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Imprinting of an evolutionarily conserved antisense transcript gene *APeg3*

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Abstract

APeg3 is an antisense transcript gene of *Peg3*, which has been recently identified from rat brain. Careful analyses of EST databases indicated that a homologous transcript also exists in other mammalian species, including mouse, cow and human. 5′- and 3′-RACE experiments have subsequently identified a 900-bp cDNA sequence of *APeg3* from mouse brain. Mouse *APeg3* is localized in the 3′UTR of *Peg3* with an intronless genomic structure. The expression of mouse *APeg3* is derived mainly from the paternal allele, indicating the imprinting of this antisense transcript gene in brain. Strand-specific RNA analyses also revealed the expression of both human and cow *APEG3* in adult brains. In sum, our study confirms that the mammalian *PEG3* locus harbors an antisense transcript gene displaying paternal allele-specific expression, and the evolutionary conservation further suggests potential roles of this transcript gene for the function of this imprinted domain.

Keywords

genomic imprinting; antisense transcript; APeg3; Peg3

1. Introduction

The two parental alleles of certain mammalian genes are not functionally equivalent due to genomic imprinting, a process by which one allele becomes epigenetically inactivated based on parental origin. About 80 imprinted genes have been isolated from human and mouse (http://www.mgu.har.mrc.ac.uk/imprinting/imprin-ref.html#impregs), and most imprinted genes are involved in controlling either fetal growth rates or animal nurturing behaviors (Tilghman, 1999). Some imprinted genes are expressed in both directions, producing sense and antisense transcripts. Also, a number of imprinted genes are transcribed without protein-coding capability (Pauler and Barlow, 2006). According to the results from several studies, these non-coding transcripts may have regulatory roles for maintaining the imprinting of a given domain (Pauler et al., 2007). Some of the well-known antisense transcript genes with this regulatory function include *Air* (antisense Igf2r RNA), *Kcnq1ot1* (Kcnq1 overlapping transcript 1) and *Tsix* (X-inactive specific transcript, antisense) (Pauler et al., 2007).

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Mouse chromosome 7 (Mmu7) contains three separate imprinted domains that are located in the proximal, central and distal regions of the chromosome (Searle and Beechey, 1990; Beechey and Cattanach, 1996). *Peg3* (paternally expressed gene 3) was the first imprinted gene identified in the proximal domain (Kuroiwa et al., 1996) and five additional imprinted genes have subsequently been isolated from the surrounding genomic region (Kim et al., 1997; Kim et al., 1999; Kim et al., 2000a; Kim et al., 2000b; Kim et al., 2001; Kim et al., 2004). These include the paternally expressed genes *Usp29* (ubiquitin-specific processing protease 29) and *Zfp264* (Kim et al., 2000b; Kim et al., 2001), and the maternally expressed genes *Zim1* (imprinted zinc-finger gene 1), *Zim2* and *Zim3* (Kim et al., 1999; Kim et al., 2004). Recent comparative genomic studies have indicated that this domain structure has been well preserved throughout mammalian evolution (Kim et al., 2004; Kim et al., 2007). However, these studies also revealed some species-specific changes that have occurred within this imprinted domain. Several imprinted genes have lost their protein-coding capability and become non-coding transcripts, which include *Zim2*, *Zim3* and *Zfp264* in mouse and *Usp29* in cow (Kim and Stubbs, 2005). In particular, maternally expressed *Zim3* has become an antisense transcript gene to a neighboring paternally expressed *Usp29* in mouse (Kim et al., 2001). Recently, an independent study has also identified another antisense transcript gene called *APeg3* (Peg3, antisense) in the rat brain (Glasgow et al., 2005). The rat *APeg3* gene is located in the 3′UTR (untranslated region) of *Peg3* and it is transcribed in the opposite direction from the *Peg3* transcription. The protein-coding capability of *APeg3* has been suggested, but is still controversial (Glasgow et al., 2005). The imprinting status and evolutionary conservation of *APeg3* in mammals are largely unknown. Thus, we have sought to determine these unknown aspects of *APeg3* in the current study.

