THE DEVELOPMENT OF A BOVINE INTERSPECIES MODEL FOR THE ANALYSIS OF GENOMIC IMPRINTING IN NORMAL AND NUCLEAR TRANSFER DERIVED FETUSES

A Dissertation

by

SCOTT VICTOR DINDOT

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2003

Major Subject: Genetics

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Approved as to style and content by:

James Derr (Co-Chair of Committee)	Jorge Piedrahita (Co-Chair of Committee)
James Womack (Member)	George Davis (Member)
Loren Skow (Member)	Evelyn Tiffany-Castiglioni (Head of Department)
Geoffrey Kappler (Chair of Genetics Faculty)	

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ABSTRACT

The Development of a Bovine Interspecies Model for the Analysis of Genomic Imprinting in Normal and Nuclear Transfer Derived Fetuses. (August 2003)

Scott Victor Dindot, B.S., Texas A&M University

Co-Chairs of Advisory Committee: Dr. Jorge Piedrahita
Dr. James Derr

The advent of somatic cell nuclear transfer in cattle has provided the opportunity for researchers to generate genetically identical animals as well as animals that possess precise genetic modifications for agriculture and biomedical purposes. However, in spite of the revolutionary impact this technology presents, problems remain which hinder the production of healthy animals on a consistent basis. Research on cloned mice implicates improper reprogramming of epigenetic modifications and genomic imprinting for the low pregnancy rates and high incidence of abnormalities that are manifested in cloned animals; however, a systematic and comprehensive analysis of nuclear reprogramming in cloned cattle remains undone.

The purpose of this research is to assess and characterize the patterns of genomic imprinting in normal and nuclear transfer derived bovine fetuses. To facilitate the identification of imprinted genes in the bovine, a *Bos gaurus/Bos taurus* interspecies model has been incorporated to maximize the genetic heterozygosity that exists between the alleles of putative imprinted genes for allelic discrimination and parental inheritance.

The sequence of twenty-six genes, previously reported as imprinted in mice and humans, was analyzed in *Bos gaurus* (Gaur) and *Bos taurus* (Angus) cattle for the presence of single nucleotide polymorphisms (SNP). SNPs were detected in the Gene trap locus 2 (*GTL2*), Insulin like growth factor 2 (*IGF2*), Wilms tumor 1 (*WT1*) and the X chromosome inactivation specific transcript (*XIST*). Allelic expression analysis in interspecies hybrids indicated maternal genomic imprinting at the *IGF2* and *XIST* loci, paternal genomic imprinting at the *GTL2* locus and no imprinting at the *WT1* locus. Analysis in cloned hybrids indicated fidelity of allelic expression at the *IGF2* and *GTL2* loci, however disruption of imprinting was observed at the *XIST* locus in the placenta of clones. These results are the largest identification of imprinted genes in the bovine and the first identification of the disruption of an imprinted gene in an animal derived from somatic cell nuclear transfer.

It is with great pride that I dedicate this work to my father. I thank you for your never-ending support of my interests and ambitions, even when you never quite understood them.

ACKNOWLEDGEMENTS

I would like to thank Dr. Jorge Piedrahita for his guidance and support during my years as an undergraduate and graduate student. I am deeply grateful for the opportunity to have completed my graduate work under your supervision. You took a chance with me when no one else was willing and for this I, as well as my family, will always be indebted. I would also like to think Dr. George Davis for his mentorship during all of my years at Texas A&M as both an undergraduate and graduate student. I spent many days both on and off the court learning lessons about life and science and I am grateful for the opportunity to have known you.

I would also like to extend my thanks to the graduate students and post-doctorates at Texas A&M. I would like to thank Gretchen Zaunbrecher, Cara Reichel, Shawn Walker, Yan-Ling Wang, Dr. Bashir Mir, Dr. Chang-Kyu Lee and Dr. Tae-Young Shin. I would also like to recognize the efforts of all the undergraduate students who had research projects under my supervision, especially KC Kent and Brett Evers. Additionally, I would like to thank the numerous individuals who helped with the generation of cattle used in my projects, in particular, Brian Mosteller, Sam Williams, Gary Hensen, Dr. Juan Romano, Dr. Peter Farin, Dr. Char Farin and Jeremy Miles. I have a deep respect and admiration for these people who helped when they had no involvement other than friendship.

My sincerest appreciation goes to my family. I thank my parents and sisters, who have encouraged me along the way and have never doubted the ambitions that I have set

forth for myself. Also to my extended family, thank you for your support. Most of all, to my wife Lisa, who has endured the trials and tribulations of a graduate student's wife, while giving me the two most precious items in my life, Payton and Tyler. I love you with all my heart and I am appreciative of your support and understanding.

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CHAPTER I

INTRODUCTION AND LITERATURE REVIEW

The agriculture and biomedical applications of nuclear transfer (NT) in cattle are promising (Piedrahita 2000; Westhusin et al. 2001). The advent of this technology provides the ability to generate animals possessing a myriad of valuable commercial and biomedical traits. To date, hundreds of calves have been produced through nuclear transfer using a variety of techniques and donor cell types (Cibelli et al. 2002). However, in spite of the revolutionary impact this technology has generated, many problems still remain which hinder the efficient and consistent production of healthy calves (Cibelli et al. 2002).

Primarily is the inefficient production of cloned calves due to embryonic and gestational loss. The first somatic cell nuclear transfer calves were generated approximately four years ago (Cibelli et al. 1998), and since this time, no significant increases in birth rate percentages have been achieved. To date, birth rate success in cloned cattle range from 1-10% on average (Hill et al. 2000; Hill et al. 2001; Hill et al. 1999; Oback et al. 2003; Wells et al. 1999); much lower than rates from other artificial *in vitro* techniques implemented in the cattle industry, such as embryo transfer and artificial insemination. Recently, it has been demonstrated that in bovine clones, a large percentage of losses occurs during the first trimester (30-60 days) and then diminishes as

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This dissertation follows the style and format of *Genome Research.*

gestation proceeds, but not without further loss of fetuses (Hill et al. 2000). The low rate of embryos developing to blastocyst, which is approximately 50%, combined with the early loss of established pregnancies are costly and time consuming. Success of cloning in the bovine will ultimately be determined by the ability to obtain and maintain pregnancies; therefore, further analysis into these events is crucial.

Secondary to loss of pregnancies are the prevalence of fetal and more often placental abnormalities that are observed in cloned calves. A recent review of animals generated through nuclear transfer indicated that more than half (64%) of all cloned cattle surviving to term manifest some form of pathological condition (Cibelli et al. 2002). Our own observations in cloned pigs, which were apparently normal at birth, lead to the discovery of subtle phenotypes that each pig possessed (Archer et al. 2003). Although the phenotypes in our cloned pigs were not pathological, they were "abnormal", considering each of the pigs was genetically identical. Aside from pathological conditions observed in cloned mice, they have been shown to be grossly obese upon maturity, which is then corrected in resulting offspring (Tamashiro et al. 2002). These reports indicate that different species of animals generated through nuclear transfer exhibit varying phenotypes, which can range from subtle changes in phenotype to extreme pathological conditions. Cloned cattle unfortunately manifest conditions that are exacerbated relative to other cloned animals (Cibelli et al. 2002). Thus, for the expansion of bovine nuclear transfer technology in biomedical and agriculture programs, the events and mechanisms influencing these problems need to be ascertained.

It has been presumed for some time that inherent problems exist in the nuclear transfer process that induce, either entirely or partially, the phenotypes and low birth rate percentages that are observed in cloned cattle. Discovery of the event or events that lead to these problems has been difficult, due to the vast technical requirements demanded by the NT process and the complexity of nuclear reprogramming. Improper epigenetic reprogramming of the donor nucleus has been suggested as a cause for these problems and has been investigated widely in mice (Humpherys et al. 2001), but in cattle a systematic and comprehensive analysis remains incomplete.

Epigenetic reprogramming is achieved by direct modifications that are imparted onto the genome and typically involve methylation of DNA at the 5 carbon position of CpG dinucleotides as well as acetylation and methylation of H3 histones (Dean et al. 2003). In the eukaryotic genome however, CpG dinucleotides are under represented as a consequence of frequent deamination of methylated cytosines into thymines; although dense regions of CpG dinucleotides are common and can be found in a number of regions including: X-linked genes in females, germ line specific and tissue specific genes, repeat elements (LINES and SINES) and imprinted genes (Jackson-Grusby and Jaenisch 1996). Recently, DNA methylation and the machinery involved in its establishment and maintenance have gained much attention because of its apparent role in nuclear reprogramming (Alberio and Campbell 2003).

Genomic imprinting, resulting from parental specific epigenetic modifications, involves the preferential expression of alleles based on parental inheritance (Barton et al. 1984). That is, imprinted genes express either the paternal or maternal allele and

regulation is presumably mediated by the presence or absence of methylated CpG islands (Reik et al. 1987). Imprinted genes identified to date are involved in fetal, placental and neurological development (Reik and Dean 2001). Establishment of these genes occurs during differentiation of germ cells and in early stages of embryonic development. Genomic imprinting is theorized to exist in all placental mammals and, at present, a number of imprinted genes have been identified in humans (Reik and Dean 2001), mice, sheep (Bidwell et al. 2001; Wylie et al. 2000; Young et al. 2001), cattle (Killian et al. 2000) and marsupials (Murphy and Jirtle 2003).

Since nuclear transfer circumvents the processes that are normally involved in establishing epigenetic modifications and genomic imprinting, it requires further examination in cloned animals. Dean et al (1998) demonstrated that mice generated from embryonic stem cells (ES cells) possess disregulation at the imprinted *Igf2, H19, Igf2R* and *U2af1-rs1* loci. Although ES cells are typically unstable with respect to methylation, these results demonstrate the plasticity of imprinted genes when not passed through the germ line and used directly for the generation of animals. Xue et al. (2002) and Eggan et al. (2000) have also demonstrated aberrant patterns of X-chromosome inactivation (XCI) in the bovine and mouse respectively, which is regulated by the imprinted *Xist* locus. Additional reports from Kang et al. (2002) show abnormal epigenetic patterns in cloned bovine embryos at repeat elements (LINES) and within tissue specific promoters (Epidermal cytokeratin). These reports are connected to Chung et al (2003) who demonstrated abnormal expression of the DNA methyltransferase, Dnmt1, and improper nuclear localization of the maternal Dnmt1o isoform in cloned

mouse embryos. Our own results indicate stochastic patterns of DNA methylation in live cloned pigs at both centromeric and euchromatic regions (Archer et al. 2003). Therefore, it is likely that the cloning process alters, through some unknown process or through the lack of a process, genomic imprinting and DNA methylation in resulting embryos and offspring.

