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Genomic organization and imprinting of the *Peg3* domain in bovine

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Abstract

Using multiple mammalian genomic sequences, we have analyzed the evolution and imprinting of several genes located in the *Peg3* domain, including *Mim1* (approved name, *Mimt1*), *Usp29*, *Zim3*, and *Zfp264*. A series of comparative analyses shows that the overall genomic structure of this 500-kb imprinted domain has been well maintained throughout mammalian evolution but that several lineage-specific changes have also occurred in each species. In the bovine domain, *Usp29* has lost its protein-coding capability, *Zim3* has been duplicated, and the expression of *Zfp264* has become biallelic in brain and testis, which differs from paternal expression of mouse *Zfp264* in brain. In contrast, the two transcript genes of cow, *Mim1* and *Usp29*, both lacking protein-coding capability, are still expressed mainly from the paternal allele, indicating the imprinting of these two genes in cow. The imprinting of *Mim1* and *Usp29* along with *Peg3* is the most evolutionarily selected feature in this imprinted domain, suggesting significant function of these three genes, either as protein-coding or as untranslated transcript genes.

Keywords: Genomic imprinting; Evolution; Peg3 domain

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 70 imprinted genes have been isolated from human and mouse (http://www.mgu.har.mrc.ac.uk/imprinting/imprinref.html#impregs), and most imprinted genes are involved in either fetal growth or animal nurturing behaviors [1]. Some imprinted genes are expressed in both directions, producing sense- and antisense transcripts, and a number of imprinted genes are transcribed yet lack protein-coding capability [2]. Known imprinted genes are clustered in discrete chromosomal regions, indicating that genomic imprinting is controlled by long-range mechanisms affecting relatively large regions of chromosomes [3–5]. Several lines of evidence suggest that small DNA elements, termed imprinting control regions, (ICRs), may serve to regulate the imprinting of a whole domain and coordinate the expression of individual genes in each region. Several studies have indicated that a gene's imprinting status is dependent upon the "genomic context" of the locus, and changes that disrupt genomic context can alter or abolish imprinting of genes within an imprinted domain [6–8]. Consistently, the known imprinted domains are well conserved with regard to gene content and order between different mammalian species [9,10].

Three imprinted domains are located in mouse chromosome 7 (Mmu7), in proximal, central, and distal regions of the chromosome [11,12]. *Peg3* (paternally expressed gene 3)/*Pw1* was the first imprinted gene identified in the proximal domain [13,14], and five additional imprinted genes have subsequently been isolated from surrounding genomic regions. These include the paternally expressed genes *Usp29* (ubiquitin-specific processing protease 29) and *Zfp264* [15,16] and the maternally expressed genes *Zim1* (imprinted zinc-finger gene 1), *Zim2*, and *Zim3* [16–18]. These six imprinted genes are clustered in a 500-kb mouse region in the following order: telomere-*Zfp264-Zim3-Usp29-Peg3-Zim1-Zim2* centromere [20]. Preliminary data derived from other species, including human and cow, indicated that this domain structure has been well preserved throughout mammalian evolution. However, these studies revealed some species-specific changes within this imprinted domain. In particular, according to the studies of others and ours

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[15,21], the upstream region of *PEG3* harbors another gene localized in a head-to-head orientation to *PEG3*. In mouse, two transcripts, *Usp29* and *Ocat* [22], have been identified from this upstream genomic interval, about 250 kb in length (Fig. 1A). *Usp29* is composed of eight exons that are spread over a 250-kb genomic region whereas the three exons of *Ocat* are located within a relatively small 20-kb region immediate upstream of *Peg3*. *Usp29* and *Ocat* share their first two exons and thus correspond to two transcripts with different 3'-ends. By contrast, the homologous regions of other mammals may have different transcripts in this genomic interval, suggesting the presence of genomic changes in a potentially critical region for the imprinting of this domain.

To elucidate the evolutionary history of this imprinted locus and the imprinting regulation mechanisms, we have carried out comparative analyses of this 250-kb genomic region using sequences derived from human, mouse, and cow. Our results indicate that this genomic locus shows a remarkable structural conservation, however, each mammal species has also accumulated lineage-specific changes, including different exon structures and imprinting status of the resident genes. More detailed analyses and discussion are presented in the current study.