2. Materials and Methods

2.1. Primer extension analysis

Total RNAs were isolated from mouse tissues including brain, liver, kidney and ovary using the Trizol RNA isolation kit (Invitrogen). APeg3 antisense primer (mAPeg3-7, 5′-CGTGTGTCTTTGAATCCTGGC -3′) was labeled with γ-^{32}P ATP and then purified by phenol/chloroform extraction. Total RNAs (10 μg) were mixed with the labeled primer and incubated at 65°C for 10 minutes (mins). The mixture was slowly cooled down and was reverse transcribed using the SuperScript First-Strand Synthesis System (Invitrogen). The prepared cDNA products were separated on a 6% acrylamide sequencing gel for 1 hour at 500 Voltage. The gel was exposed to X-ray film for 1 hour to visualize the results.

2.2. Rapid Amplification of cDNA Ends (RACE)

For the 5′-RACE of mouse *APeg3*, we first isolated the cDNA fragment derived from the primer extension experiment described above. One acrylamide gel piece containing the cDNA was dissolved in sterilized water and boiled for 15 mins. After centrifugation (5 mins at 13,000 rpm), the supernatant was transferred to a new tube and precipitated with 100% ethanol. The 5′-end of the purified cDNA was further modified by a tailing reaction using dGTPs and the terminal deoxytransferase (New England Biolabs). The tailed cDNA was amplified using two primers: the tail long primer (5′-GGTTGTGAGCTCTTCTAGATCCCCCCCCCCCCNN-3′) and the APeg3 anitsense primer 1 (5′-GCCAGGATTCAAAGACACACG-3′). The amplified DNA was re-amplified with a set of nested primers: the tail out primer (5′-GGTTGTGAGCTCTTCTAGA-3′) and the APeg3 antisense primer 2 (5′-GAATCCTGGCTGTGTGGAAAC-3′). The PCR products were subcloned into the TOPO TA vector for sequencing (Invitrogen).

For the 3′-RACE of mouse *APeg3*, total RNAs (5 μg) purified from mouse brains were first reverse transcribed with the SuperScript First-Strand Synthesis System (Invitrogen) using the

RoRiTn primer (5′-AAGGATCGGATTTGCGTAGCAACTTCGACTCACTATAGCGATTTTTTTTTTTTTTTTTTTNN-3′). The reverse-transcribed cDNA was amplified by two rounds of PCRs using the following primer sets: the first round with the Ro primer (5′-AAGGATCGGATTTGCGTAGC-3′) and the APeg3 sense primer 1 (5′-GTAGGGATGGGTTGATTTG-3′), and the second round with the Ri primer (5′-AACTTCGACTCACTATAGCG-3′) and the APeg3 sense primer 2 (5′-TTGCTCTCTTCCTCCTCAGG-3′). These PCR products were subcloned into the TOPO TA vector for sequencing (Invitrogen).

2.3. Strand-specific RT-PCR

Total RNAs were isolated from the brains of mouse and cow using the Trizol RNA isolation kit (Invitrogen). These prepared total RNAs (5 μg) were reverse transcribed using the following strand-specific primers for *Peg3* and *APeg3*: mAPeg3-9, 5′-CAATCAGTCTCAAGGGGTC-3′ for mouse *Peg3* and mAPeg3-12, 5′-GCTAGAGTGAACATCTACATC-3′ for mouse *APeg3*; bAPeg3-3, 5′-GGTTGAGGGTCAGACAGGTG-3′ for cow *Peg3* and bAPeg3-12, 5′-GCTGTTTGAACCCTATCAG-3′ for cow *APeg3*. These cDNAs were further amplified using the following sets of specific primers: the APeg3 sense primer 2 and mAPeg3-12 for mouse *APeg3*; bAPeg3-11, 5′-CCACATACAGGAAAGGCTG-3′ and bAPeg3-13, 5′-GACTAACGGACTGGTTCCTC-3′ for cow *APeg3*. For human *APEG3*, the following two primers were used individually for initial strand-specific reverse transcription: 5′-GCAACCAATCAATCTGGGTCACA-3′ for *PEG3* and 5′-ATGAGTATTCCAGACTACAGA-3′ for *APEG3*. These cDNAs were used for RT-PCR with the following two primers: 5′-CTATCATGCCTACAGCTTCAC-3′ and 5′-CTGCAGAGGTACCTTACCTGGT-3′. All PCR reactions were performed using the Maxime PCR premix kit (Intron Biotech).