The focus of this research is to develop an experimental model for the systematic characterization of genomic imprinting and DNA methylation in the bovine. Additionally, this model will facilitate analysis of nuclear reprogramming in the bovine at identified imprinted genes as well as epimutations (epigenetic disregulation) that arise in the nuclear transfer process. Development of this model will be achieved using crosses of *Bos gaurus* (Gaur) and *Bos taurus* (domestic) cattle, in an attempt to increase the frequency of single nucleotide polymorphisms (SNPs) between parents. These SNPs will be used to characterize the allelic expression and parental inheritance of putative imprinted genes. Methylation analysis of repeat elements and tissue specific promoters will be utilized so as to characterize nuclear epigenetic reprogramming.

BACKGROUND

Nuclear transfer

Attempts of nuclear transfer in animals extend well beyond the production of Dolly, the world's first animal generated through somatic cell nuclear transfer (Campbell et al. 1996). Long before, fusion of adult amphibian keratinocytes to enucleated eggs were performed and were able to support development in culture to the juvenile tadpole

stage (Gurdon et al. 1975). Afterwards, attempts in mice, sheep, cattle and rabbits generated live animals using cells from the epiblast (Stice and Robl 1988), and 8-16 cell blastomeres (Collas and Robl 1990; Prather et al. 1989; Robl et al. 1987). Recently, cattle, goats, pigs, zebra fish, rabbits, a cat and a mule have been added to the list of animals generated through somatic cell nuclear transfer (Baguisi et al. 1999; Cibelli et al. 1998; Lee et al. 2002; Polejaeva et al. 2000; Shin et al. 2002; Woods et al. 2003). However, generation of all species through cloning is hindered by low viable birth rates and high incidences of abnormalities (Cibelli et al. 2002).

At present, two methods of nuclear transfer are utilized, a) the electrofusion method, giving rise to cloned sheep, cattle, goats, cats and mice (Campbell et al. 1996) and b) the piezo driven method used primarily to generate cloned mice (Ogura et al. 2000).

Various permutations exist within the electrofusion method, but essentially metaphase II oocytes are enucleated micorsurgically and donor cells are injected into the perivittaline space. Following reconstruction, oocyte complexes are aligned within a fusion chamber and a direct current (DC) pulse is administered, which causes a temporary disorganization of cell membranes, thus allowing donor cell and cytoplasmic membrane to fuse. Fused complexes are then activated to initiate development through various methods: chemical activation consisting of ionomycin and dimethyl amino purine (DMAP), cyclohexamide or electrical activation utilizing an alternating current (AC) pulse. Reconstructed embryos are either cultured to the blastocyst (day 7) stage where they are transferred to surrogate mothers, or are transferred directly after

activation. The various protocols depend on preference of investigator or species of animal being cloned. To date, animals have been generated using all of the methods described above.

In mice, the method of choice is the piezo driven method (Wakayama et al. 1998). Metaphase II oocytes are enucleated microsurgically and donor cells are injected directly into the oocyte cytoplasm. This is achieved through the use of an injection pipette housed in a piezo-impact pipette drive unit. This unit, which drives the pipette a short distance (approximately 0.5 μm) very rapidly, allows the pipette to easily penetrate the zona pelucida and plasma membrane without lysing the oocyte. A thin-walled flush-ended pipette, approximately 7 μm in diameter, is used for injection. Additionally, the cell membrane of the donor cell is disrupted when the cell is drawn in and out of the pipette a few times; therefore, allowing the nuclei to be injected independent of donor cytoplasm and cell membrane. To date, approximately 100 cloned mice have been generated using this protocol (Perry and Wakayama 2002).

Epigenetics

DNA methylation

Methylation at cytosine-guanine nucleotides (CpG dinucleotides) exists in virtually all vertebrates, many invertebrates and most plants (Reik et al. 2003b). In vertebrate genomes, the 5-carbon position of cytosine within a CpG dinucleotide is methylated. The complementary strand of 3'GpC 5' is methylated as well and these two

methyl groups exhibit a three dimensional structure prominent in the major groove of the double stranded DNA. In most mammals, 60-90% of all CpG sequences in the genome (approximately 3 $X10^7$ methylated cytosines) are methylated and (Walsh and Bestor 1999) these CpG dinucleotides are typically clustered in GC rich regions, termed CpG islands. [An equation has been formulated for the prediction of CpG islands within the genome (observed X expected/ expected > 0.6)], but methylation is not constrained to these regions (Bestor 2000). CpG islands are found in a number of genomic regions including: repeat elements (LINES and SINES), 5' regions of promoters, inactivating centers (IC), tumor suppressor genes, proto-oncogenes and in differentially methylated regions (DMRs) of imprinted genes (Jaenisch and Bird 2003).

The sequence draft of the mouse and human genomes, indicates that of the 30,000-40,000 genes residing in the genome, as many as 20,000 are presumably associated with CpG islands (Bestor 1998a). Typically, expressing and non-expressing promoters are largely undermethylated in tissues under normal conditions, however, imprinted genes and certain inactive genes on the X chromosome are concomitant with promoter methylation (Boumil and Lee 2001). Embryonic lethality by inactivation of DNA methyltransferases (Dnmt) through knockout strategies has shown the importance of genomic methylation. Dnmt -/- mice exhibit genome wide hypomethylation and typically do not survive past E12.5 (Trasler et al. 1996). Additionally, localized disregulation of methylation can result in genomic instability and various forms of cancer (Eden et al. 2003). A number of idiopathic diseases have also been associated

with DNA methylation (Lopes et al. 2003). Thus DNA methylation in mammals magnifies the complexity of genetic regulation and disregulation.

Four DNA methyltransferases and derivatives have been identified in mice and humans: Dnmt1, Dnmt2, Dnmt 3a and Dnmt3b (Bestor 2000). Inactivation of Dnmt1, Dnmt3a or Dnmt3b by gene targeting in mice results in varying degrees of hypomethylation and embryonic lethality (Bachman et al. 2001). The biological function of Dnmt2 is unknown, but a role in centromere function is postulated (Dong et al. 2001). These methyltransferases can be further classified into two categories: (i) maintenance DNA methyltransfeases (Dnmt1 and Dnmt2) and (ii) *de novo* DNA methyltransferases (Dnmt3a and Dnmt3b) (Reik et al. 2001).

Maintenance methyltransferases (Dnmt1) are highly active against hemi-methylated DNA, such as in the case of replicating DNA. Dnmt1 provides the correct pattern of methylation to the newly synthesized daughter strand of DNA based on the methylation status of the parent strand. Improper establishment of DNA methylation during replication has been termed “epimutations” and has the potential to affect an organism in the same manner as a genetic mutation (Reik et al. 2003b). In addition to maintenance function, Dnmt1 has also been shown to repress transcription directly in cooperation with histone deacetylases (HDACs) (Fuks et al. 2000). Recently, a sex specific form of Dnmt1 (Dnmt1o) has been identified in mouse oocytes. The Dnmt1o gene possesses a sex specific promoter and a 5’ exon (exon 1) which causes translation to initiate at an ATG codon in exon 4, resulting in a shorter form of the protein than the somatic form by 118 N-terminal amino acids. Dnmt1o accumulates at high levels in the

oocyte and is nuclear only at the early stages of development. Dnmt1o is cytoplasmic in pre-implantation embryos, subsequently localizes to the nucleus for a short time at the 8-cell stage, and is later replaced by the somatic form of the protein. The biological relevance of this unusual form of nuclear/cytoplasmic trafficking is unknown, but it is intriguing as to what role it has in methylation and why it is localized in this manner (Ratnam et al. 2002).

The other class of*de novo* methyltransferases, DNMT3a and DNMT3b add a methyl group to unmethylated CpG dinucleotides. *De novo* methylation occurs during gametogenesis in both male and female germ cells, where it is believed to play an important role in the establishment of genomic imprinting in the gametes. Additionally, *de novo* methyltransferases are responsible for remethylation of the genome after the asymmetric demethylating events, which occur in the pre-implantation embryo. Evidence demonstrates that Dnmt3a is required for the establishment of methylation imprints in the oocyte (Hata et al. 2002). *De novo* methylation occurs primarily in gametes, embryonic stem cells and embryonal carcinoma cells, but is largely suppressed in differentiated somatic cells (Hajkova et al. 2002). Due to ramifications of *de novo* methylation in adult organisms (cancer, etc), regulation is thought to be under tight control and limited only to early developmental processes. Although Dnmt3a and Dnmt3b are expressed at low levels in somatic cells, high expression of the proteins is detected in early development, concomitant with the establishment of new imprints (Bachman et al. 2001). Chen et al. (1998) have recently identified a truncated form of Dnmt3a (Dnmt3a2), which exhibits differential localization patterns within the genome

relative to Dnmt3a. Dnmt3a localizes predominantly to heterochromatin while Dnmt3a2 localizes predominantly to euchromatin, thus suggesting specific biological functions for each of the proteins. Several different Dnmt3b transcripts have been detected resulting from alternative splicing and appear under the regulation of nuclear trafficking (Chen et al. 1998).

Methyl donors

Methylation of CpG dinucleotides via methyltransferases depends on the availability of methyl groups from S-adenosylmethionine (SAM). Dietary factors which are likely involved in the production of methyl groups and that influence DNA methylation include folate, vitamin B12, vitamin B6, vitamin B2, methionine, choline and alcohol. Normally in one carbon metabolism, a carbon unit from serine or glycine is transferred to tetrahydrofolate (THF) to form 5,10-methyl-enetetrahydrofolate. This compound is then subsequently used for the synthesis of thymidine, oxidized to formyl-THF for the synthesis of purines, or reduced to 5-methyltetrahydrofolate and used to methylate homocysteine to form methionine. Methionine is then converted to SAM by an ATP dependent transfer of adenosine to methionine through methionine adenosyltransferase. SAM is the primary methyl donor source for most cellular methylation reactions (DNA, RNA, proteins, histones and neurotransmitters) (Laird and Jaenisch 1994).

The demethylating activity that occurs during the generation of primordial germ cells, demethylation of sperm and oocyte genomes post fertilization and in cancers is

unknown. There are two theorized mechanisms that might remove methyl groups from CpG dinucleotides. The first is a passive mechanism whereby methylation is lost due to the absence of methyltransferases during DNA replication, resulting in a reduction in methylation over time as the genome replicates. The second is a dynamic process in which an active demethylase catalytically removes methyl groups (Reik et al. 2003b). Evidence supports both theories, especially when considering the reprogramming of the sperm and oocyte post fertilization. In mouse zygotes, the paternal (sperm) genome is rapidly demethylated upon entry into the oocyte cytoplasm whereas the maternal genome (oocyte) gradually loses methylation over time as the embryo differentiates into a blastocyst (Santos et al. 2002). It seems clear that the demethylation of the maternal genome results from absence of methyltransferases, which are localized to the cytoplasm (Oswald et al. 2000), but the mechanism that rapidly demethylates the paternal genome within minutes of fertilization is unknown. It is likely that demethytransferases, residing within the cytoplasm, obtain entry into the paternal pronuclei during decondensation and protamine to histone transition and demethylate the paternal genome. However, this is only speculation and the machinery and specificity of demethylation during this time is not known.

