Incorporating Mouse Genome
Mammalian Genome
Genes and Phenotypes

Conservation of genomic imprinting at the *XIST*, *IGF2*, and *GTL2* loci in the bovine

Scott V. Dindot,[1] Kathleen C. Kent,[1] Bret Evers,[1] Naida Loskutoff,[2] James Womack,[3] Jorge A. Piedrahita[4]

[1]Department of Veterinary Anatomy and Public Health, College of Veterinary Medicine, Texas A&M University, College Station, Texas 77843, USA
[2]Center for Conservation and Research, Henry Doorly Zoo, Omaha, Nebraska 68107, USA
[3]Department of Veterinary Pathobiology, College of Veterinary Medicine, Texas A&M University, College Station, Texas 77843, USA
[4]Molecular Biomedical Science, College of Veterinary Medicine, North Carolina State University, 617 Hutton St., Raleigh, North Carolina 27606, USA

Received: 7 April 2004 / Accepted: 20 August 2004

Abstract

Genomic imprinting is theorized to exist in all placental mammals and some marsupials; however, extensive comparative analysis of animals aside from humans and mice remains incomplete. Here we report conservation of genomic imprinting in the bovine at the X chromosome inactivation–specific transcript (*XIST*), insulin-like growth factor 2 (*IGF2*), and gene trap locus 2 (*GTL2*) loci. Coding single nucleotide polymorphisms (SNPs) between *Bos gaurus* and *Bos taurus* were detected at the *XIST*, *IGF2*, and *GTL2* loci, which have previously been identified as imprinted in either humans, mice, or sheep. Expression patterns of parental alleles in F1 hybrids indicated preferential paternal expression at the *XIST* locus solely in the chorion of females, whereas analysis of the *IGF2* and *GTL2* loci indicated preferential paternal and maternal expression of alleles, respectively, in both fetal and placental tissues. Comparative sequence analysis of the *XIST* locus and adjacent regions suggests that repression of the maternal allele in the bovine is controlled by a different mechanism than in mice, further reinforcing the importance of comparative analysis of imprinting.

Correspondence to: Jorge A. Piedrahita; E-mail: jorge_piedrahita@ncsu.edu

Genomic imprinting involves the parental control over expression of alleles of particular genes (Constancia et al. 1998). Genes affected by this rare form of allelic expression and repression, presumably 0.1%–0.2% of the total genes in the genome, are involved in a myriad of processes including fetal, placental, and neurological development (Allen et al. 1995; Reik et al. 2001). In humans, imprinted genes have been linked to a number of developmental disorders including Beckwidth–Weidemann, Prader–Willi, and Angleman syndromes, as well as a number of cancers (Falls et al. 1999). Approximately 50 imprinted genes have been identified in humans and 70 in the mouse (Surani 2001). In livestock, 11 imprinted genes have been identified in sheep (*GTL2*, *DLK1*, *DAT*, *PEG11*, *PEG1*, *MEST*, *MEG8*, *IGF2*, *H19*, and *IGF2R*) (Feil et al. 1998; Bidwell et al. 2001; Charlier et al. 2001; Young et al. 2001), 1 in cattle (*IGF2R*) (Killian et al. 2000), 2 in pigs (*IGF2* and *IGF2R*) (Jeon et al. 1999; Nezer et al. 1999; Killian et al. 2001), and none in horses or goats; although the differential phenotype exhibited between mules and hinnies is thought to be a consequence of genomic imprinting (Short 1997). In spite of the importance of imprinted genes in placental and fetal development, comparative imprinting studies are limited in scope and have failed to properly address the role imprinted genes play in placental speciation. Ruminants, with their unique form of placentation compared to humans and mice, and their ease of availability, would add valuable comparative information to the existing imprinting animal models.

Currently, the limitation of identifying imprinted genes in cattle is due to the lack of informative polymorphisms in coding regions. In mice

 DOI: 10.1007/s00335-004-2407-z • Volume 15, 966–974 (2004) •

and other species, a number of protocols have been implemented to facilitate the identification of imprinted genes including the use of parthenogenetic embryos, subtractive cDNA hybridizations assays, uniparental disomies (UPD), and interspecific hybrids (*Mus musculus* × *Mus spretus*) (Villar et al. 1995; Villar and Pedersen 1997; Feil et al. 1998; Hagemann et al. 1998). *Mus musculus* × *Mus spretus* and *Peromyscus polionotus* × *Peromyscus maniculatus* interspecific crosses of mice have been widely used to identify numerous imprinted genes and are ideal experimental models due to their high levels of heterozygosity within coding regions (Villar et al. 1995; Hemberger et al. 1998; Jong et al. 1999; Mayer et al. 2000; Schmidt et al. 2000; Yevtodiyenko et al. 2002), in spite of exhibiting parental-specific phenotypes in their offspring (Dawson 1971; Vrana et al. 1998; Hemberger et al. 1999; Vrana et al. 2000; Zechner et al. 2002).

Crosses between *Bos gaurus* (Gaur) and *Bos taurus* (domestic) cattle have been used previously to increase the genetic variation between alleles for genetic mapping purposes (Gao and Womack 1997; Yang and Womack 1997; Gallagher et al. 1998). Unlike other interspecific models, however, the gaur/taurus hybrid shows normal placentation and fetal development and survive to term with no apparent abnormalities (Gao and Womack 1997), making it an ideal model to study imprinting in the bovine. Here we demonstrate use of this interspecies model to analyze imprinting patterns in the bovine and to report similarities and differences between bovine, mouse, and humans with respect to imprinting at the *XIST*, *IGF2*, and *GTL2* loci. Further use of these hybrids will facilitate the analysis of other known imprinted genes as well as identify imprinted genes unique to the bovine.