2.4. Imprinting test

For the imprinting test of mouse *APeg3*, we used brain tissues derived from the F1 offspring of the interspecific crossing between *Mus musculus* (C3H) and *M. spretus*. One sequence polymorphism (C to T change in *M. spretus* at the nucleotide position 408 of GenBank accession No. **EF635411**) was used for differentiating two parental alleles. The cDNAs derived from F1 brains were first amplified with two primers, the APeg3 sense primer 2 and mAPeg3-12. The amplified products were digested with the restriction enzyme *Sal*I, which can differentiate the above polymorphism (New England Biolabs). The digested PCR products were separated on 2.0% agarose gels and visualized with the Ethidium Bromide staining.

3. Results

3.1. Analyses of ESTs for mammalian APeg3

Mouse EST (Expressed Sequence Tag) databases were carefully analyzed to determine whether the mouse *Peg3* locus also harbors an antisense transcript similar to the one in the rat (Glasgow et al., 2005). We used the Model Maker program within the Mapviewer browser (http://www.ncbi.nlm.nih.gov/mapview/) and the Blast program to tabulate all the EST matches that belong to a 3-kb genomic region corresponding to the 3′UTR of mouse *Peg3* (Fig. 1). All the identified ESTs (629 entries) were subsequently separated by transcriptional direction. As shown in Fig.1, the majority of ESTs (550 entries) showed an identical transcriptional direction to that of *Peg3*, indicating that these cDNAs are part of the *Peg3* transcripts. The genomic locations of these ESTs are spread throughout the entire 3′UTR of *Peg3*. However, a small fraction of the transcripts (79 entries) appear to have the opposite transcriptional direction relative to that of *Peg3*. The major fraction of these transcripts (59/79)

are all derived from a 1-kb genomic interval located in the 5′-side of the 3′UTR. The minor fraction (20 entries) of these transcripts is also derived from the 3′-end (3.0-3.5kb interval) of *Peg3*′s UTR. This evidence suggests the presence of potential antisense transcripts within this 3-kb genomic interval. To rule out any artifacts stemming from mis-annotation of ESTs, we have also analyzed 126 ESTs belonging to the coding region of mouse *Peg3*. However, none of these ESTs showed an antisense orientation.

We also repeated this series of analyses using human and cow EST databases. Like those in the mouse, the 3′UTRs of both human and cow *PEG3* also have a large number of EST matches, some of which appears to be transcribed in the opposite direction of *PEG3* (46/326 for human and 7/164 for cow). The identified ESTs from the two species also show similar patterns as the mouse ESTs in terms of their sense-to-antisense ratios and genomic positions within the 3′ UTR of *PEG3* (Fig.1B). This suggests that all three mammals analyzed most likely have antisense transcript gene(s) within the 3′UTR of *PEG3*. The location of the rat *APeg3* transcript corresponds to the major group of antisense direction ESTs that are localized in the 5′-side of the 3′UTR, which will be further analyzed in the following section.

3.2. Isolation of mouse APeg3

The observations described above prompted us to isolate cDNAs corresponding to the predicted antisense transcript. The relative expression levels of this antisense transcript is expected to be much lower than those of the sense transcript of *Peg3* based on sheer number differences between the two types of ESTs. Thus, we decided to employ strand-specific cDNA cloning schemes to avoid potential contamination problems stemming from the dominant, sense direction transcript of *Peg3*. First, we performed primer extension analyses using a set of sense direction oligonucleotides to target antisense transcripts. This experiment used the total RNAs isolated from four different tissues of one-month-old mice, including brain, liver, kidney and ovary. As shown in Fig.2A, one extended product was detected only in the brain RNA. This detected single-stranded DNA fragment was isolated from a gel and used for subsequent 5′-RACE experiment (described in Materials and Methods). Sequencing of several clones confirmed that the isolated single-stranded DNA was indeed derived from an antisense transcript gene *APeg3*. This also allowed us to map the 5′-end of this transcript as shown in Fig.2B. Another set of sense direction oligonucleotides were also used for 3′-RACE experiments, which subsequently mapped the two 3′-ends of *APeg3*. Sequencing of these cDNA clones resulted in the identification of a 900-bp cDNA sequence for mouse *APeg3* (GenBank accession No. **EF635411**).