Results

Isolation of human and cow MIM1

Our previous study [15] identified two EST matches in the upstream region of human *PEG3* (GenBank Accession Nos. H80201 and H79292) and confirmed that these cDNAs are derived from an unknown gene distinct from human *USP29*, implying that the 250-kb upstream region of human *PEG3* is occupied by at least two separate genes (Fig. 1A). To isolate the transcripts of this unknown gene, we have performed a series of 5'- and 3'-rapid amplification of cDNA end (RACE) experiments with adult testis RNAs. These experiments identified a 1.3 kb transcript (GenBank Accession No. EF110915). The sequence of this transcript does not code for an open reading frame (ORF) and is composed of two exons that are spread over a 7-kb genomic region. Inspection of this cDNA sequence revealed that the first and second exons contain a 120-bp-long LINE1 element and 260-bp-long MER1 repeat sequences, respectively. Thus, this gene was named *MIM1* (MER1 repeat-containing Imprinted transcript 1, approved name *MIMT1*). Sequence comparison of human *MIM1* with the 5'-side exons of mouse *Usp29* revealed that the 120-bp LINE1 and 260-bp MER1 sequences are also found in the first and third exons of mouse *Usp29*, respectively. This indicates the evolutionary conservation of two repeat-containing regions, as part of transcripts, in both species (Fig. 3A).

A series of cDNA cloning experiments was also conducted using the 60-kb genomic sequence derived from the upstream region of cow *Peg3* [18] (GenBank Accession No. AC073666). We have identified three cow EST matches that belong to this genomic region (GenBank Accession Nos. DT852251, DT859167, DR712777) and subsequently sequenced the entire cDNA portions of two of these EST clones (DT852251, DT859167). One remaining EST clone (DR712777) could not be analyzed due to a large deletion in its cDNA region. Sequencing of these clones derived a 1.3-kb cDNA sequence (GenBank Accession No. EF110916). Inspection of this sequence

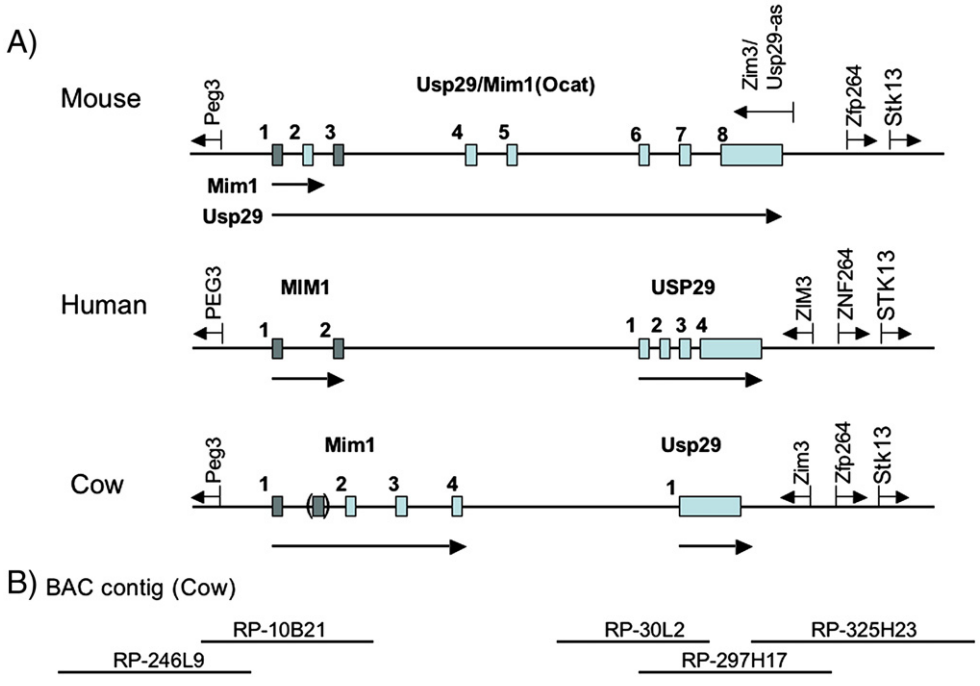

Fig. 1. Genomic organization of the *MIM1-USP29* intervals of mouse, human, and cow. (A) The exons of *MIM1* and *USP29* are depicted by boxes with numbers. The transcriptional direction of each gene is indicated by an arrow. Conserved *MIM1* exons are indicated by a darker color. The second conserved region in cow is not transcribed and is thus marked by parentheses. (B) Two cow BAC contigs covering the *Peg3* domain are shown with five representative BACs.

indicated that cow *Mim1* is made of four different exons that are spread over a 70-kb genomic distance (Fig. 1A). The 3'-ends of the two EST clones also contain polyadenylation signals and poly(A) tails, suggesting that these two EST clones were derived from a full-length mRNA of cow *Mim1*. Our detailed examination also revealed that exons 2 and 3 are alternatively spliced between these two EST clones, resulting in two forms of cDNAs: EST DT852251 with an 1-2-4 exon combination and EST DT859167 with an 1-3-4 exon combination. As seen in human and mouse *MIM1*, cow *Mim1* also contains a 120-bp LINE1 sequence in its first exon. The 260-bp MER1 repeat sequence is also detected in a similar genomic position, 10 kb upstream of cow *Peg3*, but this region was not detected as part of the transcribed region of cow *Mim1*. In addition to the two repeat regions, the first exon of *MIM1*, 600 bp in length, is overall well conserved with sequence similarity ranging from 69 to 74% among three different species.