Materials and methods

Identification of SNPs. Genomic DNA was extracted from male and female *Bos gaurus* and *Bos taurus* fibroblast cell lines using a DNA isolation kit (Promega, Madison, WI). Primers used to amplify the *XIST*, *IGF2*, and *GTL2* (Table 1) genes were designed using sequence obtained by performing BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) searches of the mouse *Igf2* (NM 010514) and sheep *GTL2* (AY017220, AY017221, and AY017222) cDNA sequences against bovine expressed sequence tag (EST) libraries in GenBank and from the published bovine *XIST* sequence (AJ421481 and NR001464). Fifty-microliter PCR reactions were run in duplicate and consisted of 5 μl 10× PCR buffer (Promega), 4 μl of 25 mM $MgCl_2$, 1.25 μl of 10 mM dNTPs, 2.5 μl of 3 μM forward primer, 2.5 μl of 3 μM reverse primer, 2 μl of 50 ng/μl DNA, and 1 μl Taq (Promega) PCR. All reactions were performed with cycling parameters of as follows: 94°C (5 min); 94°C (30 sec), 60°C (30 sec), 72°C (3 min) [10 cycles]; 94°C (30 sec), 60°C (30 sec), 72°C (3 min) [25 cycles]. Amplicons resulting from PCR were resolved on a 2% ethidium bromide (EthBr) agarose gel and gel purified using a Gel Purification Kit (Qiagen, Valencia, CA). Two to four microliters of purified product was used as template for sequencing reactions. Forward primers used to amplify regions were used as the sequencing primer. Sequencing reactions consisted of 25 cycles at 94°C (30 sec), 50°C (30 sec), 60°C (4 min). Sequences obtained for each of the genes from *Bos gaurus* and *Bos taurus* genomic DNA were aligned and analyzed for polymorphisms between sequences.

Generation of Bos gaurus/B. taurus hybrids. Heifers and mature (1.5–3 year old) Angus and Angus-cross cows were used to generate day-72 hybrid fetuses. Estrus was synchronized by serial injections of 25 mg Lutalyse (Pharmacia, Exton, PA) administered at 11-day intervals. Twelve hours (h) after detection of estrus, heifers were artificially inseminated with semen from a gaur bull. Heifers were then checked at day 28 of gestation for establishment of pregnancy using transrectal ultrasonography. At day 72 of gestation, hybrid fetuses were isolated. Weights and measurements were taken so as to monitor development of hybrid animals. Chorion, allantois, liver, lung, and brain samples were isolated and flash frozen in liquid nitrogen to preserve RNA and DNA.

RNA and DNA extraction. RNA was extracted from frozen samples utilizing the RNA aqueous kit according to the manufacturer's directions (Ambion, Austin, TX). Two micrograms of RNA for each sample was treated using the Ambion DNAse I kit and subsequently converted to cDNA through the Ambion First Strand Synthesis kit according to the manufacturer's directions. DNA was extracted from frozen tissues using the Wizard DNA Extraction kit according to the manufacturer's directions (Promega).

Analysis of allelic expression through direct sequencing method. RT-PCR of the *XIST*, *IGF2*, and *GTL2* loci was performed on samples obtained from chorion, allantois, liver, lung, and brain. Amplicons were resolved on 2% EthBr agarose gels, were gel extracted, resuspended in 50 μl of ddH_2O, and used directly as a sequencing template. Sequencing

Table 1. Primer sequences and positions of single nucleotide polymorphisms (SNPs) identified between *Bos gaurus* (*Bg*) and *Bos taurus* (*Bt*)

Locus	*Primers*	*SNP*	*SNP position*
XIST	F: GAACATTTTCCAGACCCCAAC	*Bt*(C)	+353
	R: AAACCAGGTATCCACAGCCG	*Bg*(T)	
IGF2	F: CAAGGCATCCAGCGATTAG	*Bt*(A)	+767
	R: TTCAAGGGGGCTGATTGAG	*Bg*(C)	
GTL2	F: CCCACCAGCAAACAAAGCAAC	*Bt*(A)	+352
	R: CATCAAGGCAAAAAGCACATCG	*Bg*(C)	

primers consisted of the forward primer used to amplify the product. Sequences were visually analyzed for the presence or absence of each single nucleotide polymorphism (SNP). RT-PCR and sequencing reactions were run in triplicate. To confirm the absence of genomic contamination in cDNA samples, an internal control was utilized through the *IGF2* amplicon, which spans intron 6. Genomic contamination results in the presence of an additional 1-kb band (data not shown).

Bisulfite treatment of genomic DNA. Genomic DNA was isolated using a Promega Wizard DNA isolation kit from samples of chorions and liver. The sodium bisulfite reaction was carried out with 1 μg of DNA from each sample using the CpG DNA conversion kit (Intergenco, Norcross, GA) according to the manufacturer's directions.