Sequence inspection revealed that this cDNA sequence is co-linear without any interruptions when compared to the genomic region, indicating intronless genomic structure. The sequence of mouse *APeg3* cDNA codes for several ORFs (Open Reading Frame) with relatively short lengths, ranging from 20 to 34. However, none of these potential ORFs show reasonable sequence similarity with the ORF of rat *APeg3* (Glasgow et al., 2005). Sequence comparison analyses also indicated an overall 88% identity between the sequences of rat and mouse *APeg3* with many small insertions/deletions. This further supports the possibility that *APeg3* of rat and mouse most likely functions as a non-coding RNA gene. Sequence inspection also indicated that mouse *APeg3* does not contain ant repeat as well as inverted repeat. This is contracting to one inverted repeat region found in rat *APeg3*. Sequence comparison revealed that one small region of the transcribed region of *APeg3*, marked in red in Fig.2B, is relatively well conserved among different mammals, 80% sequence identity. Since this regions is immediate downstream of the STOP codon for *Peg3*, a sense transcript, the observed evolutionary conservation within this region might be selected for some unknown functions for *Peg3*, but not for *APeg3*. However, the functional significance of this region is not known at this moment.

3.3. Imprinting and brain-specific expression of mammalian APeg3

The imprinting of mouse *APeg3* was analyzed using the tissues derived from two different crosses: 1) the F1 hybrid offspring of an interspecific crossing between female *Mus musculus* (C3H) and male *M. spretus* and 2) the F2 backcross offspring between male *M. musculus* and female F1. Two oligonucleotides in sense and antisense directions were individually used for the reverse transcription reaction of total RNAs isolated from the brains of one-month-old mice. The two pools of cDNAs were subsequently used for PCR amplification (Lanes 2&4 in Fig.3A). The amount of the amplified product from the antisense-directed reverse transcription, which targeted the *Peg3* transcript (Lane 2 in Fig.3A), is much greater than that targeting *APeg3* (Lane 4 in Fig.3A). This is consistent with the expected expression level difference between the two transcripts. For the imprinting test, we identified one sequence polymorphism between two parental species, *M. musculus* and *M. spretus* (C versus T at the nucleotide position 408 in GenBank accession No. **EF635411**). The surrounding bases of this polymorphism are a recognition site for the restriction enzyme *Sal*I in *M. musculus*, but not in *M. spretus*. Thus, a restriction enzyme digestion with *Sal*I was used for the imprinting test of mouse *APeg3* (Fig.3B). As shown in Fig.3B, the amplified products derived from both *Peg3* and *APeg3* transcripts correspond to the *M. spretus* allele for the F1 hybrid offspring while the *M. musculus* allele for the F2 backcross offspring. This shows that the expression of *APeg3* is derived mainly from the paternal allele, which is the same parental allele specificity as the sense transcript *Peg3*. A series of different trials, from reverse transcription to restriction enzyme digestion, consistently indicated that *APeg3* was expressed paternally in the mouse brain.

We also performed a similar series of strand-specific RT-PCR using total RNAs derived from the F1 hybrid offspring of the interspecific crossing of female *Bos taurus* and male *B. indicus* to test the imprinting of cow *APeg3* (Fig.3C). The results derived from strand-specific RT-PCR showed a similar result as that seen in mouse *APeg3*. The cow *APeg3* expression was detected in adult brains, and was seen at much lower levels than the *Peg3* expression. However, due to the lack of available polymorphisms, we were not able to determine the imprinting of cow *APeg3*. We have also performed strand-specific RT-PCR for human *APEG3*, and we were able to confirm the presence of *APEG3* in total RNAs from brain thalamus and testis (Fig.3D). The detection of human *APEG3* in thalamus is somewhat consistent with the expression of rat *APeg3* in a very specialized cell type, magnocellular neurons of hypothalamus (Glasgow et al., 2005). In sum, this series of experiments confirm the expression of *APeg3* in the brain tissues of mouse, cow and human, and also the paternal allele-specific expression (imprinting) in mouse brain.