Several rounds of RT-PCR were also performed to test whether cow *Mim1* shares some exons with *Usp29*, as seen in mouse. However, our attempts did not yield any data supporting this possibility in cow (not shown). Therefore, it is most likely that the 250-kb genomic region of cow is occupied by two separate genes, *Mim1* and *Usp29*, which is more similar to the human region than to the mouse region (Fig. 1A). Overall, our cDNA cloning experiments identified a homologous transcript gene *MIM1* in the upstream region of *PEG3* in both human and cow. This transcript also appears to be an equivalent of mouse *Ocat* or *Mim1* based on similar sequence and genomic location.

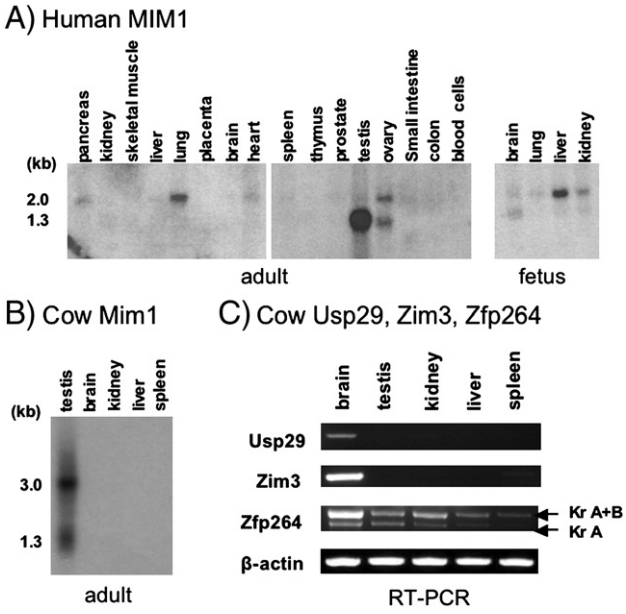

Fig. 2. Expression patterns of human *MIM1* and cow *Mim1, Usp29, Zim3*, and *Zfp264*. (A) Northern blot analysis of human *MIM1* detected two different-sized transcripts, 1.3 and 2.0 kb in length. A smaller-sized transcript (1.3 kb in length) is highly expressed at adult testis. (B) Northern blot analysis of cow *Mim1* also detected two different-sized transcripts, 1.3 and 3.0 kb in length, at adult testis. (C) RT-PCR analyses of cow *Usp29*, *Zim3*, and *Zfp264* expression. RT-PCR analyses demonstrate expression of cow *Usp29* and *Zim3* at adult brain. The expression of cow *Zfp264* was detected ubiquitously with high levels at adult brain. Two alternatively spliced forms of *Zfp264* transcripts were detected: Kr A +B and Kr A forms.

Expression patterns of human and cow MIM1

The expression patterns of human *MIM1* were analyzed with Northern blot analyses using one probe derived from the second exon of *MIM1*. As shown in Fig. 2A, two different-sized transcripts, 1.3 and 2.0 kb in length, were detected in several human tissues. The 1.3-kb transcript of *MIM1* was detected in adult testis with very high levels of expression. Relatively low levels of expression were also detected in ovary and in fetal brain and kidney. In contrast, the expression of the 2.0-kb transcript was detected more ubiquitously in several tissues of adults and fetus but with much lower levels than those of the smaller-sized transcript. It is important to note that the germ-cell-specific expression pattern of the smaller-sized transcript is somewhat similar to the patterns observed in human *PEG3* [23]. This agrees well with the fact that human *PEG3* and *MIM1* share a bidirectional promoter (Fig. 1A).

The expression patterns of cow *Mim1* were similarly analyzed with Northern blotting using poly(A)+ RNAs derived from adult tissues (Fig. 2B). One probe derived from the first exon of cow *Mim1* detected two different-sized transcripts, 1.3 and 3.0 kb in length, but only in adult testis. We have not been able to detect the expression of cow *Mim1* in other adult tissues. This is consistent with the fact that two of the three EST clones were derived from adult testis. In both human and cow *MIM1*, the sizes of the reported cDNAs in the current study correspond to the smaller-sized transcripts. The larger-sized transcripts of both human and cow *MIM1* are currently being investigated in the lab.

Isolation of cow Usp29, Zim3, and Zfp264

Several imprinted genes within the *Peg3* domain are part of the zinc finger gene families that have undergone many lineage-specific changes during recent evolutionary times [24–26], which could be problematic for isolating orthologous sequences from different species. To avoid this problem, we have used two non-zinc-finger genes, *Usp29* and *Stk13* (approved name, *Aurkc*), as primary mapping probes for isolating imprinted genes from the cow genome. We screened cow BAC libraries generated from *Bos taurus* using a conserved probe derived from cow *Stk13* and subsequently constructed one genomic contig that is represented by three BAC clones (Fig. 1B). Among these BAC clones, two BACs have been sequenced at the draft stage at the Joint Genome Institute of DOE (http://genome.jgi-psf.org/). Our initial examination of these sequences confirmed that these BACs indeed contain cow *Stk13* and *Zfp264*. We also confirmed the presence of cow *Usp29* and *Zim3* in an adjacent BAC, RP42-30L2, by performing individual subcloning and sequencing of the DNA fragments that had been hybridized with probes derived from human *USP29* and *ZIM3*.