Comparative sequence analysis of* Xist/XIST *regulatory regions. At the *Xist* locus in mice, regulation of expression is associated with differential methylation of CpG dinucleotides located in the promoter (−44 to −36) and in the 5′ region of exon 1 (+828 to +1183), thereby allowing comparative analysis in the bovine through available sequences of these regions (AF104906 and AJ4214811). Sequence was obtained from GenBank for the *XIST*/*Xist* promoter, all exons and introns, and 3′ regions extending approximately 45 kb downstream in the human (U50908), mouse (AJ421479), and bovine (AJ421481). Each region was analyzed for the presence of CpG dinucleotides through the European Bioinformatics Institute, CpG plot/CpG report/Isochore software program (www.ebi.ac.uk/emboss/cpgplot/). This program identifies CpG islands within large sequences (40 kb), based on the observed number of CpG dinucleotides relative to the expected number of CpG dinucleotides in a given sequence. For comparative sequence analysis between the bovine and mouse, sequences were aligned using PipMaker software (http:/bio.cse.psu.edu/cgi-bin/pipmaker?basic) (Schwartz et al. 2000). PipMaker software allows for the alignment of two sequences over a considerable length (>100 kb) and summarizes the homology as a ''percent in plots'' (PIP) graph ranging from 50% to 100%.

DNA methylation analysis of the* XIST *DMR. PCR primers were designed flanking the bovine *XIST* CpG island at +1477 to +1683, which was detected using CpG prediction software (F: TTTGTTGTAGGGATAATATGGTTGAT, R: GG TGGGAAAGATTAATTTATTTTGTG). Primers flanking the region were designed by converting all cytosines in the sequence that were not adjacent to guanines to thymines. This is the predicted sequence after bisulfite conversion of DNA with all CpG dinucleotides protected (methylated). PCR reactions were performed for 35 cycles at 95°C (5 min); 95°C (30 sec), 52°C (30 sec), 72°C (2 min 30 sec); 72°C (10 min). Products were resolved on a 2% EthBr agarose gel and gel purified using a Qiagen Gel Purification kit. Purified products were then cloned into TOPO4 sequencing vectors (Invitrogen, Carlsbad, CA). Plasmids from an average of 20 colonies were extracted using Plasmid Mini Prep (Qiagen) and sequenced separately. Sequencing reactions were performed as previously described, with annealing temperatures ranging from 50°C to 55°C.

Results

***Identification of SNPs between* Bos gaurus *and* Bos taurus.** Genomic DNA was isolated from independent *Bos gaurus* and *Bos taurus* fibroblast cell lines and used to amplify coding regions from the *XIST*, *IGF2*, and *GTL2* loci. Partial bovine sequences were obtained by performing BLAST searches of the mouse *Igf2* (NM 010514) and sheep *GTL2* (AY017220, AY017221, and AY017222) cDNA sequences against bovine EST libraries in GenBank and from the published bovine *XIST* sequence (AJ421481 and NR001464). Sequence analysis of these genes in the bovine resulted in the identification of informative SNPs for each gene between the *Bos gaurus* and *Bos taurus* (Table 1). The X inactivation–specific transcript (*XIST*), a RNA transcript

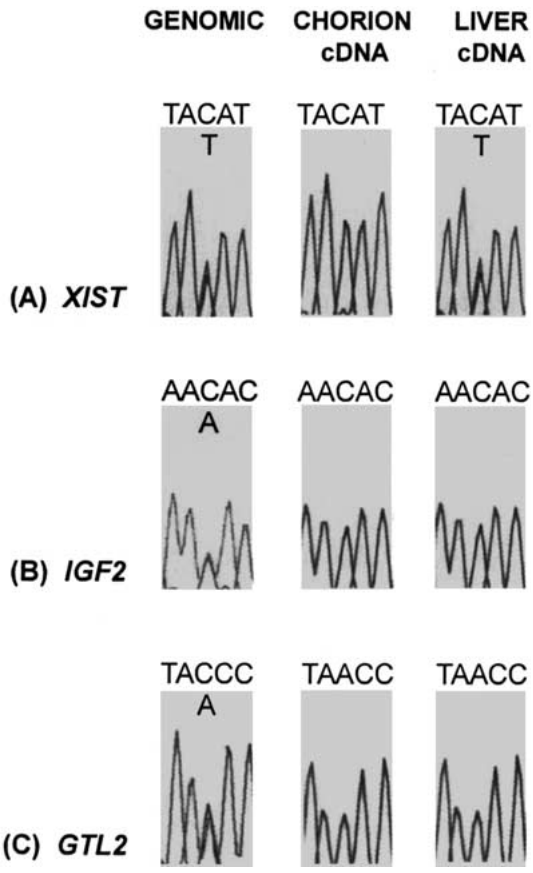

Fig. 1. Identification of SNPs at the *XIST*, *IGF2*, and *GTL2* loci and subsequent characterization of allelic expression. (**A**) Sequence chromatogram of *XIST* amplified from genomic DNA demonstrates the presence of the C/T SNP in hybrids. Sequence chromatogram obtained from chorion RT-PCR demonstrates preferential expression of the paternal (allele C) allele. In liver, lung (not shown), and brain (not shown) RT-PCR sequence chromatograms demonstrate expression of both paternal (C) and maternal (T) alleles. (**B**) *IGF2* amplified from genomic DNA shows the A/C SNP in hybrids. RT-PCR sequences of chorion, liver, lung (not shown), and brain (not shown) demonstrate preferential paternal (allele C) expression. (**C**) *GTL2* amplified from genomic DNA shows the C/A SNP in hybrids. RT-PCR sequences of chorion, liver, lung (not shown), and brain demonstrate preferential maternal (allele A) expression.

directing inactivation of one of the two X chromosomes in females; the insulin-like growth factor 2 (*IGF2*), the major somatomedin in fetal development; and the gene trap locus 2 (*GTL2*), an untranslated transcript associated with the callipyge overgrowth, all contained a polymorphism between the two species (Fig. 1).