4. Discussion

The current study has identified an antisense transcript gene *APeg3* from mouse, and confirmed the existence of a homologous transcript in other mammals, including cow and human. The detection of this transcript in several mammals suggests the conservation of *APeg3* during mammalian evolution. This observed evolutionary conservation of *APeg3* is quite different from that seen in all the other non-coding (nc) transcripts found in the *Peg3* domain. In mouse, *Zim2*, *Zim3*, and *Zfp264* are expressed as ncRNA transcripts (Kim et al., 2001; Kim et al., 2004), while in cow *Usp29* is expressed as another ncRNA transcript (Kim et al., 2007). It is clear that all these ncRNA transcripts have been derived from protein-coding genes, and also that the conversion of protein-coding genes into these ncRNA genes may have occurred in relatively recent evolutionary times. By contrast, the location and antisense direction transcription of *APeg3* in the 3′UTR of *Peg3* clearly indicate that *APeg3* has always been an ncRNA gene from the time of its formation. Also, based on the detection of *APeg3* in several mammals, *APeg3* is thought to have been formed at a much earlier evolutionary time than any

other ncRNA genes in this domain. Nonetheless, *APeg3* appears to be the most evolutionarily selected ncRNA gene in this imprinted domain, which suggests significant functional roles played by this antisense transcript gene.

APeg3 shows several differences from the other well-known ncRNA genes of imprinted domains, such as *Air* and *Kcnq1ot1*. First, the genomic interval harboring *APeg3* is only about 1 kb in length and the entire locus is contained within the 3′UTR of another gene *Peg3*. In contrast, the genomic intervals harboring *Air* and *Kcnq1ot1* are usually greater than 100 kb in length and cover the entire genomic regions of the corresponding sense direction genes and sometimes even neighbor genes. Second, both *APeg3* and *Peg3* are expressed from the same paternal allele. This is also different from the reciprocal imprinting pattern that is usually observed from sense and antisense gene pairs within imprinted domains, such as maternally expressed *Igf2r*/ paternally expressed *Air* and maternally expressed *Kcnq1*/ paternally expressed *Kcnq1ot1*. These differences hint at a possibility that the functional roles played by *APeg3* be different from those by other antisense ncRNA genes. Both *Air* and *Kcnq1ot1* are known to play a regulatory role for the imprinting of their surrounding genes (Pauler et al., 2007). Apparently, a key component for this regulatory role is the process of transcription itself of the long genomic intervals of these antisense genes, as opposed to their transcription products, i.e. 'ncRNAs' (Pauler et al., 2007). Based on the much smaller genomic interval of *APeg3*, it is unlikely that *APeg3* is involved in a similar global imprinting control for its surrounding genes. Since *APeg3* is localized in the 3′UTR of *Peg3* and the *APeg3* transcript can base-pair with the *Peg3* transcript, it is more likely that *APeg3* is involved in post-transcriptional regulation of the *Peg3* transcript. This scenario remains to be studied in the near future, but is consistent with a growing number of protein-coding sense/ncRNA antisense gene pairs found in human and mouse genomes (Sun et al., 2005).

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Abbreviations

EST, expressed sequence tag; RACE, rapid amplification of cDNA ends; RT-PCR, reverse transcriptase-polymerase chain reaction; UTR, untranslated region; ORF, open reading frame.