Understanding the mechanisms of DNA methylation raises another question: why is DNA methylated in the first place? The involvement of DNA methylation in so many different types of organisms, especially prokaryotes, provides a link to genome defense (Jahner and Jaenisch 1985). The homology between prokaryotic and eukaryotic methyltransferase enzymes suggests that these enzymes have evolved to disable foreign

or invasive DNA sequences. In eukaryotic genomes, transposable elements are constantly bombarding DNA moving about without regard to sequence location. As much as 35% of the human genome is composed of mobile elements and plant genomes possess even more. Therefore, to contain the spread of transposable elements and to suppress transcription, such as with viral components, it appears that the host genome has acquired the ability to methylate and thus inactivate such elements (Bestor 1998b). Interestingly, CpG islands possess elements reminiscent of ancestral retroviruses (Yoder et al. 1997). Promoters and introns also contain repeat elements indicative of transposons and many of these have been shown to affect regulation by their methylation (Constancia et al. 1998). It appears that these elements have co-evolved with mammals and regulate important biological functions.

Genomic imprinting

Genomic imprinting is a phenomenon whereby alleles of particular genes are repressed or expressed on the basis of parental inheritance. Although it has been known for some time that DNA methylation is involved in the mechanisms of imprinting, it is still unclear as to how exactly these modifications interact with genes to regulate expression (Lopes et al. 2003). Regions residing in all imprinted genes identified to date, termed differentially methylated regions (DMRs), are believed to regulate the expression of alleles based on the presence or absence of CpG methylation that resides as islands within these DMRs (Constancia et al. 1998). Thus, differential methylation of DMRs is coincident with the differential expression of alleles.

Characteristic of imprinted genes is their tendency to be found in clusters. In humans, two major clusters have been reported on chromosome 11p15.5 and 15q11-13. In mice both clusters are located on chromosome 7 (Reik and Maher 1997). One important aspect of clustering is enhancer competition, whereby adjacent promoters compete for access to enhancers, such as in the *Igf2/H19* domain located on chromosome 11p15 in humans. *H19* is expressed exclusively from the maternal allele (Bartolomei et al. 1991) whereas *Igf2* is restricted to the paternal allele (Lopes et al. 2003). Further analysis of the region demonstrates an imprinting control region (ICR), located 2kb upstream of the *H19* gene (Bell and Felsenfeld 2000) and its differential methylation originates from the gametes (Tremblay et al. 1995). A zinc finger protein binding the sequence CCCTC, termed CTCF, binds to the unmethylated maternal allele and activates the boundary function of this unit which further prevents a common set of enhancers from interacting with the *Igf2* locus. On the paternal allele, methylation interferes with the binding of CTCF; therefore, the enhancer element, which has a higher affinity towards *Igf2*, interacts and mediates transcription of *Igf2* and not *H19* (Bell and Felsenfeld 2000).

Another unique characteristic of imprinted genes is regional control or "spreading". A classic example is the X inactivation specific transcript (*Xist*), which is located within the X chromosome inactivation center (*Xce*) (Constancia et al. 1998). The *Xist* locus in mice is maternally imprinted in the trophectoderm (extraembryonic membrane of the placenta), whereas the paternal allele is preferentially expressed (Lyon 1999). Expression of *Xist* from the paternal allele results in inactivation of all but a few

genes within the respective X chromosome (Lyon 1999). Inactivation of the X linked genes on the paternal chromosome is accompanied by methylation of CpG islands located within promoters. Therefore, expression of the imprinted *Xist* locus acts in *trans* on the other genes and effectively induces inactivation. XCI is the most extreme form of "spreading" amongst imprinted genes, but it provides evidence to the capabilities of imprinted elements and their regulation of other genes (Constancia et al. 1998).

The germ line has a critical role erasing imprints inherited from the previous generation and in establishing imprints in the next generation, according to the sex of the germ line (Fig 1.1). In the mouse, primordial germ cells (PGCs) migrate through extraembryonic regions and the hindgut to the gonads in the genital ridge by E10.5-11.5 (Buehr 1997). At E13.5 female germ cells enter the meiotic prophase, whereas male germ cells undergo mitotic arrest until after birth, which is then followed by meiotic differentiation. Global demethylation and remethylation events occur in germ cells by E12.5-13.5. All non-imprinted genes tested to date are demethylated in both sexes (Kafri et al. 1993; Sanford et al. 1987) as well as reactivation of the inactive X chromosome in females (Csankovszki et al. 2001), although it is still uncertain if complete demethylation of some regions occurs. Hinting to this idea are few instances of incomplete demethylation, which occur at repeat elements of ancestral intracisternal A particles (IAP). It has been demonstrated that alleles of the agouti locus in mice (A^{iapy}, A^{hvy} and A^{vy}), containing long terminal repeats (LTR) of IAP retrotransposons upstream of the agouti promoter, maintain variable levels of methylation during

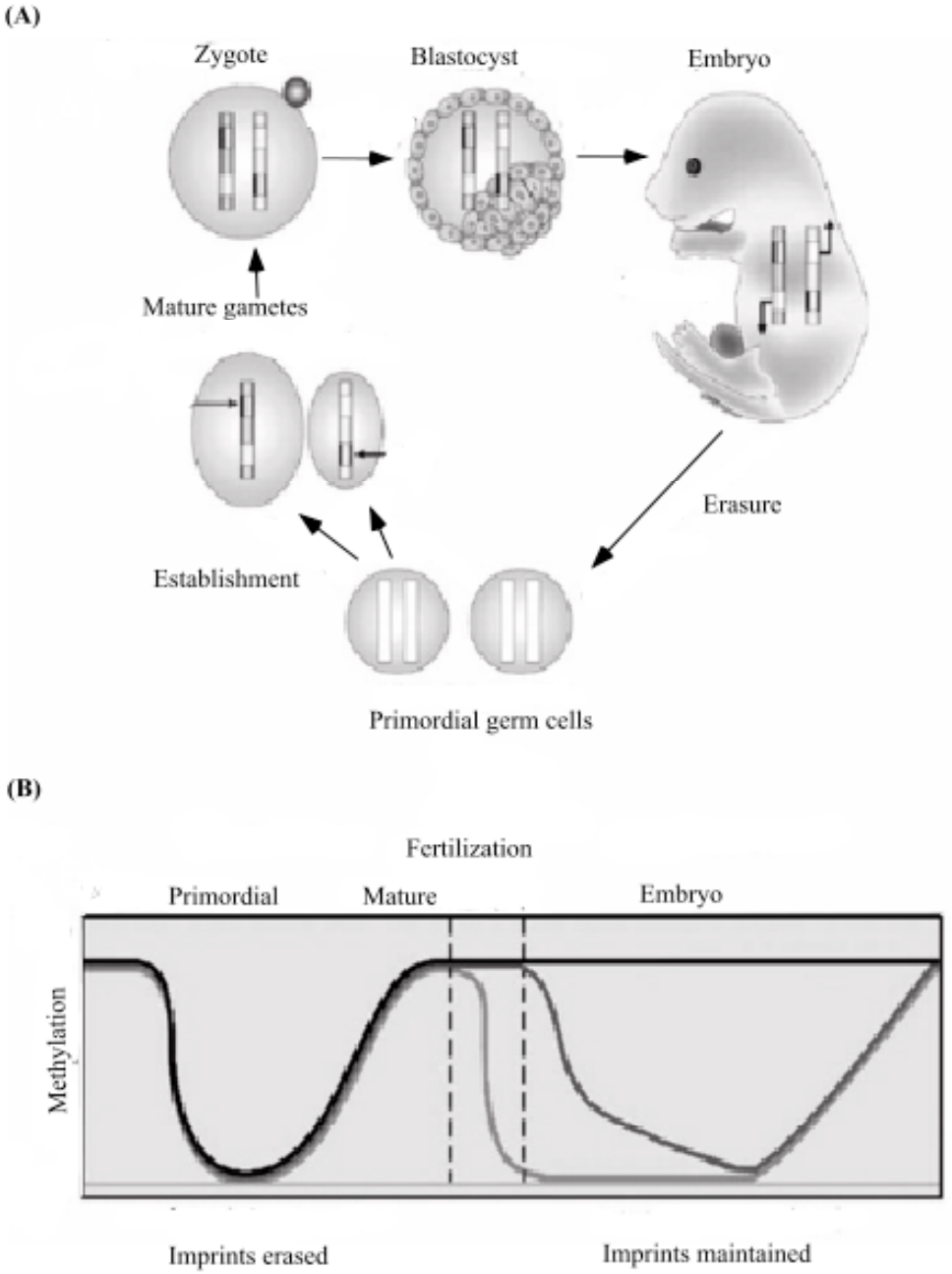

Fig 1.1. The life cycle of imprints. **(A)** Methylation imprints are introduced in oocytes and sperm in different imprinted genes. Differential methylation is maintained following fertilization. Methylation imprints are erased during development of the primordial germ cells. **(B)** DNA methylation and reprogramming. Methylation patterns are reprogrammed genome-wide in primordial germ cells (and imprints are erased at this time) and reestablished in mature gametes. After fertilization, the paternal genome is actively demethylated (dark), whereas the maternal genome is passively demethylated during cleavage divisions of the preimplantation embryo (grey). De novo methylation begins in inner cell mass cells of the blastocyst. (Reik et al. 2002)

gametogenesis when passed through the maternal germ line (Rakyan et al. 2003). This results in variable expression of the agouti locus and is manifested in the pups by differences in the agouti coat color. This observation demonstrates that either these regions are protected from demethylation due to inherent properties of the LTR or that the machinery has evolved not to demethylate these regions due to the consequences of active IAPs within the genome. Either way, this supports the idea of heritable traits influencing phenotype independent of genetics.