Generation of Bos gaurus/B. taurus interspecific hybrids. A *Bos gaurus* (Gaur) bull was crossed to six *Bos taurus* (Angus) cows to generate the hybrid fetuses and placentas used for analysis. Fetal and placental components were obtained at day 72 of gestation, By which day placental and fetal components are entirely established. Samples derived from the placenta (chorion and allantois) and fetus (lung, liver, and brain) were isolated to determine parental expression of alleles in these tissues. A total of six hybrid fetuses were produced, four female and two male.

Characterization of imprinting at the XIST *locus*

XIST allelic expression. A (T/C) SNP at +353 of the *XIST* locus was identified in all hybrid female fetuses generated, whereas the males possessed only the maternal (C) allele, as would be expected since the *XIST* gene is located on the X chromosome. Expression of the *XIST* locus in females was detected in samples obtained from chorion, allantois, liver, lung, and brain by RT-PCR. Sequences generated by directly sequencing RT-PCR products amplified from samples of chorion (placenta), allantois, liver, lung, and brain were analyzed for the expression of the maternal (C) and paternal (T) allele. Analysis of allelic expression in each tissue revealed that the chorion preferentially expressed the paternal allele whereas sequences generated from allantois, liver, lung, and brain contained both the maternal and paternal alleles (Fig. 1). These results demonstrate tissue-specific maternal genomic imprinting of the *XIST* locus in the bovine.

DNA methylation analysis of the bovine XIST DMR. In exon 1 of the *XIST* locus, a CpG island was detected at +1477 to +1683, as was determined by CpG island prediction software (www.ebi.ac.uk/emboss/cpgplot/) and a primer set was designed for amplification of bisulfite-treated DNA encompassing this area. Additionally, a (T/A) polymorphism was detected at +1569 thereby allowing discrimination between paternal and maternal X chromosomes within this region. Liver and chorion samples from an individual female were chosen since these tissues had previously exhibited biallelic and monoallelic expression, respectively, in all females analyzed. Bisulfite sequencing of the CpG island, however, proved to be difficult; it became apparent after analyzing multiple sequences (>100), that possibly a secondary structure or a high AT-rich repeat sequence had formed from the bisulfite conversion and inhibited sequencing from proceeding past the first 30–50 base pairs of the transcript. After modifying cycling parameters, four full-length sequences were obtained from the liver sample but not from chorion, which indicated reciprocal methylation of the CpG island between the paternal and maternal X chro-

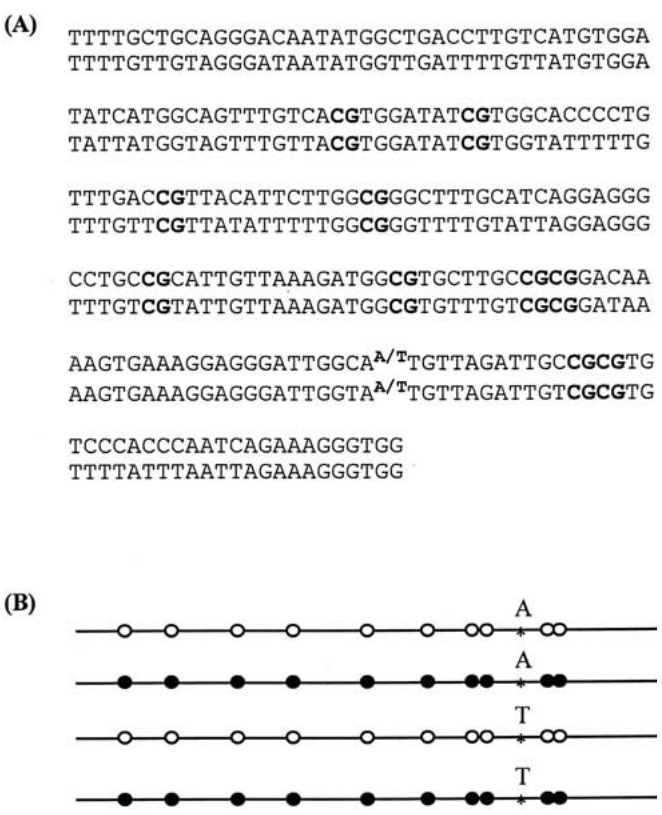

Fig. 2. (**A**) Sequence of the bovine *XIST* DMR, +1477 to +1683 (upper strand) aligned to predicted sequence after bisulfite modification (lower strand). Potentially methylated CG dinucleotides are represented in bold. (**B**) Distribution of methylated and unmethylated CpG dinucleotides on the paternal and maternal X chromosomes within the *XIST* DMR region.

mosomes (Fig. 2). These results demonstrate conservation of differential methylation at the *XIST* CpG island in exon 1 of the bovine, which corresponds with random monoallelic expression of the maternal and paternal *XIST* alleles within somatic tissues.

Comparative sequence analysis of the XIST locus. In an attempt to identify a putative imprinting center that controls *XIST* expression, a comparative sequence analysis was performed between the mouse, bovine, and human at regions suggested as regulating imprinting in mice. Recently, a *XIST* (–44 to –36) minimal promoter (GCGCCGCGG) in mice was identified and shown to be differentially methylated and bound by a 100-kDa methylation-dependent protein; this region is necessary for transcription of the locus in its unmethylated form (Huntriss et al. 1997). We performed sequence analysis of this region in the mouse, bovine, and human, where imprinting is not observed, and demonstrated that no CG-rich minimal promoter exists in the bovine (Fig. 3), suggesting that the minimal promoter and its role in imprinting is unique to the mouse.