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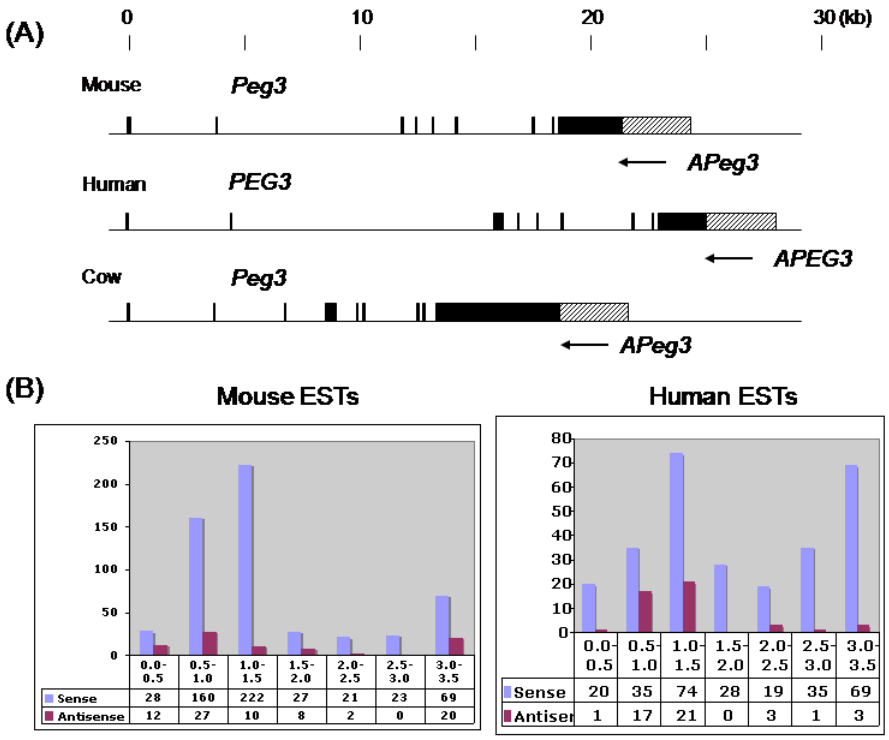

Figure 1. Genomic structures of *PEG3* and *APEG3* in mouse, human and cow

(A) The exons and 3′UTR of *PEG3* were represented by solid and hatched boxes, respectively. Arrows indicate the relative position and transcriptional direction of *APeg3*. **(B)** Distribution of sense and antisense direction ESTs within the 3′UTR of human and mouse *PEG3*. A large number of ESTs have been categorized based on their directions, sense (blue) and antisense (red), and their locations within the 3-kb UTR of *PEG3* (X-axis). The value on the Y-axis represents the total number of ESTs that belong to a given genomic interval (500-bp increment) within the 3′UTR of *PEG3*.