According to our initial examination of the genomic sequences derived from the BAC contig, this genomic interval

of bovine has gene content and order very similar to those of human and mouse (Fig. 1A), indicating structural conservation of this imprinted domain during mammalian evolution. However, we have also observed several lineage-specific changes that are unique to the bovine genome. First, cow *Usp29* localized within BAC RP42-30L2 shows 73 and 67% sequence similarity to human and mouse *Usp29*, respectively (GenBank Accession No. EF110917). However, this bovine sequence contains many insertions/deletions compared to the sequences of human and mouse *Usp29* and thus appears to lack an ORF for ubiquitin-specific proteases (data not shown). These results suggest that cow *Usp29* may have lost an ORF capability in recent evolutionary times. Our RT-PCR analyses, however, revealed that the cow *Usp29* is still expressed in adult brain (Fig. 2C). Similar cases of protein-coding capability loss have often been observed among imprinted genes. Mouse *Zim2* and *Zim3* both have lost ORFs and, in particular, mouse *Zim3* has been converted into an antisense transcript gene of its neighbor *Usp29* (Fig. 1A) [16].

Second, cow *Zim3* located within the same BAC (RP42-30L2) as cow *Usp29* shows 76% sequence similarity to human *ZIM3*, indicating syntenic conservation of the two genes in the bovine genome. However, database searches with this cow sequence (GenBank Accession No. EF110919) identified another very similar sequence (XM_870718) in the bovine genome. This similar sequence shows 96–97% sequence identity to cow *Zim3*, suggesting that the genomic interval containing cow *Zim3* has been duplicated in recent evolutionary times. Interestingly, similar genomic duplications have also been observed in the surrounding regions of the *Peg3* imprinted domain in other species, such as multiple rounds of mouse *Zfp264* duplications [16]. Duplication of *Zim3* in cow and *Zfp264* in mouse is consistent with the nature of this zinc finger gene family, with recent formation in mammalian genomes [24–26]. According to our RT-PCR analyses, both cow *Zim3* and its duplicated copy are expressed, and major expression of these two sequences was detected in adult brain (Fig. 2C).

By contrast, cow *Zfp264* does not show any major species-specific changes (GenBank Accession No. EF110918; Fig. 3B). Cow *Zfp264* show 84 and 85% sequence similarity with human *ZNF264* at the nucleotide and protein sequence levels (data not shown). This zinc finger gene shows the most sequence similarity among the resident genes in the *Peg3* domain. Our expression analyses also indicated that cow *Zfp264* is expressed ubiquitously in adult tissues with the highest expression levels being detected at adult brain (Fig. 2C). According to our RT-PCR analyses, cow *Zfp264* is expressed in two different transcript forms due to alternative splicing, which involves the 5'-side, two exons of cow *Zfp264*. The larger form contains both of these two exons, which are predicted to code for KRAB (Kruppel-Associated Box) A and B domains. In contrast, the smaller form contains only one exon corresponding to the KRAB A domain. A similar alternative splicing involving KRAB A and B has often been detected in this type of zinc finger gene family [24–26]. In sum, our analyses indicate that the cow domain has maintained a gene content overall similar to those of human and mouse, suggesting structural conservation during mammalian evolution (Fig. 1A).