Further analysis was extended to include the *Xist/XIST* antisense transcript, *Tsix/TSIX*, which has also been implicated as an element responsible for the imprinting phenomenon in the mouse (Migeonp et al. 2002). Using the PipMaker dot plot program (http://bio.cse.psu.edu/cgi-bin/pipmaker?basic), for long-range (>100 kb) homology query, and CpG island prediction software, we analyzed the region encompassing the bovine *TSIX* and found that no conservation existed with the mouse *Tsix* and, furthermore, no CpG island was detected in the bovine *TSIX* promoter or adjacent regions (Fig. 3).

IGF2 *and* GTL2 *allelic expression analysis*

Insulin like growth factor 2. A (C/A) SNP previously detected between *Bos gaurus* and *Bos taurus* cell lines was detected in all hybrid fetuses generated (n = 6). RT-PCR products of *IGF2* in samples obtained from chorion, allantois, liver, lung, and brain were directly sequenced and preferential expression of the paternal allele (C allele) was detected for all samples in each animal (Fig. 1). These results indicate maternal genomic imprinting at the *IGF2* locus in the bovine.

Gene trap locus 2. A (C/A) SNP was detected in each hybrid fetus generated and RT-PCR products of *GTL2* were detected in brain, liver, lung, chorions, and allantois. The maternally inherited *GTL2* allele (A allele) was detected, in the absence of the paternal allele, in sequences obtained from samples of chorion, allantois, liver, lung, and brain. These results indicate paternal imprinting at the *GTL2* locus (Fig. 1).

Discussion

Our results validate the use of *Bos gaurus/B. taurus* interspecies hybrids for the analysis of allelic expression. Although *Mus musculus* × *Mus spretus* and *Peromyscus polionotus* × *Peromyscus maniculatus* exhibit parental-specific phenotypes in their offspring (Dawson 1971; Vrana et al. 1998; Hemberger et al. 1999; Vrana et al. 2000; Zechner et al. 2002), no apparent abnormalities were detected in day-72 *Bos gaurus/B. taurus* hybrids. Placental structure (cotyledon number, chorio-allantoic fusion), placental fluid, and fetal weights and lengths were consistent with measurements from intraspecies crosses in the bovine (data not shown). There-

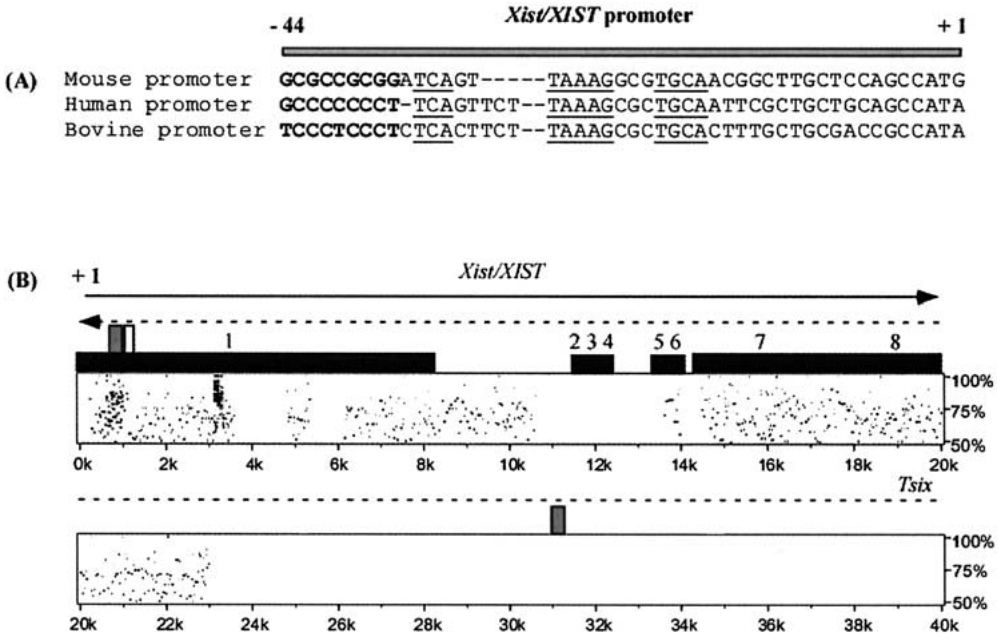

Fig. 3. Comparative sequence analysis of the *XIST/Xist* minimal promoter and *TSIX/Tsix* antisense between the mouse, human, and bovine. (**A**) Comparative sequence analysis of the *XIST/Xist* minimal promoter region between mouse, bovine, and human. Homologous promoter elements are denoted by underlined sequences. The −44 minimal promoter is denoted by bold sequence and demonstrates lack of CpG dinucleotides at this region in both bovine and human. (**B**) PipMaker dot plot schematic of the bovine and mouse *XIST/Xist* locus. PipMaker percent identity plot of the bovine *XIST* region and 3′ sequence relative to the mouse *XIST* and *TSIX* genes. Nucleotides 0–40,000 are shown of the X chromosome sequence ranging from 116,296 to 156,296 in the bovine compared to the corresponding region in the mouse. The dot patterns show the percent homology (50%–100%) with the comparable mouse *Xist* and *Tsix* region. The *XIST* sequence is denoted with the black line and exons 1–8 are represented by black boxes. The dashed line denotes the mouse *Tsix* antisense. CpG islands found present in the bovine are denoted by white boxes and mouse CpG islands are denoted by gray boxes.

fore, the *B. gaurus/B. taurus* hybrid is an ideal experimental model for allelic expression analysis of genes because of a lack of phenotypic abnormalities in offspring and the presence of coding SNPs. The use of these animals can be further expanded into a wide-scale systematic and comprehensive analysis of genomic imprinting as well as a model for nuclear reprogramming in the bovine.

