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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).
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-32P 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′-GGTTGTGACGTCTCTTCTAGATCCCCCCCCCC-3′) and the APeg3 antisense primer 1 (5′-GCCCAGATTCAAAGACACACG-3′). The amplified DNA was re-amplified with a set of nested primers: the tail out primer (5′-GGTTGTGACGTCTCTTCTAGA-3′) and the APeg3 antisense primer 2 (5′-GAATTCTGGCTGTGGAAAAC-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
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′-GGTTGTTGTAACCCCTCATCAG-3′ for cow Peg3 and bAPeg3-12, 5′-GCTGTGTTGTAACCCCTCATCAG-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](http://www.ncbi.nlm.nih.gov/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 SalI, 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 SalI in M. musculus, but not in M. spretus. Thus, a restriction enzyme digestion with SalI 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).

**Acknowledgements**

We thank Jennifer M Huang for critical reading of our manuscript. This research was supported by National Institutes of Health R01 GM66225.

**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.

**References**


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 SalI site that has been used for the imprinting test described in Fig.3.
Figure 3. Strand-specific RT-PCR and imprinting test of ApG3

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 ApG3 transcripts. The two reactions (Lanes 1&3) were performed as negative controls without reverse transcriptase. (B) Paternal expression of ApG3 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 SalI 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 SalI-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).