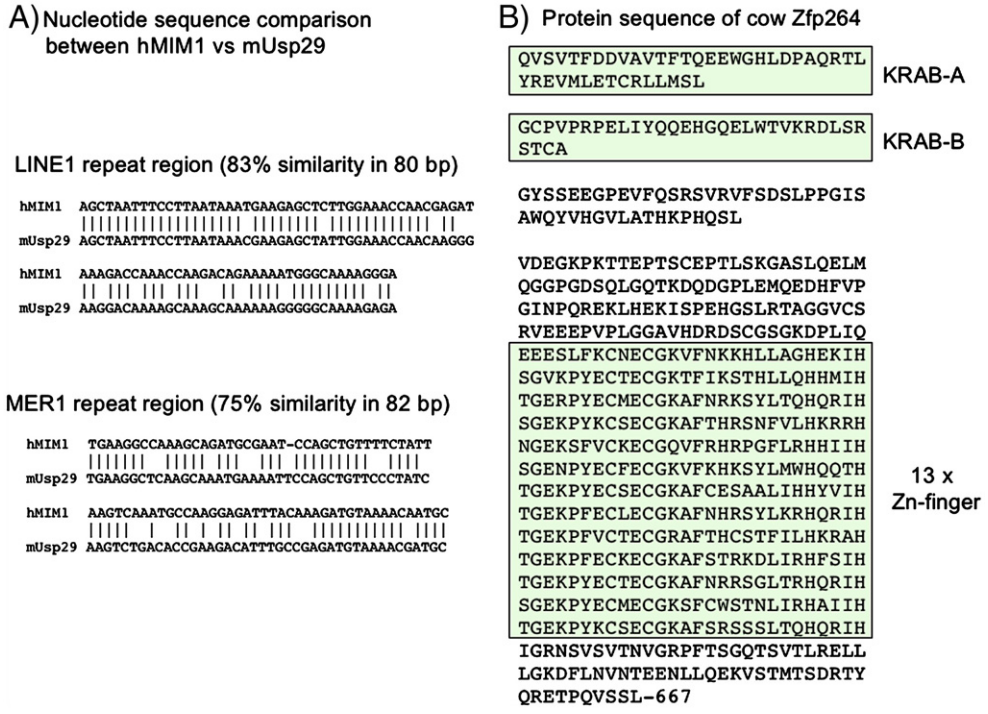

Fig. 3. Comparison of human *Mim1* and mouse *Usp29* (A) and protein sequence of cow *Zfp264* (B). (A) Conservation of two repeat-containing regions in human *MIM1* and mouse *Usp29*. The upper sequence represents human *MIM1* and the lower sequence mouse *Usp29*. Sequence similarities in the two species are shown inside of parentheses. (B) Protein sequence of cow *Zfp264*. Two Kruppel-associated boxes (KRAB-A and B) are indicated by boxes, and 13 zinc finger units are also boxed.

Imprinting tests of cow Mim1, Usp29, and Zfp264

The imprinting of cow genes was tested using tissues derived from hybrid animals of interspecific crossing between two bovine species, *B. taurus* and *Bos indicus*. The sequence polymorphisms in these two species were first identified and used for differentiating the parental alleles of each gene. For the imprinting test of cow *Mim1*, we have used the offspring of backcross between F1 hybrid males and *B. indicus* females. As shown in Fig. 4A, the expression of cow *Mim1* in testis is mainly derived from the paternal allele. The paternal allele expression of cow *Mim1* is consistent with the paternal expression of an immediate neighbor, *Peg3*. These results indicate the evolutionary conservation of imprinting of the immediate neighbor gene pair, *Peg3* and *Mim1*, in all three mammals [15,21,22].

The imprinting status of cow *Usp29* was also similarly tested using hybrid animal tissues (Fig. 4B). Since the *Usp29* expression was detected only in brains, we have used total RNAs derived from the brain of F1 hybrid (*B. indicus* males x *B. taurus* females) for this imprinting test. Cow *Usp29* expression was also mainly derived from the paternal allele. The paternal expression of cow *Usp29* in brain is also consistent with the paternal expression of mouse *Usp29* [15].

In the case of cow *Zfp264*, we have used brain and testis for assessing imprinting status. In both tissues, cow *Zfp264* was expressed equally from two parental alleles, indicating biallelic expression of *Zfp264* in cow. In contrast, the imprinting of *Zfp264* in mice was tissue specific: paternal expression of *Zfp264* was observed in brain but biallelic expression was evident in testis [16]. The imprinting status of *Zfp264*, therefore, does not appear to be strictly conserved between mouse and cow. However, since we have not analyzed exhaustively the imprinting status of cow *Zfp264*, it is possible that cow *Zfp264* might be still imprinted in other adult tissues or embryonic tissues. In the case of cow *Zim3*, we have not been able to confirm its imprinting status mainly due to the technical difficulties stemming from the presence of two very similar sequences in each species of two bovine genomes. In conclusion, our analyses revealed that the two bovine genes, *Mim1* and *Usp29*, are imprinted with paternal-specific expression.

Discussion

The current study has identified and analyzed the evolution and imprinting of several genes located in the *Peg3* domain, including *Mim1*, *Usp29*, *Zim3*, and *Zfp264*. According to the results, the overall genomic structure of this imprinted domain has been well maintained throughout mammalian evolution, consistent with the prediction that the bovine 500-kb domain may be controlled by shared controlling mechanisms similar to those in human and mouse. However, several lineage-specific changes have also been detected in the bovine *Peg3* region, including the loss of protein-coding capability for *Usp29*, gene duplication of *Zim3*, and biallelic expression of *Zfp264*. Despite these changes, the two transcript genes of cow, *Mim1* and *Usp29*, have maintained their imprinting throughout mammalian evolution irrespective of protein-coding capability, suggesting that the main function of these transcript genes is related to imprinting regulatory mechanisms for this domain.