Our findings of genomic imprinting at the *XIST* locus in cattle is especially intriguing, since this is the only other placental mammal reported to be imprinted other than the mouse (Graves 1996). In females, X chromosome inactivation is initiated by expression of the *Xist* locus, whereas in males this locus is silent. In females, expression of the *Xist* gene, which is regulated in part by methylation of a CpG island in exon 1 and in conjunction with other epigenetic modifications such as hypoacetylation of lysine residues of histone H3, induces the bidirectional inactivation of one of the two chromosomes (Csankovszki et al. 2001). Allelic expression patterns of the *Xist/XIST* locus have been examined in mice, humans, and marsupials, and imprinting at this locus is observed only in the mouse preimplantation embryo and polar trophectoderm. Analysis in the bovine reveals that the *XIST* gene is preferentially paternally expressed in the chorions of females but is expressed randomly in the allantois, liver, lung, and brain, demonstrating conservation of genomic imprinting with the mouse but not with the human. Furthermore, expression at the *Xist/XIST* locus in females is inversely correlated with DNA methylation of the promoter and 5′ region of exon 1 in mice and humans. Bisulfite sequencing of the CpG island in exon 1 of the bovine established that this region is reciprocally methylated in somatic tissue, where random monoallelic expression occurs.

Identification of genomic imprinting at this bovine locus presented us with a unique opportunity to compare between mice and bovine two regions believed to induce imprinting. Huntriss et al. (1997) have identified a minimal promoter region in the mouse (5′-GCGCCGCG-3′) located at −44 to −36. This element is differentially methylated in gametes and is bound by a nuclear protein in the presence of methylation, which inhibits transcription. In humans, this region has been replaced by a (5′-GCCCCCCT-3′), which is not subjected to methyl-

ation due to the lack of any CpG dinucleotides. We compared the corresponding region in the bovine and found no conservation of this region (Fig. 3), indicating that this site is unique to the mouse. Although this is not a proven site for imprinting in mice, its binding activity to a nuclear protein in a methylation-dependent manner has implicated it as one (Huntriss et al. 1997). Our results suggest, however, that this region is likely not to be the element responsible for imprinting in the bovine and may need further clarification in the mouse.

Others reports have demonstrated that a *Xist* antisense transcript, termed *Tsix*, exhibits reciprocal expression with *Xist* and regulates monoallelic expression (Fig. 3). In mice, *Tsix* has been shown to inhibit the maternal *Xist* allele in placental cells and the future active X chromosome in embryonic stem cells (Migeon et al. 2001, 2002; Migeon 2003). Antisense transcripts are commonly identified with imprinted genes, but their exact role in suppressing one allele in the presence of another is unclear. In mice, the *Tsix* promoter region contains a CpG island that is differentially methylated, whereas the human *TSIX* does not. Additionally, the human *TSIX* does not span into the *XIST* promoter but prematurely terminates in exon 5. Analysis in the bovine reveals that there is no corresponding CpG island in the *TSIX* promoter and no apparent homology over the entire transcript (Fig. 3). Evidence from other reports suggests that the bovine *TSIX* does not span into the *XIST* promoter (Chureau et al. 2002). This would further suggest that *TSIX* in the bovine does not regulate the maternal-specific silencing that was observed in the chorions of our F1 female hybrids. These findings suggest that a different mechanism might be involved in establishing and maintaining the maternal-specific silencing of the *XIST*/*Xist* allele in the placenta of the bovine and mouse. Moreover, we are confident that the silencing of the maternal *XIST* allele observed in our hybrid females is not a consequence of the interspecies cross, since preferential paternal X chromosome inactivation has already been demonstrated in cattle (Xue et al. 2002). Furthermore, it is unlikely that the bovine and mouse *XIST*/*Xist* have evolved to show similar patterns of tissue-specific allelic expression but by different mechanisms. Therefore, the bovine *XIST* presents a unique experimental model for the identification and analysis of genomic imprinting at this locus.

The *Igf2*/*IGF2* locus has been the most widely investigated imprinted gene in all mammals and is located within the human and mouse imprinting cluster on Chromosomes 11p15 and 7, respectively (Reik et al. 2003). In the bovine, conservation of this region has been demonstrated by radiation hybrid mapping and is found on Chromosome 29. Preferential paternal expression of the locus has been identified in humans, mice, sheep, pigs, rats, and opossums (Feil et al. 1998; Killian et al. 2000; Nolan et al. 2001). In all species investigated to date, maternal silencing has been demonstrated in all tissues analyzed except for the choroid plexus and leptomeninges in mice and the liver of adult humans and sheep (Pham et al. 1998; McLaren and Montgomery 1999). *IGF2* transcripts analyzed in tissues obtained from prenatal day 72 chorion, allantois, liver, lung, and brain demonstrated preferential paternal expression. In contrast to findings in the mouse, where the *Igf2* locus in the choroid plexus and leptomeninges is biallelic, allelic expression of the *IGF2* in prenatal bovine brain was determined to be preferentially paternal. It should be noted, however, that analysis was on the whole fetal bovine brain not specific regions and it is possible that biallelic expression of *IGF2* in the choroid plexus and leptomeninges went undetected due to the prevalence of other monoallelically expressed brain tissues in the samples used for analysis.