Remethylation of the mouse genome begins at approximately E15.5 in many gene sequences, with certain sequences maintaining differential methylation between sperm and oocyte (Piedrahita 2000; Sanford et al. 1987). In the female, oocytes in dictyate stage arrest are apparently not methylated until after birth, when methylation occurs during oocyte growth (Lucifero et al. 2002). This has been observed in repeat sequences (Howlett and Reik 1991), *Igf2R* (Stoger et al. 1993), imprinted transgenes (Chaillet et al. 1991) and inferred for the imprinted *Peg1, Peg3* (Paternally expressed genes 1 and 3) and *Snrpn* from functional studies (Constancia et al. 1998). Establishment of methylation in these previously mentioned genes is in conjunction with high levels of expression of Dnmt1, although it is unclear whether all other imprinted sequences initiate methylation at this time as well (Biniszkiewicz et al. 2002). In the male germ line it is also unclear when imprints are erased and reestablished. In the paternal germ line, evidence points to remethylation of the *Igf2* DMR at approximately E18.5 and of *H19* at approximately E21.5, however, complete demethylation of this

region is not observed (Constancia et al. 1998). Additionally, the establishment of imprints at chromosome 15q11-13 in round spermatids, elongated spermatids and ejaculated spermatozoa have been identified indicating the timing of imprint establishment in differentiation of spermatagonia (Manning et al. 2001). Thus it is clear that reprogramming of the paternal and maternal genomes occurs before fertilization, but at different times during development between parents and at different locations in the genome.

Following imprinting in the germ line, the parental genomes exhibit differential modes of demethylation in the newly formed zygote. Upon entry of the sperm into the oocyte, maternal cytoplasmic factors obtain access to paternal chromosomes for approximately 5 hours, at which time decondensation of the tightly packaged sperm DNA occurs resulting from the protamine to histone exchange required of the paternal chromosomes. During this time the paternal genome undergoes dramatic demethylation, although some are protected such as *H19* (but not *Igf2*), *Ras, Grf1* and some repeat elements, while the maternal genome, which contains most of the marks associated with imprints, undergoes further *de novo* methylation up to the blastocyst stage and is most likely regulated by passive demethylation via lack of maintenance methyltransferases (Oswald et al. 2000; Rougier et al. 1998). Afterwards, the paternal pronucleus forms, thereby regulating access of cytoplasmic factors to the genome. Asymmetric demethylation of the parental genomes has also been observed in cattle, pigs and humans (Dean et al. 2001).

Remethylation of the paternal and maternal genome resumes after implantation and formation of the epiblast and trophectoderm (Santos et al. 2002). Postzygotic *de novo* methylation requires functional Dnmt1, Dnmt3a and Dnmt3b transcripts (Hata et al. 2002), however, the Dnmt1 and Dnmt3 knockout mice exhibit low levels of methylation (Fuks et al. 2000; Rhee et al. 2002; Robertson and Wolffe 2000). A number of cytoplasmic factors with the potential to modify the epigenetic states of the parental genomes have been identified (Arney et al. 2001). Heterochromatin protein HP1, can bind methylated histone H3 (meH3) via a chromodomain and there is evidence that this interaction can lead to *de novo* DNA methylation (Surani 2001). Mager et al (2003) have also identified a mouse Polycomb group (PcG) gene *Eed* (embryonic ectoderm development), which acts to maintain the imprinted repression of one X chromosome in females as well as a subset of autosomal imprinted genes. *Eed* -/- fetuses exhibited biallelic expression (of normal maternally monoallelic) of the *Cdkn1c, Ascl2, Grb10* and *Meg3*, whereas four other paternally repressed genes (*Kcnq1, slc22a1l, Tssc3 and Igf2R*) and six maternally repressed genes (*Kcnqlot1, Snrpn, Peg3, Dlk1, Nnat and Plagl1*) maintained monoallelic expression. These results indicate specific *trans* acting factors, which are responsible for, or are involved in, inactivation of imprinted loci. Together with previous results, this demonstrates the complexity of imprinted gene regulation and that all sites, although imprinted, are not equal.

Abnormalities associated with nuclear transfer

Presently, cloning by somatic cell nuclear transfer is an inefficient process. Percent efficiencies of live clones per fused embryos among sheep, cattle, goats, pigs,

mice, cats and rabbits are 0.4, 0.2, 0.7, 0.5, 0.6, 1.1 and 0.7% respectively (Baguisi et al. 1999; Campbell et al. 1996; Chesne et al. 2002; Onishi et al. 2000; Shin et al. 2002; Wakayama et al. 1998), although recent efforts in some species report much higher pregnancy rates (Walker et al. 2002; Wells et al. 2003) .

In a recent article, a comprehensive analysis of the health profiles of cloned animals produced to date indicates that 64% of cattle, 40% of sheep, 100% of pigs and 93% of mice exhibit some form of abnormality (Cibelli et al. 2002). A large percentage of these animals, not reported (100-90%, depending on cell line and species), die during gestation or shortly after birth (Campbell et al. 1996; Hill et al. 2000; Hill et al. 1999; Young et al. 1998). Various abnormalities are manifested in animals derived from nuclear transfer. In cattle they include "Large Offspring Syndrome (LOS), diabetes, pulmonary hypertension, dilated cardiomyopathy, internal hemorrhaging umbilical artery, viral infection, dystocia, kidney problems, leg malformations, pneumonia, heart defects, liver fibrosis, osteoporosis, joint defects, anemia and placental abnormalities. In sheep abnormalities include LOS, arthritis, kidney, liver and brain defects. Mice exhibit obesity, LOS, enlarged placentas (attributed to expansion of the spongiotrophoblast), umbilical hernias, respiratory failure as well as failure to foster pups. Goats and pigs exhibit relatively few abnormalities, but they include bacterial infections of the lungs (goats) and abnormal teat numbers, cleft lips and malformed limbs (pigs) (Archer et al. 2003; Cibelli et al. 2002).

In cattle, where extensive loss of pregnancies and abnormalities are prevalent, it has been demonstrated that more than 80% of cloned pregnancies were lost in the bovine

between days 30-60 and this was attributed to placental aberrations. Observations in these experiments indicated a reduction in the number of expected cotyledons (placental units responsible for fetal/maternal exchange of nutrients, oxygen, etc.) and marked decrease in allantoic blood vessels (Hill et al. 2000; Hill et al. 2001). These findings are consistent with other reports in cloned cattle, (Stice et al. 1996), where no placentome formation was observed in the placentas of NT fetuses that died in utero between days 35-55. These findings were also consistent other first trimester losses reported in previous cloning experiments (Kubota et al. 2000; Wells et al. 1997; Wells et al. 1998; Wilmut et al. 1997).

Aside from gross physical abnormalities prevalent in nuclear transfer, genetic, epigenetic and mitochondrial aberrations have been observed in cloned animals as well. Humpherys et al (2001) demonstrated, through use of microarray technology, that of 10,000 genes examined in the placentas of cloned mice derived form embryonic stem cells and cumulus cells, approximately 4% of genes differed dramatically in expression levels from those in controls, and this was consistent for animals derived from both cell types. Dean et al (1998) also demonstrated abnormal expression of imprinted genes (*Igf2R, H19, Igf2 and U2af1-rs1*) in ES cell derived fetuses and this was accompanied by changes in methylation patterns as well as gross physical abnormalities (polyhydramnios, poor mandible development and interstitial bleeding). Kang et al and Reik et al (2002) examined the methylation patterns of repeat elements in cloned bovine embryos, donor cells and control embryos, produced *in vitro* and *in vivo*. This experiment determined that the methylation patterns observed in cloned embryos were

similar to that present in the donor cells, and this pattern was dramatically different than that exhibited in both *in vitro* and *in vivo* derived control embryos (Fig 1.2). The abnormal methylation patterns observed in cloned animals and embryos prompted others to examine the expression of methyltransferases in cloned mouse embryos. Reports demonstrate expression of the somatic Dnmt1 methyltransferase during embryonic development, which is normally repressed during this time, and instead the improper nuclear trafficking of the oocyte specific Dnmt1o during embryonic development (Chung et al. 2003). Other experiments in cattle (Steinborn et al. 2002) demonstrate the coexistence of donor cell and recipient cytoblast mitochondrial DNA within cloned calves.

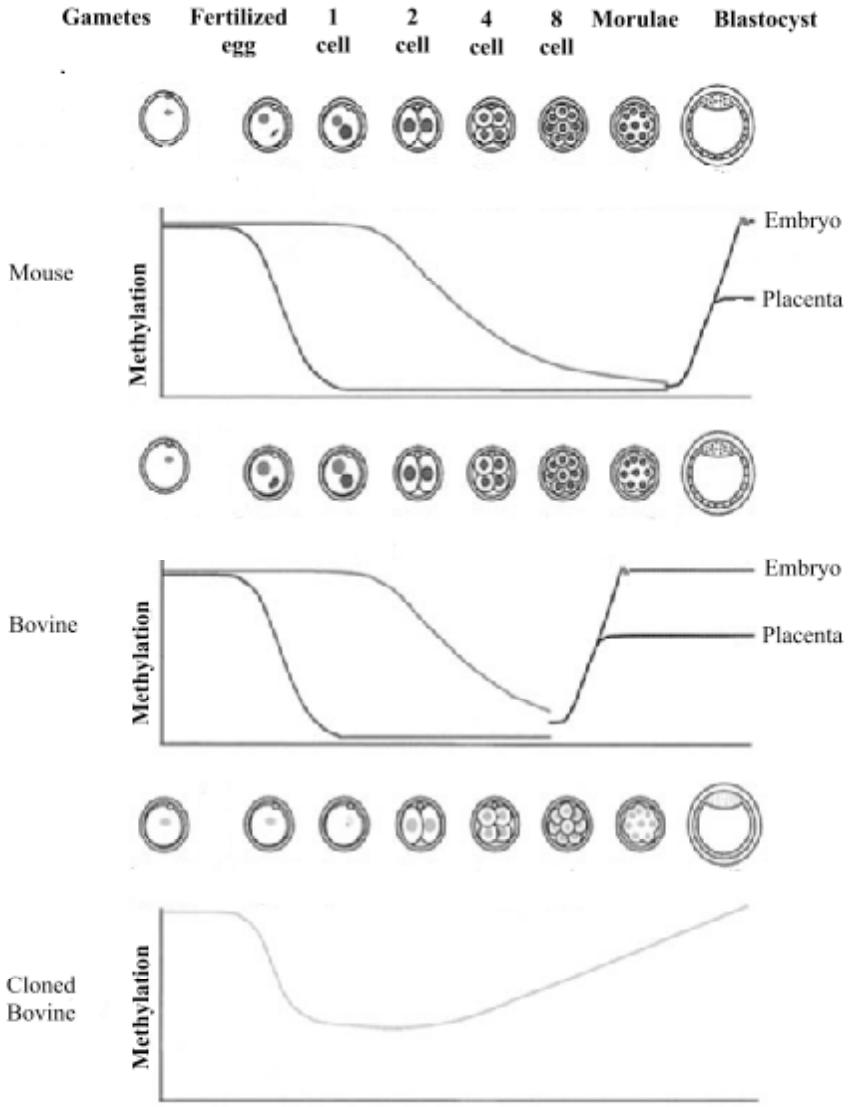

Figure 1.2 Genomic imprinting in normal and nuclear transfer derived bovine embryos. Deficient reprogramming in cloned embryos. A number of studies have shown that in cloned embryos, the reprogramming process is aberrant. Although there is some rapid demethylation of donor nuclei initially, further demethylation does not occur and nuclei end up with abnormally high levels of methylation and a nuclear organization reminiscent of the donor nucleus. (Reik et al. 2002)

Bovine interspecies hybrid

Over the past ten years, several strategies have been utilized to investigate genomic imprinting in various species ranging from disruption of parental alleles to subtractive hybridization between cDNAs from normal and parthenogenetic embryos (Feil et al. 1998). Recently, Villar and Pedersen et al (1997) described a relatively simple interspecies approach to "mRNA phenotyping", which discriminates parental alleles based on single nucleotide polymorphisms (SNPs) that exist in coding regions of genes under investigation. Through the use of SNPs and subsequent direct sequencing of products obtained through RT-PCR reactions, the parental allelic expression profiles of genes can be characterized. Thus, interspecies models maximize the heterogeneity between parents and facilitate the wide scale identification of SNPs in resulting offspring. To perform a systematic analysis of imprinted genes within an individual experimental model, interspecies approaches are ideal candidates to utilize.