In contrast to other well-known imprinted domains [3,9,10], the evolution patterns observed in the *Peg3* domain are very unusual in many ways. First, many genes in this domain have undergone very drastic changes as protein-coding genes, such as loss of protein-coding capability, as seen in cow *Usp29* and

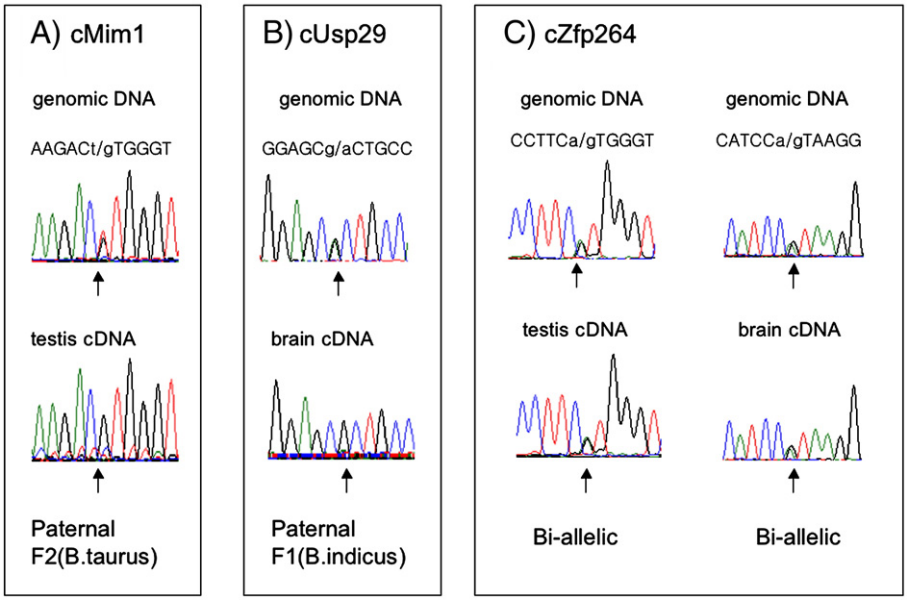

Fig. 4. Imprinting test of cow *Mim1, Usp29*, and *Zfp264*. The imprinting of cow genes was tested using either F1 or F2 offspring of the interspecific cross between *Bos indicus* and *B. taurus*. The PCR products derived from either genomic DNAs or cDNAs were isolated and sequenced. (A) Paternal expression of cow *Mim1*. The two polymorphisms (G or T) are shown as a mixed base in F2 genomic DNA, but the paternal allele (G) is detected only in cDNA from adult testis, indicating the paternal-specific expression of cow *Mim1*. (B) Paternal-specific expression of cow *Usp29* at brain. (C) Biallelic expression of cow *Zfp264* at both testis and brain.

in mouse *Zim2*, *Zim3*, and *Zfp264* [16,18]. In particular, mouse *Zim3* has become an antisense transcript gene to a neighbor gene *Usp29* [16]. We have also analyzed two additional genome sequences, rat and dog, to further validate our observations (Table 1). These results confirm again that most variations observed in this and previous studies [18] represent authentic, lineage-specific changes that have occurred during mammalian evolution. It is thought that the purpose of genomic imprinting in placental mammals is to control the dosage of a subset of genes that are involved in determining fetal growth rates and nurturing behaviors [27]. In this regard, losing protein-coding capability in several genes of the *Peg3* domain is very unusual and enigmatic. Second, most imprinted genes in this domain, except for *Usp29* and *Mim1*, are DNA-binding zinc finger genes, including *Peg3*, *Zim1*, *Zim2*, *Zim3*, and *Zfp264*. This gene family tends to be clustered in specific regions of chromosomes [24–26]. In fact, the 500-kb *Peg3* domain is part of and is surrounded by tandem arrays of zinc finger gene clusters [16]. The vast majority of this gene family is also known to have duplicated in recent evolutionary times. The recent formation of this gene family in mammalian genomes might have contributed to the species-specific variations observed in the imprinted zinc finger genes in the *Peg3* domain, such as loss of protein-coding capability and differences in gene content of species (Fig. 1A and Table 1; deletion of *Zim1* in human and cow). However, most of these imprinted zinc finger genes are still expressed without protein-coding capability but with imprinting, suggesting that these genes might have adapted into new roles, as transcript genes, for the imprinting of this domain.

The middle 250-kb genomic interval of the *Peg3* domain is relatively gene poor but has been well conserved among different mammals (Fig. 5). This structural conservation of the 250-kb gene-poor region is a stark contrast to the gene-rich surrounding regions that harbor most imprinted genes. In mouse, this large region is occupied by two transcripts, *Usp29* and *Mim1*. The current study revealed that the homologous regions of human and cow are also occupied by two genes, *MIM1* and *USP29* (Fig. 5). However, the detailed exon