The *Gtl2*/*GTL2* locus has been reported as imprinted in humans, mice, and sheep and resembles the organization and regulation of the *Igf2*/*H19* locus, where it is reciprocally imprinted with the downstream *Dlk1* gene (Wylie et al. 2000; Bidwell et al. 2001). The bovine *GTL2* locus maps to Chromosome 18 and it has been demonstrated that the organization of this region is similar to that of sheep, suggesting high levels of conservation with other species (Shay et al. 2001). Our results show preferential maternal expression of the *GTL2* locus in the chorion, allantois, liver, lung, and brain.

In summary, our results validate the use of an interspecies bovine model for the study of imprinting. This model will facilitate the analysis of imprinted genes in the bovine, as identification of expressed SNPs is greatly enhanced in this interspecies model compared to intraspecies crosses (data not shown). The results also support the importance of comparative analysis of imprinting and demonstrate the utility of comparative approaches for elucidating the mechanisms that regulate imprinting in genes such as the *XIST*.

Acknowledgments

We thank Gary Hansen and members of Dr. Piedrahita's laboratory for assistance with generating and maintenance of the experimental cattle. This research was supported by NIH grant HL51587 and a

Texas A&M University, College of Veterinary Medicine Signature grant.

References

1. Allen ND, Logan K, Lally G, Drage DJ, Norris ML, et al. (1995) Distribution of parthenogenetic cells in the mouse brain and their influence on brain development and behavior. Proc Natl Acad Sci USA 92, 10782–10786
2. Bidwell CA, Shay TL, Georges M, Beever JE, Berghmans S, et al. (2001) Differential expression of the GTL2 gene within the callipyge region of ovine chromosome 18. Anim Genet 32, 248–256
3. Charlier C, Segers K, Wagenaar D, Karim L, Berghmans S, et al. (2001) Human–ovine comparative sequencing of a 250-kb imprinted domain encompassing the callipyge (clpg) locus and identification of six imprinted transcripts: DLK1, DAT, GTL2, PEG11, antiPEG11, and MEG8. Genome Res 11, 850–860
4. Chureau C, Prissette M, Bourdet A, Barbe V, Cattolico L, et al. (2002) Comparative sequence analysis of the X-inactivation center region in mouse, human, and bovine. Genome Res 12, 894–908
5. Constancia M, Pickard B, Kelsey G, Reik W (1998) Imprinting mechanisms. Genome Res 8, 881–900
6. Csankovszki G, Nagy A, Jaenisch R (2001) Synergism of Xist RNA, DNA methylation, and histone hypoacetylation in maintaining X chromosome inactivation. J Cell Biol 153, 773–784
7. Dawson WD (1971) Postnatal development in Peromyscus maniculatus-polionotus hybrids. II. Tail and hind foot growth. Growth 35, 359–367
8. Falls JG, Pulford DJ, Wylie AA, Jirtle RL (1999) Genomic imprinting, implications for human disease. Am J Pathol 154, 635–647
9. Feil R, Khosla S, Cappai P, Loi P (1998) Genomic imprinting in ruminants: allele specific gene expression in parthenogenetic sheep. Mamm Genome 9, 831–834
10. Gallagher DS Jr, Yang YP, Burzlaff JD, Womack JE, Stelly DM, et al. (1998) Physical assignment of six type I anchor loci to bovine chromosome 19 by fluorescence in situ hybridization. Anim Genet 29, 130–134
11. Gao Q, Womack JE (1997) A genetic map of bovine chromosome 7 with an interspecific hybrid backcross panel. Mamm Genome 8, 258–261
12. Graves JA (1996) Mammals that break the rules: genetics of marsupials and monotremes. Annu Rev Genet 30, 233–260
13. Hagemann LJ, Peterson AJ, Weilert LL, Lee RS, Tervit HR (1998) In vitro and early in vivo development of sheep gynogenomes and putative androgenones. Mol Reprod Dev 50, 154–162
14. Hemberger M, Redies C, Krause R, Oswald J, Walter J, et al. (1998) H19 and Igf2 are expressed and differentially imprinted in neuroectoderm-derived cells in the mouse brain. Dev Genes Evol 208, 393–402
15. Hemberger MC, Pearsall RS, Zechner U, Orth A, Otto S, et al. (1999) Genetic dissection of X-linked interspecific hybrid placental dysplasia in congenic mouse strains. Genetics 153, 383–390
16. Huntriss J, Lorenzi R, Purewal A, Monk M (1997) A methylation-dependent DNA-binding activity recognising the methylated promoter region of the mouse Xist gene. Biochem Biophys Res Commun 235, 730–738
17. Jeon JT, Carlborg O, Tornsten A, Giuffra E, Amarger V, et al. (1999) A paternally expressed QTL affecting skeletal and cardiac muscle mass in pigs maps to the IGF2 locus. Nat Genet 21, 157–158
18. Jong MT, Carey AH, Caldwell KA, Lau MH, Handel MA, et al. (1999) Imprinting of a RING zinc-finger encoding gene in the mouse chromosome region homologous to the Prader–Willi syndrome genetic region. Hum Mol Genet 8, 795–803
19. Killian JK, Byrd JC, Jirtle JV, Munday BL, Stoskopf MK, et al. (2000) M6P/IGF2R imprinting evolution in mammals. Mol cell 5, 707–716
20. Killian JK, Nolan CM, Wylie AA, Li T, Vu TH, et al. (2001) Divergent evolution m M6P/IGF2R imprinting from the Jurassic to the Quaternary. Hum Mol Genet 10, 1721–1728
21. Mayer W, Hemberger M, Frank HG, Grummer R, Winterhager E, et al. (2000) Expression of the imprinted genes MEST/Mest in human and murine placenta suggests a role in angiogenesis. Dev Dyn 217, 1–10
22. McLaren RJ, Montgomery GW (1999) Genomic imprinting of the insulin-like growth factor 2 gene in sheep. Mamm Genome 10, 588–591
23. Migeon BR (2003) Is Tsix repression of Xist specific to mouse?. Nat Genet 33, 337 author reply 337–338
24. Migeon BR, Chowdhury AK, Dunston JA, McIntosh I (2001) Identification of TSIX, encoding an RNA antisense to human XIST, reveals differences from its murine counterpart: implications for X inactivation. Am J Hum Genet 69, 951–960
25. Migeon BR, Lee CH, Chowdhury AK, Carpenter H (2002) Species differences in TSIX/Tsix reveal the roles of these genes in X-chromosome inactivation. Am J Hum Genet 71, 286–293
26. Nezer C, Moreau L, Brouwers B, Coppieters W, Detilleux J, et al. (1999) An imprinted QTL with major effect on muscle mass and fat deposition maps to the IGF2 locus in pigs. Nat Genet 21, 155–156
27. Nolan CM, Killian JK, Petitte JN, Jirtle RL (2001) Imprint status of M6P/IGF2R and IGF2 in chickens. Dev Genes Evol 211, 179–183
28. Pham NV, Nguyen MT, Hu JF, Vu TH, Hoffman AR (1998) Dissociation of IGF2 and H19 imprinting in human brain. Brain Res 810, 1–8
29. Reik W, Davies K, Dean W, Kelsey G, Constancia M (2001) Imprinted genes and the coordination of fetal and postnatal growth in mammals. Novartis Found Symp 237, 19–31; discussion 31–42
30. Reik W, Constancia M, Fowden A, Anderson N, Dean W (2003) Regulation of supply and demand for mater-