In mice, where approximately 80 imprinted genes have been identified to date, use of interspecies hybrids (*Mus musculus*, *Mus spretus and Mus casteneus*) to facilitate identification of SNPs for parental allelic expression is prevalent (Adler et al. 1997; Hardt et al. 1999; Hemberger et al. 1998; Hu et al. 1995; Jong et al. 1999; Leco et al. 1997; Mayer et al. 2000; Pulford et al. 1999; Schmidt et al. 2000; Villar et al. 1995; Villar and Pedersen 1997; Vrana et al. 2000; Vrana et al. 1998; Vrana et al. 2001; Yevtodiyenko et al. 2002)

Interspecific hybrids of cattle have also been widely utilized as experimental models to maximize the genetic heterogeneity and allow for the discrimination of alleles and chromosomes. *Bos gaurus* (Gaur) and *Bos taurus* (domestic cattle) interspecific hybrids have recently been utilized for backcross panels as a method in optimizing the resolution of the bovine gene map *Bos gaurus gaurus* (Gaur) and *Bos gaurus laosienies* (Seledang) cattle (2n=58) (are native to South and Southeast Asia and are currently endangered) are characterized karyotypically by a 2;28 Robertsonian translocation. Crosses between Gaur and domestic cattle (2n=60) have been reported to produce apparently normal offspring (Bongso et al. 1988; Gao and Womack 1997; Riggs et al. 1997; Yang and Womack 1997).

CHAPTER II

DEVELOPMENT OF A *Bos gaurus/Bos taurus* INTERSPECIES MODEL FOR THE ANALYSIS OF GENOMIC IMPRINTING IN THE BOVINE

INTRODUCTION

Genomic imprinting is the parental control over expression of alleles of particular genes. Genes involved in 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 (Reik et al. 2003b). A large proportion of imprinted genes in humans and mice reside within clusters and exhibit enhancer competition and regional inactivation through "spreading" from *cis* acting regulatory elements (Lopes et al. 2003). Evidence to date suggests that genomic imprinting is regulated in part by DNA methylation, which is achieved by sequence and developmental stage specific DNA methyltransferases (Dnmt1, Dnmt1o, Dnmt2, Dnmt3a, Dnmt3a2, Dnmt3b) (Ehrlich 2003). Differential expression of alleles within a cell is achieved by differential methylation of CpG islands located within or near imprinted genes (Constancia et al. 1998). Allelic repression, in the presence of transcription factors, results from the recruitment of methylation specific proteins (MeCP2, MBD2, MBD3 and HDACS) that insulate regions from the initiation of transcription (Fuks et al. 2003).

Genomic imprinting is believed to exist in all placental mammals and has been shown to exist in some marsupials (Murphy and Jirtle 2003). Approximately fifty imprinted genes have been identified in humans and seventy in the mouse (Surani 2001). In livestock, eleven imprinted genes have been identified in sheep (*Gtl2, Dlk1, Dat, Peg11, Peg1, Mest, Meg8, Igf2, H19* and *Igf2R*) (Bidwell et al. 2001; Charlier et al. 2001; Feil et al. 1998; Young et al. 2001), one in cattle (*Igf2R*) (Killian et al. 2000) two in pigs (*Igf2* and *Igf2R*) (Jeon et al. 1999; Killian et al. 2001; Nezer et al. 1999), 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 somatic cell clones, improper reprogramming of genome wide DNA methylation and imprinted genes has been implicated as the reason for the increased incidences of death and placental and fetal abnormalities. Recent reports of abnormal genomic imprinting in cloned mice derived from cumulus and embryonic stem cells as well as aberrant patterns of X chromosome inactivation in female bovine clones support this hypothesis (Dean et al. 2001; Eggan et al. 2000; Xue et al. 2002).

Currently, the limitation of identifying imprinted genes in cattle is due to the lack of informative polymorphisms in coding regions. In mice 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* X *Mus spretus*) (Feil et al. 1998; Hagemann et al. 1998; Villar et al. 1995; Villar and Pedersen 1997).

To facilitate the identification of imprinted genes in the bovine, we have utilized interspecific crosses between *Bos gaurus* (Gaur) and *Bos taurus* (domestic) cattle.

Crosses between Gaur and domestic cattle have been used extensively to increase the genetic variation that exists between alleles. Riggs et al. (1997), Gao et al. (1997), and Ya-Ping and Womack (1997), have utilized offspring from Gaur bulls crossed with Limousine and Holstein cows (*Bos taurus*) for the generation of backcross panels for linkage analysis studies. The use of interspecific hybrids in this model maximizes heterozygosity within coding regions, so that the frequencies of single nucleotide polymorphisms (SNPs) between parents are increased. Parental SNPs can then be used to assess allelic expression and parental inheritance of genes in resulting offspring. Information obtained from this *Bos gaurus/Bos taurus* interspecies hybrid will facilitate the wide scale identification of imprinted genes in the bovine as well as serve as an experimental model to better understand nuclear reprogramming in the bovine.

The purpose of this experiment is to characterize the allelic expression patterns of genes previously identified as imprinted in humans and mice, and to serve as a model for analysis of genomic imprinting in cloned pregnancies.

Materials and Methods

Identification of single nucleotide polymorphisms within putative imprinted genes in *Bos gaurus* and *Bos taurus* cell lines

Male and female *Bos gaurus* and *Bos taurus* cell lines were obtained as a generous gift from Dr. James Womack (Texas A&M University). Cell lines were

cultured until confluent and genomic DNA was extracted (Promega DNA isolation kit). A total of twenty-six candidate genes were chosen from those previously identified as imprinted in humans and mice. Additionally, 3 genes were chosen that reside on the X chromosome (Table 2.1), and undergo inactivation in females. Bovine sequence for the *IGF2* (Accession # AJ320234, AF416605, Z68151), *WT1* (Accession # AF202074, AF201738), *IGF2R* (Accession # NM_174352, AF342811), *SNURF* (Accession # NM_174463, AF101040), *SNRPN* (Accession # NM_174463, AF101040), GABRB3 (Accession # NM_17542), *CDKN1C* (Accession # NM_174016, L26548, L26547), *XIST* (Accession # AF104906, AJ421481l) and *DLK1* (Accession # NM-174037) loci are published in Gene Bank, whereas sequence for the remaining genes was obtained through BLAST (Gene Bank) searches of bovine Expressed Sequence Tags (ESTs) using known genes in either mouse, human or sheep and include *GTL2* (Accession # gi24333950), *MEG3* (Accession # gi29736097), *PLAGL1* (Accession # gi4505854), PEG9 (Accession # gi30054643), MAGEL2 (Accession # gi18765721), *GABRA5* (Accession # gi6031207), *NCD* (Accession # gi6754805), *UBE3A* (Accession # gi19718763), *ARH1* (Accession # gi10835048), *P73* (Accession # gi4885644), *HTR2A* (Accession # gi10835174) and *ZNF215* (Accession # gi 7019582). Positive EST matches were subsequently aligned to all known available sequences to ensure that the correct sequences were utilized for primer design.

Primers were designed on MacVector 6.0 (Table 2.1), and if possible, designed so as to flank introns. 50 □l PCR reactions were run in duplicates and consisted of 5 □ls 10X PCR buffer (Promega), 4 □ls 25 mM MgCl2, 1.25 □ls 10mM dNTPs, 2.5 □ls 3 M

forward primer, 2.5 µl 3 M reverse primer, 2 µls DNA and 1 µls Taq (Promega) PCR. All reactions were initially run with cycling parameters of: 94°C (5min) (denaturation); 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]. Those primer sets that did not amplify under these conditions were then amplified on a Techne Thermocycler that is capable of cycling reactions on an annealing temperature gradient. The cycle parameters were: 94°C 5min (denaturation);94°C (30 sec), 55°C Δ 12°C (30 sec), 72°C (3 min) [25 cycles]. Amplicons resulting from PCR were resolved on a 2% EthBr agarose gel and gel purified (Qiagen Gel Purification Kit). Two to four µls of purified product, depending on intensity of bands, were then used as templates for sequencing reactions. Forward primers used to amplify regions were also used as sequencing primers. Twenty-five µl sequencing reactions using Big Dye Terminator mix (ABI Biosystems) and forward primers used for amplification were run on a Perkin Elmer Thermocycler at 94°C (30 sec), 50°C (30 sec), 60°C (4 min) [25 cycles]. Sequences were then analyzed on either an ABI 370 or 3700. Sequences obtained for each of the genes from DNA extracted separately from *Bos gaurus* and *Bos taurus* cell lines were aligned on MacVector Pustell (Oxford Molecular) query and analyzed for polymorphisms between sequences.

Table 2.1. Putative imprinted genes in the bovine selected for the analysis of SNPs between *Bos gaurus* and *Bos taurus* species of cattle. Forward and reverse primers used for amplification in the bovine.