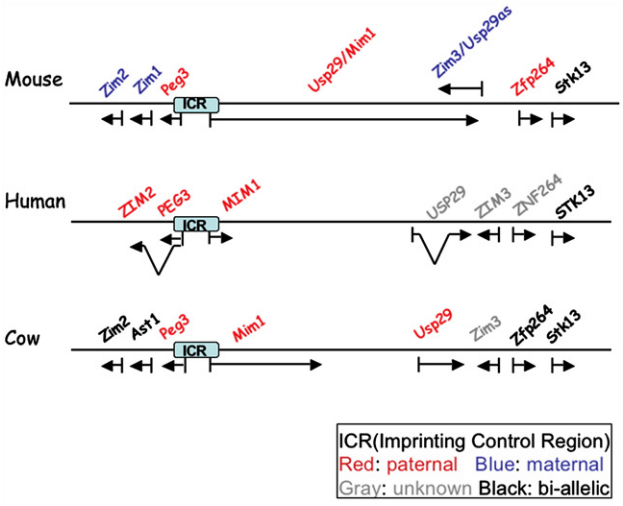

Fig. 5. Genomic structure and imprinting of mammalian *Peg3* domains. This diagram summarizes comparative analyses of mammalian *Peg3* domains. The transcriptional direction of each gene is indicated by an arrow. The imprinting status of each gene is indicated by the color of each name: red (paternalspecific), blue (maternalspecific), black (biallelic expression). The gray-colored genes are unknown with regard to their imprinting status. The 4-kb CpG island surrounding the first exons of *Peg3* and *Usp29* or *Mim1* is known to be differentially methylated between parental alleles and is predicted to be an imprinting control region (ICR) for this domain. This potential ICR is indicated by a box.

structure of *MIM1* and *USP29* shows several species-specific differences. In mouse, *Mim1* and *Usp29* share 5'-side exons, whereas this exon sharing has not been detected in either cow or human, in which *Mim1* and *Usp29* are expressed as separate units. This difference may be related to another lineage-specific variation detected in this domain, imprinting relaxation of the surrounding genes, as observed with cow *Zfp264* and *Zim2* (Fig. 5). According to the observations drawn from other imprinted domains that have similar large-transcript genes, such as *Air* and *Kcnq1ot1*, the disruption of these large genes' transcription tends to affect the imprinting of neighboring genes, suggesting a role of these large genes' transcription for imprinting regulation [2]. If this is the case, the transcription of

Table 1
Coding and imprinting status of the *Peg3* imprinted domain among mammals

Gene	Human	Mouse	Cow	Rat	Dog	Mammals[a]	Changes in animals[b]	References
OLFRs[d]	**absent**	coding	coding	coding	coding	coding	deletion in human	this
ZIM2	coding paternal	**noncoding** maternal	coding biallelic	**noncoding**	coding	coding	ORF lost in rodents	[18,19,30]
ZIM1	absent	coding maternal	absent	coding	coding	coding	deletion in human, cow	[17]
AST1	absent	absent	**noncoding biallelic**	absent	absent	absent	insertion in cow	[18]
PEG3	coding paternal	coding paternal	coding paternal	coding	coding	coding		[13,14,18,23]
MIM1	noncoding paternal	noncoding paternal	noncoding paternal	noncoding	noncoding	noncoding		[15,21,22,]
USP29	coding	coding paternal	**noncoding** paternal	coding	(gap)[c]	coding	ORF lost in cow	[15], this
ZIM3	coding	**noncoding** maternal	coding	**noncoding**	(gap)[c]	coding	ORF lost in rodents	[16], this
ZNF264	coding	**noncoding** paternal	coding biallelic	coding	coding	coding	ORF lost in mouse	[16], this
STK13[d]	coding	coding biallelic	coding	coding	coding	coding		[16], this

Boldface type: lineage-specific features; paternal, paternally expressed genes; materal, maternally expressed genes.
[a] Shared feature among mammals.
[b] Lineage-specific change.
[c] Gap in genomic contigs.
[d] Mammalian Peg3 domain is flanked by one OLFR (Olfactory Receptor) cluster and STK13 (Ser/Thr kinase 13).

the 250-kb genomic region of the *Peg3* domain might be similarly required for the imprinting of the *Peg3* domain. Losing this large transcriptional context and yielding two separate transcripts, *Mim1* and *Usp29* in cow, might have resulted in the imprinting relaxation of cow *Zfp264*. This also agrees well with the prediction that the 4-kb genomic region surrounding the first exons of *Mim1* and *Usp29* is a potential ICR for this domain. A disruption of interaction with an ICR, resulting from the establishment of individual transcripts for *Mim1* and *Usp29* in cow, might have affected the imprinting of downstream genes, such as *Zfp264*. To further confirm this mechanistic connection between the transcription of this 250-kb region and the imprinting status of neighbor genes, we need to analyze this region more thoroughly, including determining the imprinting status of human genes in this domain. Nevertheless, several variations observed through our comparative analyses of the *Peg3* domain provide a somewhat corroborating evolutionary demonstration for the regulatory roles of the large transcripts located within mammalian imprinted domains.

