yolk sac and also observed in day 10 embryo to a lesser degree. Note that the B6 allele was expressed more strongly than the JF1 allele in all cases.



mice, using ISOGEN (Nippon Gene), as described previously (Kaneko-Ishino et al. 1995). cDNA was synthesized from 1  $\mu$ g of total RNA using Superscript II reverse transcriptase (Life Technologies) with an oligodT primer. Gene expression profiles were deduced by agarose gel electrophoresis of RT-PCR products with ethidium bromide (EtBr) staining. The primers used for the expression profiles were the same as those described in the next section. A SMART RACE cDNA Amplification Kit (Clontech) was used for 5'-RACE, following the manufacturer's protocol. RNA was prepared from B6 day 16 embryos, and the gene-specific primer used was GSP: 5'-GTGAGAGGGGCTTCACTCCCCTG-3'. The amplified DNA fragments were purified and sequenced directly.

### Allelic Analysis of Gene Expression Combined With Hot-Stop PCR

DNA polymorphisms in six genes between JF1 and C57BL/6 were detected in restriction fragment length polymorphism (RFLP) and single site polymorphism (SSP) analyses. For RT-PCR, 10 ng of cDNA in a 100- $\mu$ L reaction mixture containing 1  $\times$  ExTaq buffer (TaKaRa), 2.5 mM dNTP mixture, primers, and 2.5 U of ExTaq (TaKaRa) was subjected to 30–35 PCR cycles. In the case of *Pon3*, we performed nested PCR and added 10 PCR cycles in the second amplification. PCR was carried out on a Perkin Elmer GeneAmp PCR system 2400 under the following cycle conditions: 96°C for 15 sec, 65°C for 30 sec, and 72°C for 30 sec. The final cycle of PCR was performed in the presence of a primer labeled with [ $\gamma$ -<sup>32</sup>P] ATP. The PCR product was digested with the appropriate restriction endonuclease and electrophoresed on a 10% polyacrylamide gel. The intensity of the PCR products was measured with a BAS2000 Bioimaging Analyzer (Fuji Film). Experiments were carried out three times and average ratios were shown. Errors in observed values were within 5% in all cases except J  $\times$  B yolk sac (day 13) of *Cas1*. The following primers were used for DNA amplification: *Cas1*, 5'-AGCAGAGTGTAACGAACTCCAC-3' and 5'-CACAGTGGACGGGTGAATGTGC-3' \*; *Neurabin*, 5'-ACTCTCCTGCCGAGGCTG-3' and 5'-CAGTTTCAGGGGCTCTCACT-3' \*; *Peg10*, 5'-GGGTAGATAATCATAAGTATTTTGGGC-3' and 5'-CAACATTCTAAACTTTATTCCAGCAAC-3'; *Pon1*, 5'-ACAAGAACCATCGGTCTTCC-3' \* and 5'-CCTTCTGCTACACCTGGAC-3'; *Pon2*, 5'-ACGAGCTCCTTCCAAGTGTG-3' and 5'-ACCTCTGATGCAGGAGGATG-3' \*; *Pon3* (the first amplification), 5'-TCAGAAGTACTACGCATCCAGG-3' and 5'-CATGGCTGAAGGTAAGTCTCC-3'; *Pon3* (the second amplification), 5'-GAACAACGGCTCTGTGCTTC-3' \* and 5'-ATGCACCAAGCTAGCTGATG-3'. Asterisks indicate labeled primers with [ $\gamma$ -<sup>32</sup>P] ATP used in the last PCR cycles.

For RFLP analysis of *Cas1*, *Neurabin*, *Pon1*, *Pon2*, and *Pon3*, the PCR products were digested with *DdeI*, *AccI*, *MspI*, *HaeIII*, and *PleI*. For SSP analysis of *Peg10*, the PCR product was sequenced on an ABI 3100 sequencer using Big-Dye terminator chemistry (Applied Biosystems).

### Methylation Analyses of Embryos, Placentas, Eggs, and Sperm

Genomic DNA and RNA were isolated from both day 10 embryos and placentas, as well as eggs and sperm, using ISOGEN, as described in the RT-PCR section. Purified genomic DNA was treated with a sodium bisulfite solution, as described previously (Raizis et al. 1995). During this process, cytosine was converted to uracil, except methylated cytosine. The sodium bisulfite-treated DNA was amplified with following the primers: *Peg10-Sgce* CGI, 5'-GTAAAGTGATTGGTTTGTATTTTAA GTG-3' and 5'-TTAATTACTCTCCTACAACCTTCCAAATT-3'; *Cas1* CGI, 5'-GTTTAGGTAGTTGTTAGTTTATTTGGGTA TAG-3' and 5'-CCTCCTAATAACCTCCTACCTTAATAAC-3'; *Neurabin* CGI, 5'-GGTGTTTTGGTATTAGGTTAGATTG-3'

and 5'-ATAAACACCCTCCCCTCTCC-3'; *Pon2* CGI, 5'-AGTGTTTAGGTTTGGTGGAGTG-3' and 5'-CCCAAACCTA AACTAATTAATAAAAACTCC-3'.

The DNA fragments were amplified using ExTaq (TaKaRa) for 35–40 cycles under the following cycle conditions: 96°C for 15 sec, 60°C for 30 sec, and 72°C for 1 min. The amplified fragments were cloned into plasmids and sequenced. DNA polymorphisms in *Peg10* promoter region (T/A; B6/JF1;1453020 bp; NW\_000272) and DNA polymorphisms in *Cas1* promoter region (A/G; B6/JF1;1304769 bp; NW\_000272) and in *Pon2* promoter region (T/G; B6/JF1; 2009669 bp; NW\_000272) were used to determine paternal and maternal alleles of *Cas1* CGI and *Pon2* CGI, respectively.

### Northern Blot Analysis

To analyze *Peg10* and *Neurabin* expression, we used membranes of numerous adult tissues in Northern blot (Seegene, Korea) experiments. The 3' part of *Peg10* was amplified with primers 5'-GGGTAGATAATCATAAGTATTTTGGGC-3' and 5'-CAACATTCTAAACTTTATTCCAGCAAC-3' and *Neurabin* was amplified with primers 5'-ACTCTCCTGCCGAGGCTG-3' and 5'-CAGTTTCAGGGGCTCTCACT-3', and used as a DNA probe. Hybridization was performed at 42°C in Ultrasensitive Hybridization Buffer (Ambion) for 18 h. The membrane was then washed with a solution containing SSC and 0.1% SDS at 42°C to a final stringency of 0.1  $\times$  SSC.

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