nal nutrients in mammals by imprinted genes. J Physiol 547, 35–44
31. Schmidt JV, Matteson PG, Jones BK, Guan XJ, Tilghman S (2000) The Dlk1 and Gtl2 genes are linked and reciprocally imprinted. Genes Dev 14, 1997–2002
32. Schwartz S, Zhang Z, Frazer KA, Smit A, Riemer C, et al. (2000) PipMaker—a web server for aligning two genomic DNA sequences,. Genome Res 10, 577–586
33. Shay TL, Berghmans S, Segers K, Meyers S, Beever JE, et al. (2001) Fine-mapping and construction of a bovine contig spanning the ovine callipyge locus. Mamm Genome 12, 141–149
34. Short RV (1997) An introduction to mammalian interspecific hybrids. J Hered 88, 355–357
35. Surani MA (2001) Reprogramming of genome function through epigenetic inheritance. Nature 414, 122–128
36. Villar AJ, Eddy EM, Pedersen RA (1995) Developmental regulation of genomic imprinting during gametogenesis. Dev Biol 172, 264–271
37. Villar AJ, Pedersen RA (1997) Interspecies approaches for the analysis of parental imprinting during mouse development. J Hered 88, 401–407
38. Vrana PB, Guan XJ, Ingram RS, Tilghman SM (1998) Genomic imprinting is disrupted in interspecific Peromyscus hybrids. Nat Genet 20, 362–365
39. Vrana PB, Fossella JA, Matteson P, del Rio T, O'Neill MJ, et al. (2000) Genetic and epigenetic incompatibilities underlie hybrid dysgenesis in Peromyscus. Nat Genet 25, 120–124
40. Wylie AA, Murphy SK, Orton TC, Jirtle RL (2000) Novel imprinted DLK1/GTL2 domain on human chromosome 14 contains motifs that mimic those implicated in IGF2/H19 regulation. Genome Res 10, 1711–1718
41. Xue F, Tian XC, Du F, Kubota C, Taneja M, et al. (2002) Aberrant patterns of X chromosome inactivation in bovine clones. Nat Genet 31, 216–266
42. Yang YP, Womack JE (1997) Construction of a bovine chromosome 19 linkage map with an interspecies hybrid backcross. Mamm Genome 8, 262–266
43. Yevtodiyenko A, Carr MS, Patel N, Schmidt Jt (2002) Analysis of candidate imprinted genes linked to Dlk1-Gtl2 using a congenic mouse line. Mamm Genome 13, 633–638
44. Young LE, Fernandes K, McEvoy TG, Butterwith SC, Gutierrez CG, et al. (2001) Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture. Nat Genet 27, 153–154
45. Zechner U, Hemberger M, Constancia M, Orth A, Dragatsis I, et al. (2002) Proliferation and growth factor expression in abnormally enlarged placentas of mouse interspecific hybrids. Dev Dyn 224, 125–134