Materials and methods

Isolation and sequencing of cow BACs

Cow BAC libraries, RPCI-42, were screened using a series of P^{32}-labeled probes generated through the Overgo labeling method [28], and the isolated BAC were further analyzed with the restriction-finger printing method [28] to build BAC contigs for the cow *Peg3* domain. Of 30 isolated BAC clones, 3 BAC clones have been mainly used for analyzing cow imprinted genes in this study, including RP-30L2, RP-297H17, and RP-325H23. Two BACs, RP-297H17 and RP-325H23, have been sequenced at the draft stage at the DOE Joint Genome Institute. One remaining BAC, RP-30L2, has been used for individual subcloning and sequencing for the isolation of cow *Usp29* and *Zim3*.

cDNA isolation and sequencing

A 1.3-kb human *MIM1* cDNA has been amplified from a testis cDNA template (Human testis Marathon cDNA template; Clontech, Palo Alto, CA) by two rounds of 5′- and 3′-RACE experiments. The cDNA sequence for cow *Mim1* has been obtained through assembling the sequences of two EST clones, which were obtained from the IMAGE consortium at Lawrence Livermore National Laboratory. This assembled sequence has been later confirmed by a series of independent RT-PCR reactions. Cow genomic sequences for *Usp29* and *Zim3* were obtained through individual subcloning with the pBluescript vector. The genomic extent of the transcribed regions for *Usp29* and *Zim3* were confirmed by performing RT-PCR with the following primer sets: *Usp29* (cUsp29-1, 5′-CACGTGCCTGACCCAGCTACTTG-3′ and cUsp29-2, 5′-AGCTATAGCGTTTCAGATGGA-3′) and *Zim3* (cZim3-5, 5′-CCGAGCACA-GACAGTGTTC-3′ and cZim3-6, 5′-CTGTGCACCACAATACTGAG-3′). The genomic sequences for cow *Zfp264* was obtained from the draft stage BAC sequence of RP-325H23, and later the extent of the transcribed region was determined through RT-PCR using the following primer sets: *Zfp264* (cZfp264-1.1, 5′-CAGGTGTCTGTGACCTTTGATG-3′ and cZfp264-2, 5′-CTCTCCC-GACACTGGATAAC-3′).

Amplified RACE and RT-PCR products were separated and isolated from 0.8% agarose gels using a gel extraction column (QIAquick gel extraction kit; Qiagen, Valencia, CA). The isolated PCR products were subcloned into the TA cloning vector (TA cloning kit; Invitrogen, Carlsbad, CA). Subcloned cDNA fragments were sequenced from both directions using a fluorescence-based cycle-sequencing DNA sequencing kit (Dye terminator sequencing core kit; PE Applied Biosystems, Warrington, UK) and reactions analyzed on an ABI 377 automated sequencer. Sequence alignments and database searches were performed using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/).

Northern blot and RT-PCR analyses

The expression of human *MIM1* was analyzed with Northern blots containing fetal and adult tissue poly(A)+RNAs (Clontech). These blots were hybridized with one cDNA probe derived from the second exon of the gene. The expression of cow *Mim1* was similarly analyzed using poly(A)+ RNAs derived from several adult tissues. Detailed procedures and conditions for generating probes and for performing hybridizations were previously described [29]. The position information of these cDNA probes is available upon request.

The expression of cow *Usp29*, *Zim3*, and *Zfp264* was analyzed with RT-PCR using cDNA templates derived from different adult tissues (1 year old). For these RT-PCRs, we used the following oligonucleotides: the same primer set as described above for *Usp29* (c*Usp29*-1 and c*Usp29*-2), *Zim3* (cZim3-5, 5′-CACATTGAAGAGAAACCCTTTTGA-3′ and cZim3-6, 5′-GGTCAA-TGGCATTGGATTTCTG-3′), and *Zfp264* (cZfp264-3, 5′-GGCTTCTCC-CCGGTGTGGGTCT-3′and cZfp264-1.1). The detailed PCR conditions for these RT-PCRs are available upon request, but the annealing temperatures for all three genes were 60 °C.

Imprinting test

The imprinting (or monoallelic expression) of cow *Mim1*, *Usp29*, and *Zfp264* was tested with either F1 hybrid offspring produced by crossing *B. taurus* females with *B. indicus* male or backcross offspring produced by crossing of *B. indicus* females with F1 males. The sequences of oligonucleotides used for imprinting tests of cow genes are as follows: *Mim1* (cMim1-3, 5′-GCTAATAA-GACGATGGAAATTCT-3′ and cMim1-4, 5′-CTCTCCCCACACCACGT-CATC-3′), *Usp29* (cUsp29-1 and cUsp29-2), and *Zfp264* (cZfp264-1, 5′-TGCAGGCTCCTGATGTCTTTG-3′ and cZfp264-2). The RT-PCR products amplified with the above primer sets were purified using the gel extraction kit (QIAquick gel extraction kit; Qiagen) and used as a template DNA for sequencing.

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