Locus	Forward primer (5'-3')	Reverse primer (5'-3')
Gene trap locus 2 *(GTL2)*	CCCACCAGCAAACAAAGCAAC	CATCAAGGCAAAAAGCACATCG
Insulin like growth factor 2 *(IGF2)*	CAAGGCATCCAGCGATTAG	TTCAAGGGGGCTGATTGAG
Maternally expressed gene 3 *(MEG3)*	CCCATCATTATTGCTAAGCGTCC	CCATCATCTGGAATCCTCCGTG
Impact *(IMPT)*	CCTGCCTTCCAAACAGTATCTGC	TGCCAGTATGAAAGAGCCAGTAGC
Wilms tumor 1 *(WT1)*	ATCACAAGCAACCCCATTCAAC	GGAGAGCAAAGTCCCATCTGTAGTG
Pleiomorphic adenoma gene-like 1 *(PLAGL1)*	GCTATCCTGCCTCATTTCCAACC	TCCAAACCTTCCACAGTTCCC
Insulin like growth factor 2 receptor *(IGF2R)*	TCCCCCACCACCAACACTC	ACGGCGACGAGCAGGATAG
Small nuclear RING finger protein *(SNURF)*	GGGACCGTTTACACTTGAGAC	TGAGTTCTGCCTGGAAATCC
Small nuclear ribonucloprotein polypeptide N *(SNRPN)*	GTTTTGGGTCTGGTGTTGTTGC	GGGTCATTACCTGTTGAGATGGC
Paternally expressed gene 9 *(PEG9)*	CCCTCCCACTACATTTGCATAG	GCTGCATGTTCTGCTGC
MAGE like protein 2 *(MAGEL2)*	GYGGGCAGGTGTGTCCTATTTG	TGGTGGGGTCATCGGTTTTATC
Gamma amino butyric receptor acid 5 *(GABRA5)*	CGCCTTCCACTCAACAATCTCC	ATCTTCTGCCACCACCACTGAC
Gamma amino butyric receptor base 3 *(GABRB3)*	GGGCTGCTTTGTCTTTGTGTTTC	GTAGATGGGTCTTCTTGTGCGG
Neruonatin *(NNT)*	GAAACCTACCAGCAGTTCTTGGAC	CTTGCCATTCTTCTTCCGATTG
Necdin *(NCD)*	GCTTTGACCAGCGAAAAC	CATCGGCAGTTACAAGAAGTG
E6-ubiquitin-protein ligase gene *(UBE3A)*	TCTGAGGGGCAATGTGTATGTTC	ATTTTCCATCGGGTCACTGGGCAG

Table 2.1 continued

Locus	Forward primer (5'-3')	Reverse primer (5'-3')
P73	CCCAGCTCCACCTTCGACACC	CAGGCATGGCCCGGATGGC
H19	CCAGGCATGAGCTGGGTAGC	CACTTCACCCACTGTAATTCC
X- chromosome inactivation specific transcript *(XIST)*	GAACATTTTCCAGACCCCAAC	AAACCAGGTATCCACAGCCG
Serotonin 2-A receptor *(HTR2A)*	CCTGTTTGTGGTGATGTGGTGC	TTGACTGCTGAAAAGAGGTAACCG
Delta like gene 1 *(DLK1)*	TTCTGCGACGATGACAGTTGTTGC	TTCCTGACAATCCTTTCCTGAG
Zinc finger 215 *(ZNF215)*	AAATGTCAGGAATGTGAGAGAGCC	CGTTTGTGCTTTTGGAAGGAAGTG
Growth factor bound receptor 10 *(GRB10)*	AGAAGATGCTGCTGTGGTGGAG	CCAGTAGTTGTCCTGAGATTCAAGG
CD81	TGTGGGCATCTACATCCTTATCG	ACAGAGGTGGTCAGTGTCATCAGC
Aplysia ras homolog 1 *(ARH1)*	TTCCTCTCCTCCTCCAATGTCC	ACCCTCTTCTTTAGTGTGCCG
Cyclin dependent kinase inhibitor *(CDKN1C)*	CCGACAGCCAGCACATTGG	GCCCCGAAATCCCTGAGTG
Three prime repair exonuclease 2 *(TREX2)*	GGCAAGTACATGGGCTCGATG	CTCTTCCACCGCTACTTCC
L1 cell adhesion molecule *(L1CAM)*	GGTGTAGTGGACACATAGGG	GAGACCTCCAGGCCAATGACAC
Androgen receptor *(AR)*	GCCTCAATGAACTGGGTGAAAG	GCAGGTCAAAAGTGAACTGATGC

Generation of day 72 control *Bos gaurus /Bos taurus* hybrids

Bos gaurus semen was obtained as a generous gift from the Dr. Nadia Luskotoff at the Henry Doorly Zoo in Omaha, NE. Six *Bos taurus* (Angus) cross heifers of approximately 18 months of age (7/8 Angus 1/8 Brangus) were synchronized for estrous by serial injections of Prostaglandin F2alpha (Lutalyse) (2 shots 11 days apart with estrous detection 3 days post second shot). Upon detection of estrus, heifers were transvaginally inseminated following the am/pm rule (a.m. detection of estrous, pm insemination/ p.m. detection of estrous, a.m. insemination). Heifers were checked for establishment of pregnancy at day 28 through transrectal ultrasonography. Pregnancy of the six heifers was established after two rounds of insemination. At day 72 of gestation, pregnant heifers were slaughtered at the Texas A&M Meat Science Center, at Texas A&M University, College Station, TX, and reproductive tracts removed. Reproductive tracts were transported to a lab on ice for processing and tissue collection. 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 Ambion RNA aqueous kit and resuspended in 10 μg aliquots in DEPC H_2O and stored at – 80 ^oC. 2 μg of RNA for each sample was DNase I treated using the Ambion DNase I Kit and subsequently converted to cDNA through the Ambion First Strand Synthesis Kit for all RT-PCR reactions. DNA was extracted from frozen tissues using the Promega Wizard DNA Extraction Kit and resuspended at 20 ng/μl and stored at –20 ^oC until further use.

Analysis of allelic expression through direct sequencing method

RT-PCR of the *IGF2*, *GTL2*, *WT1* and *XIST* loci was performed on chorion, allantois, liver, lung and brain. Amplicons resolved on 2% EthBr agarose gels were gel extracted (Qiagen Gel Extraction Kit), resuspended in 50 μls of dH_20 and used directly as sequencing template. Sequencing primers consisted of forward primers used in the amplification of each of the four RT-PCR reactions. Twenty-five μl sequencing reactions using Big Dye Terminator mix were run on a Perkin Elmer Thermocycler at 94^{o}C (30 sec), 50^{o}C (30 sec), 60^{o}C (4 min) [25 cycles]. Cleanup of sequencing reactions was performed in 800 μl Sephadex columns (Sigma). Sequences were then run on either an ABI 370 or 3700 and sequence chromatograms were visually analyzed for the presence or absence of both SNPs. RT-PCR and sequencing reactions were run in triplicates. To confirm the absence of genomic contamination in cDNA samples, an internal control was utilized through the *IGF2* amplicon, which spanned intron 6.

Genomic contamination results in the presence of an additional 1kb band (Figure on pg. 48).

Bisulfite treatment of genomic DNA

Genomic DNA was isolated (Promega Wizard DNA Isolation Kit) from the chorion of hybrid females (n=4). The sodium bisulfite reaction was carried out with one μg of DNA from each sample using the CpG DNA Conversion Kit (Intergenco). Sodium bisulfite catalyzes the deamination of cytosines to uracils (thymines), where as methylated cytosines (m5C) are protected. This technique allows for the rapid identification of m5C in genomic DNA. Genomic DNA was denatured through incubation of 3M NaOH at 37^{o}C. Denatured DNA was then incubated for 16-20 hrs at 50^{o}C in the presence of 3 M Sodium bisulfite and 0.5 mM hydroquinone. Carrier glycogen was added to bisulfite treated DNA and incubated at room temp for 5 min. DNA was then washed, centrifuged (13,000 rpm) and vortexed in successive (3X) volumes of 90 % and 70% ethanol. DNA was then resuspended in 50 μl TAE and incubated at 60^{o}C for 15 min. The DNA samples were centrifuged at 13,000 rpm for 30 sec and the supernatant containing DNA removed to a new tube. 1.5 μl of the supernatant was used in subsequent PCR reactions.

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 (Accession # AF104906, AJ4214811). Sequence was obtained from Gene Bank for the *XIST* promoter, gene and 3' regions extending approximately 45 kb downstream in the human (Accession # U50908), mouse (Accession # AJ421479), horse (Accession # U50911), rabbit (Accession # U50910) and bovine (Accession #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 (expected = # Cs X # Gs) [observed / expected = < 0.60]. 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 50-100%.

DNA methylation analysis of the *XIST* Differentially Methylated Region (DMR) in exon1

PCR primers were designed flanking the bovine *XIST* CpG island at +828 to +1183, which was previously detected using the CpG prediction software (*XIST5Bis1*, *XIST5Bis2* and *XIST5Bis3*). Primers flanking the region were designed by converting

all cytosines in the sequence that are not adjacent to guanines to thymines. This is the predicted sequence after bisulfite conversion of DNA with all CpG dinucleotides protected (methylated). Cycle sequencing parameters were: 95°C (5 min) (denaturation); 95°C (30 sec), 52°C (30 sec) 72°C (2min, 30 sec) [35 cycles]; 72°C (10 min). Products were resolved on a 2% EthBr agarose gel and gel purified (Qiagen Gel Purification kit). Purified products were then cloned into TOPO4 sequencing vectors. Plasmids from an average of 20 colonies were extracted (Qiagen Mini prep) and sequenced separately. Sequencing reactions consisted of varying primer concentrations (3 μM to 3 pM) of M13 universal primers (5'-CTGGCCGTCGTTTTAC-3'). 25 μl sequencing reactions using Big Dye Terminator mix were run on a Perkin Elmer Thermocycler at 94°C (30 sec), 50°C (30 sec), 60°C (4 min) [25 cycles] and products were subsequently sequenced on an ABI 3700 sequencer.

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Table 2.2. Sequence of the CpG island in exon 1 of the bovine *XIST*. **(A)** Sequence of the bovine *XIST* DMR (top strand) and bisulfite converted sequence (bottom strand). CpG dinucleotides are in bold. **(B)** Primer information for *XIST5Bis1, XIST5Bis2* and *XIST5Bis3* used to amplify the *XIST* DMR.