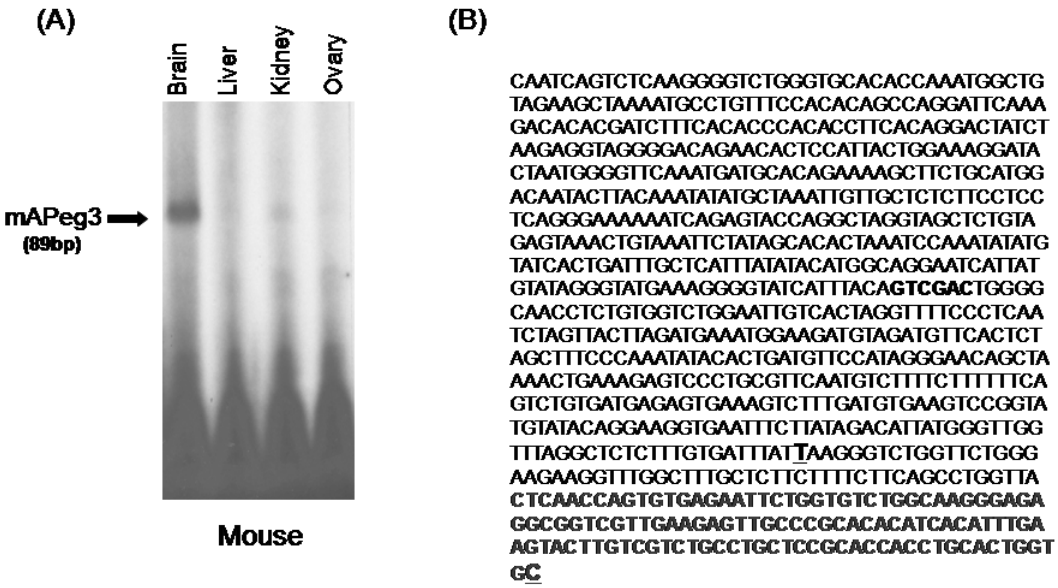

Figure 2. Expression and sequence structure of mouse *APeg3*

(A) Primer extension analysis of mouse *APeg3* expression using total RNAs from brain, liver, kidney and ovary. Total RNA of each tissue (10 μg) was first reverse-transcribed with a labeled sense strand-specific primer and separated on a 6% acrylamide gel. The picture shown above is the autoradiogram image that resulted from exposure of X-ray film to the acrylamide gel. The arrow indicates the extended product derived from mouse *APeg3*. The *APeg3* expression was detected only in brains. **(B)** Nucleotide sequence of the mouse *APeg3*. The conserved region among different mammals is shown in red. The two different polyadenylation sites are marked with underlines. The bold-typed region is the *Sal*I site that has been used for the imprinting test described in Fig.3.

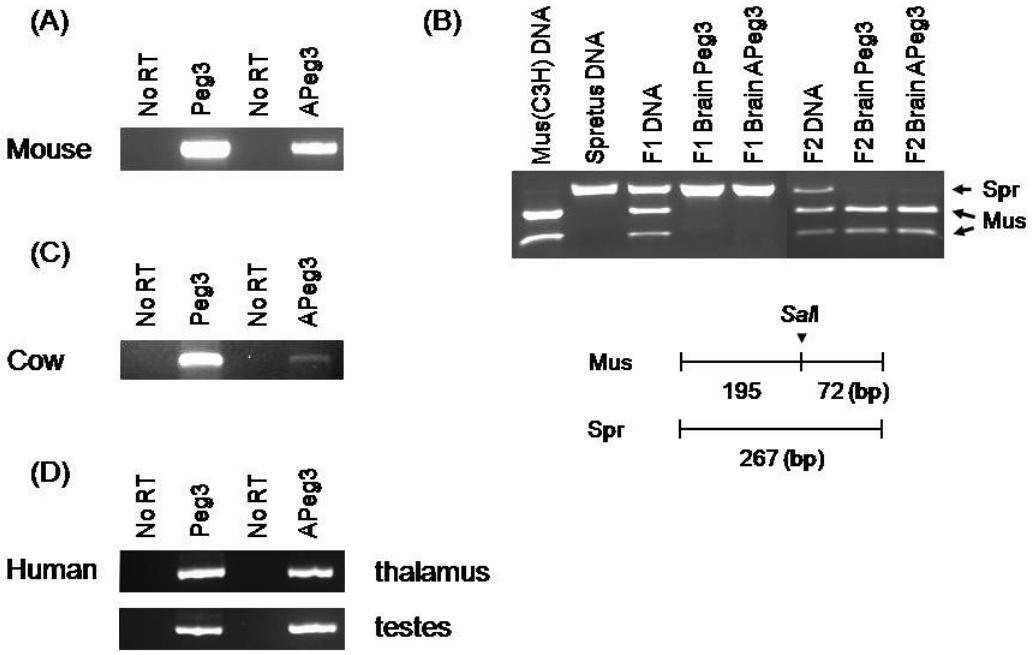

Figure 3. Strand-specific RT-PCR and imprinting test of *APeg3*

Total RNA (10 μg) from the brains of mouse **(A)** and cow **(C)**, and the thalamus and testes of human **(D)** was used for each individual RT-PCR reaction. Two reactions (Lanes 1&2) were reverse-transcribed with antisense direction primers, which target the *Peg3* transcripts, whereas the two remaining reactions (Lanes 3&4) were with sense direction primers, which target the *APeg3* transcripts. The two reactions (Lanes 1&3) were performed as negative controls without reverse transcriptase. **(B)** Paternal expression of *APeg3* in mouse brain. Strand-specific RT-PCRs were first performed using the total RNA from the F1 hybrid offspring of the interspecific crossing between female *M. musculus* (C3H) and male *M. spretus* (Lanes 4&5). The amplified PCR products were digested with *Sal*I to differentiate parental alleles. For the reciprocal imprinting test, the total RNAs from F2 offspring of the backcross between female F1 and male C3H were also used (Lanes 7&8). The diagram underneath the gel picture schematically represents *Sal*I-digested products from different parental alleles. PCR products from four genomic DNAs, including *M. musculus*, *M. spretus* and F1, were also digested as positive control reactions (Lanes 1-3 & 7).