(A)

TGGATATCATGGCAGTTTGTCA**CG**TGGATAT**CG**TGGCAGGGGTGTTTGAC
TGGATATTATGGTAGTTTGTTA**CG**TGGATAT**CG**TGGTAGGGGTGTTTGAT

CGTTACATTCTTGG**CG**GGCTTTGCATCAGGAGGGCCTGC**CG**CATTGTTAA
CGTTATATTTTTGG**CG**GGTTTTGTATTAGGAGGGTTTGT**CG**TATTGTTAA

AGATGG**CG**TGCTTTGC**CGCG**GACAAAGTGAAAGGAGGGATTGGCAATGTT
AGATGG**CG**TGTTTTGT**CGCG**GATAAAGTGAAAGGAGGGATTGGTAATGTT

AGATTGC**CGCG**TGTCCCACCCAATCAGAAAGGGTGGTAGAAT**CG**GTCACA
AGATTGT**CGCG**TGTTTTATTTAATTAGAAAGGGTGGTAGAAT**CG**GTTATA

GCCAGTT
GTTAGTT

(B)

Locus	Forward primer (5'-3')	Reverse primer (5'-3')
XIST5Bis1	GGAAGGTAAGATGAATAATGYG	AACAATACRACAAACCCTCC
XIST5Bis2	GGGTTTTTGTTTTTGTYGTGT	CCTCCTTTCACTTTATCCRC
XIST5Bis3	TTTGTTGTAGGGATAATATGGT	CCACCCTTTCTAATTAAATAAAA

RESULTS

Identification of Single Nucleotide Polymorphisms (SNPs) within candidate imprinted genes in the bovine

A total of twenty-six genes were randomly selected from a list of previously identified imprinted genes in other species (Table 2.1). Bovine sequences of candidate genes were obtained from published sequences (Gene Bank), or human, mouse and sheep sequences were obtained from Gene Bank and a BLAST search was performed against bovine EST libraries. High percent homology matches were selected and aligned with the sequences of other species to ensure the correct sequences were chosen for primer design. Two independent *Bos gaurus* and two independent *Bos taurus* fibroblast cell lines served as genomic templates for amplification and determination of SNPs. Amplification of genomic DNA using the 29 primer pairs resulted in products from approximately 24 genes and readable sequences were obtained from 20 of these genes (*GTL2, IGF2, MEG3, IMPT, WT1, IGF2R, SNRPN, PEG9, MAGEL2, GABRA5, GABRB3, NNT, NCD, P73, H19, XIST, HTR2A, DLK1, ZNF215* and *AR*).

Sequence analysis of the coding regions of twenty putative imprinted genes in the bovine identified four informative SNPs between the *Bos gaurus* and *Bos Taurus,* (Fig 2.1). Insulin like growth factor 2 *(IGF2)* the major somatomedin in fetal development, Gene trap locus 2 *(GTL2)* an untranslated transcript associated with the callipyge overgrowth syndrome in sheep, Wilms tumor 1 *(WT1)* associated with fetal kidney tumors and X inactivation specific transcript *(XIST)*, a RNA transcript directing inactivation of one of the two X chromosomes in females, all contained at least one or more polymorphisms between the two species.

Sequence of the bovine *IGF2* gene is available on Gene Bank (accession AF416605), however only partial sequence of mRNA exists in this database, therefore, a BLAST search was performed against bovine EST libraries using the mouse *Igf2* sequence and a more complete sequence of the gene was obtained. Three primer sets (*IGF2-1, IGF2-2* and *IGF2-3, IGF2-4*) were designed within exon 5, 6 and the 3' untranslated region (UTR) in an attempt to locate SNPs between species. A SNP was located in primer set *IGF2-3* and *IGF2-4* at position 226 of the sequence chromatogram in the 3' UTR and was utilized for parental discrimination between alleles (Fig 2.1).

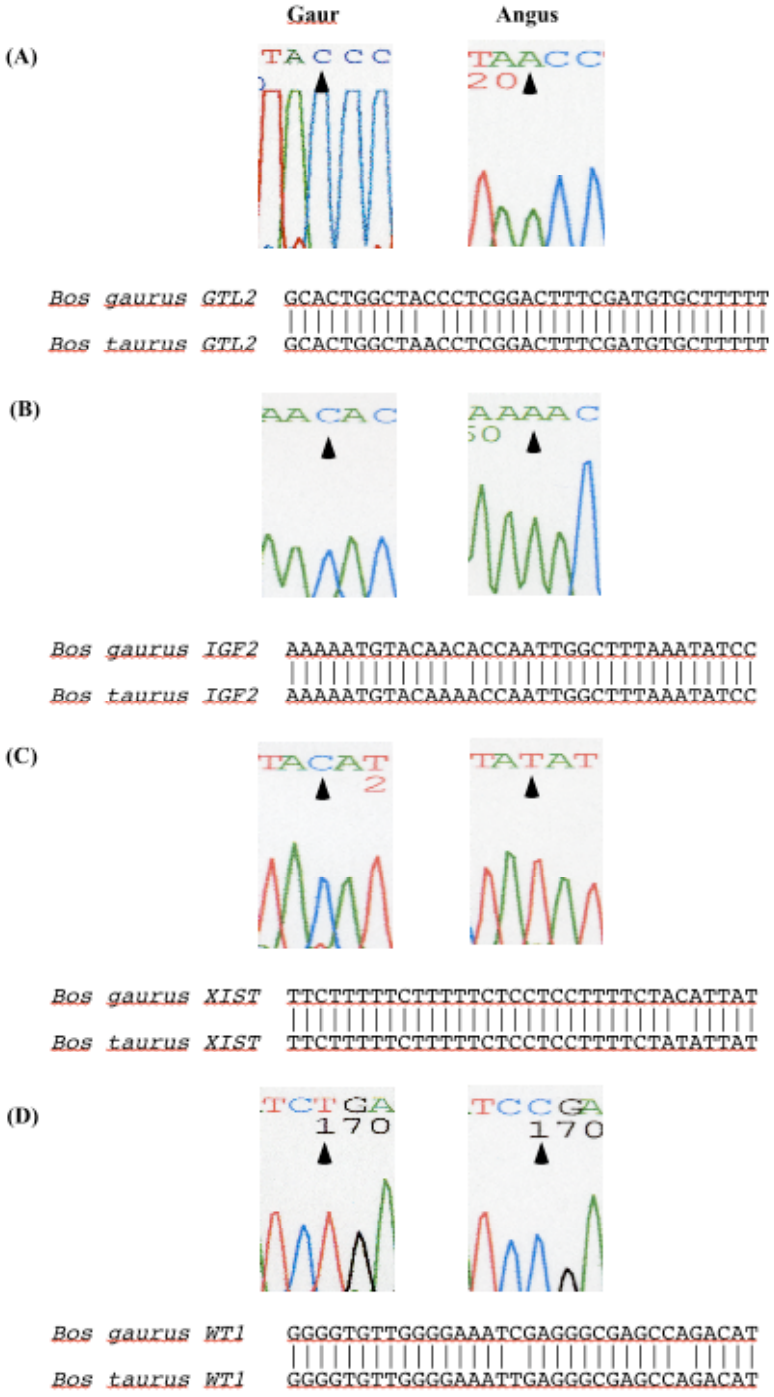


Figure 2.1. Sequence chromatograms of the *IGF2, GTL2, WT1* and *XIST* loci. Sequence chromatograms of genomic amplified **(A)** *GTL2*: *Bos gaurus* (C allele) *Bos taurus* (A allele), **(B)** *IGF2*: *Bos gaurus* (C allele) *Bos taurus* (A allele), **(C)** *XIST*: *Bos gaurus* (C allele) *Bos taurus* (T allele) and **(D)** *WT1 Bos gaurus* (C allele) *Bos Taurus* (T allele) loci from *Bos gaurus/ Bos taurus* hybrid fetuses.

The allele possessing an (C) polymorphism, termed *IGF2* allele C, is inherited from the Gaur (paternal transmission) and the (A) polymorphism, termed *IGF2* allele A, is inherited from the Angus (maternal), as was confirmed through subsequent sequencing of Gaur and fetus *IGF2-4* (Fig 2.1). *IGF2-4* primers span exon 5 through exon 6 (as was determined through homology with known mouse and human exon/intron boundaries) into the 3' UTR. The size of genomic amplification is approximately 1kb (Fig 2.4) and when RNA is spliced, results in product of approximately 750 bp (Fig 2.4). This primer set (*IGF2-4*) provided a genomic contamination control for all remaining reactions in all tissues.

Sequence for the *GTL2* locus in the bovine is not published in Gene Bank, therefore, a BLAST search was performed against bovine EST libraries using the published sheep sequence (Accession # AY017222) and then confirmed with the human and mouse sequence. One primer set was designed within exon 1 of the *GTL2* gene and C/A polymorphism was detected at position 123 of sequence chromatograms between the *Bos gaurus* and *Bos taurus* cell lines as well as animals utilized for model. The (C) polymorphism, termed *GTL2* allele C, is inherited from the Gaur (paternal) and an (A) polymorphism, termed *GTL2* allele A, is inherited from the Angus (maternal) (Fig 2.1).

Sequence for the bovine *WT 1* exons 8 and 9 are published in Gene Bank (Accession # AF202074, AF201738) and a single primer set was designed in exon 9. A C/T polymorphism was detected at position 160 of the *WT1* sequence chromatogram with the paternal allele, termed *WT1* allele T, and maternal allele, *WT1* allele C.

The bovine *XIST* sequence and adjoining regions are available on Gene Bank (accession # AF104906). Four primer sets were designed within exon 1 (*XIST1, XIST2, XIST3* and *XIST4*) and a T/C SNP was detected between the Gaur and Angus within *XIST4* primers. The (C) polymorphism, termed *XIST* allele C, is paternally inherited (Gaur) and the (T) polymorphism, termed *XIST* allele T is maternally inherited (Angus).

Generation of *Bos gaurus/Bos taurus* interspecies hybrids

Bos gaurus (Gaur) and *Bos taurus* (Angus) hybrid fetuses and placentas were obtained at day 72 of gestation (Fig 2.2). Samples derived from the placenta (chorion and allantois), and fetus (lung, liver and brain) were isolated, weighed and frozen for future analysis of allelic expression of candidate genes possessing SNPs. A total of six hybrid fetuses were produced: four female and two male. By day 72 of gestation, placental and fetal components are entirely established. Table 2.3 summarizes weights and measurements obtained from each of the hybrid fetuses.

Additionally, biparietal (BP) and crown rump length (CRL) growth curves of three of the fetuses (fetuses A, B and C) were obtained starting at day 36 of gestation (day 0= day of insemination) through trans rectal ultrasonography and were taken again every 6 days until day 60, and are summarized in Table 2.4.

Table 2.3. Weights and measurements of the *Bos gaurus/Bos taurus* day 72 hybrids (Fetus A, B, C, D, E and F).

Fetus	Fetus Weight (g)	Placental Weight (g)	CRL (cm)	Allantoic fluid (mls)	Amniotic fluid (mls)	Cotyledon number	Brain Weight (g)	Liver Weight (g)	Lung Weight (g)	Heart weight (g)	Sex	Dam Weight (lbs)
A	X	X	11	400	220	69	X	X	X	X	F	900
B	52.2	60.8	11.7	420	295	52	X	X	X	X	F	920
C	61.7	66.7	12.3	350	240	87	X	X	X	X	F	945
D	55.7	76.2	11	485	235	67	2.01	1.66	2.0	.34	F	960
E	62.9	75.7	11.7	360	260	47	2.28	2.19	1.83	.635	M	970
F	66.82	125.0	11.9	450	270	96	2.11	1.42	2.38	.445	M	1158

*** X indicates no measurement was obtained.**

Table 2.4. Biparietal and crown rump length measurements of the *Bos gaurus/Bos taurus* day 72 hybrid control fetuses. **(A)** Biparietal diameter and **(B)** crown rump length measurements of hybrid fetuses from day 25 (day 36 biparietal) to day 60 of gestation.

Crown-Rump(mm)	Fetus A	Fetus B	Fetus C
Day 25	3.5	3.6	4
Day 30	11.2	8.9	8
Day 36	16.8	15.8	17
Day 42	28.8	25.1	26
Day 48	37.1	37.5	36
Day 54	53.5	57.8	57
Day 60	68	69.2	62.5

Biparietal (mm)	Fetus A	Fetus B	Fetus C
Day 25	X	X	X
Day 30	X	X	X
Day 36	8.3	8	8
Day 42	9.9	9.5	9
Day 48	13.9	15	12
Day 54	15.1	15.8	14
Day 60	18.7	19.2	20.3

*** X indicates measurements were not obtained.**

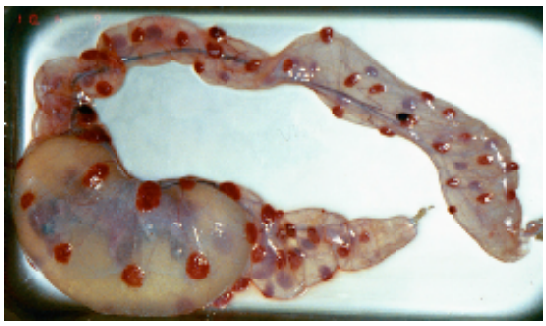
(A)

Bos gaurus (Gaur)

(B)

Bos taurus (angus)

(C)



Day 72 hybrid fetus and placenta

Figure 2.2. Schematic of *Bos gaurus* and *Bos taurus* animals utilized for the production of day 72 hybrid fetuses. **(A)** *Bos gaurus* sire (Gladys Porter Zoo, Brownsville TX) and **(B)** *Bos taurus* Angus dam (Texas A&M University) utilized for the production of interspecific hybrids. **(C)** Day 72 *Bos gaurus/Bos taurus* placenta and fetus isolated for the analysis of genomic imprinting in the bovine. Six fetuses (4 female and 2 male) were generated.

Allelic expression analysis of putative imprinted genes in the bovine

Insulin like growth factor 2

Amplification of the each *IGF2* primer set (*IGF2-1, IGF2-2, IGF2-3* and *IGF2-4*) produced strong bands for tissues examined. *IGF2-4* spans intron 6 and thus gave two different size bands when amplified from cDNA and genomic DNA (Fig 2.4). The C/A SNP was detected in genomic amplified products for all of the fetuses (n=6). Allelic expression analysis of directly sequenced RT-PCR products using the *IGF2-3* primer set in chorion, allantois, liver, lung and brain in all fetuses showed preferential expression of the paternal allele (C allele). These results indicate maternal genomic imprinting at the *IGF2* locus in the bovine. Figure 2.3 demonstrates the inheritance of the C allele from the Gaur (A) and the presence of the C/A SNP in genomic amplified DNA from hybrids (B). Differential expression of the locus is demonstrated in (C), where the A allele (maternal) is absent from the sequence chromatogram.

(A) Gaur genomic PCR **(B)** Hybrid genomic PCR **(C)** Chorion RT-PCR

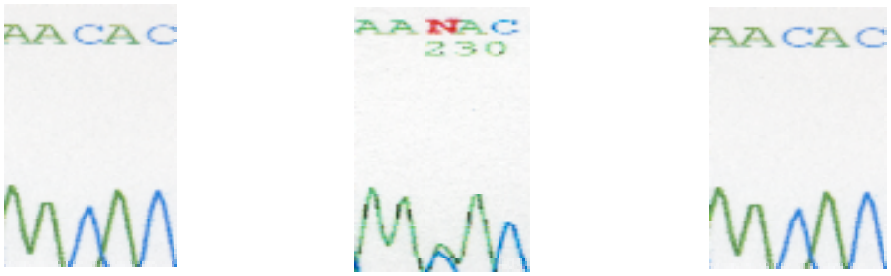


Figure 2.3. Sequence chromatogram of the *IGF2* transcript. **(A)** Sequence chromatogram of *IGF2-3* amplified from *Bos gaurus* genomic DNA. **(B)** Sequence chromatogram of hybrid fetus A *IGF2-3* amplified from genomic DNA. A *A/C* SNP exists between the *Bos gaurus* (C) and *Bos taurus* (A) parents. **(C)** Sequence chromatogram of hybrid fetus A liver *IGF2-3* from cDNA. The paternal (A allele) is preferentially expressed in liver (shown), lung, brain, chorion and allantois (data not shown).

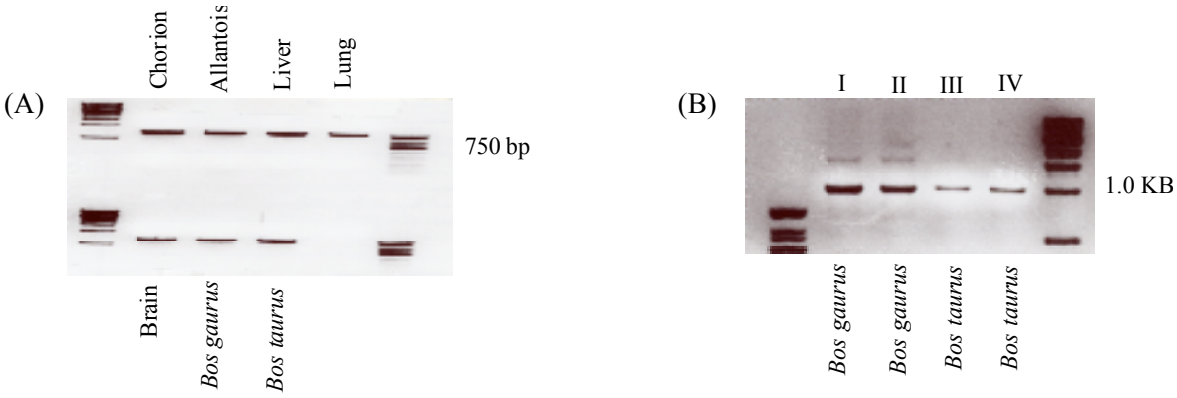


Figure 2.4. RT-PCR amplicons of the *IGF2* transcript. **(A)** 2% agarose gel of *IGF2-4* amplified from: Hybrid chorion, allantois, liver lung brain, *Bos gaurus* cultured fibroblast cells and *Bos taurus* cultured fibroblast cells. **(B)** 2% agarose gel of amplified *IGF2-4* from genomic DNA (lanes I and II amplified from *Bos gaurus* fibroblast cell line and lanes III and IV amplified from *Bos taurus* fibroblast cell line). Primers flank intron 5 and can be used as an internal control for genomic contamination.

Gene trap locus 2

High levels of expression of *GTL2* were detected in brain, liver, lung, chorion and allantois. The C/A SNP was detected in all animals investigated (n=6). The maternally inherited *GTL2* allele A, was preferentially expressed in all sequences for all tissues examined (chorion, allantois, liver, lung and brain) thus indicating paternal imprinting at this locus (Fig 2.5).

(A) Gaur genomic PCR **(B)** Hybrid genomic PCR **(C)** Chorion RT-PCR

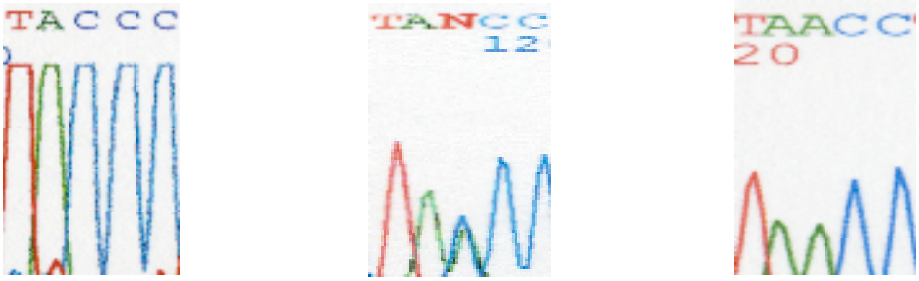


Figure 2.5. Sequence chromatograms of the *GTL2* transcript **(A)** Sequence chromatogram of *GTL2* amplified from *Bos gaurus* genomic DNA. **(B)** Sequence chromatogram of *GTL2* amplified from hybrid fetus A genomic DNA. An *A/C* single nucleotide polymorphism exists at position 119 between the *Bos gaurus* (C) and *Bos taurus* (A). **(C)** Sequence chromatogram of *GTL2* amplified from hybrid fetus A cDNA. The maternal A allele is preferentially expressed in liver (shown), brain, lung, chorion and allantois (data not shown) (paternal imprinting).

Wilms tumor 1

Expression of *WT1* was detected faintly in chorion and allantois, but exhibited strong bands in liver, lung and brain. Serial rounds of PCR were performed on chorion and allantois so that sufficient template could be obtained for the sequencing reaction.

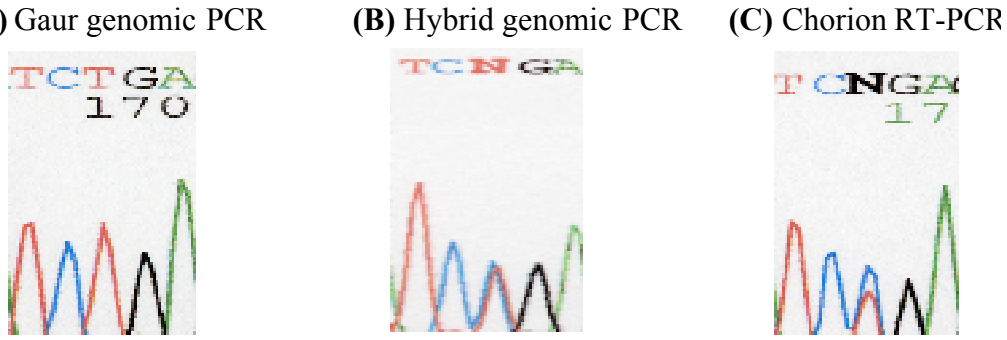


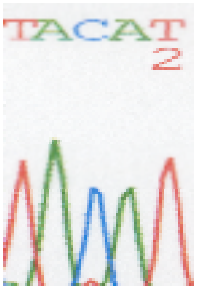
Figure 2.6. Sequence chromatograms of the *WT1* transcript. **(A)** *Bos gaurus WT1* amplified from genomic DNA, **(B)** Fetus A hybrid *WT1* amplified from genomic DNA and **(C)** Fetus A chorion amplified from cDNA. A *T* SNP is inherited paternally inherited (Gaur) and *C* SNP is inherited maternally (Angus). Chromatogram analysis of the *WT1* in chorion (shown), allantois, liver, lung and brain (data not shown) indicates lack of genomic imprinting at the *WT1* locus in the bovine.

The T/C SNP was detected in all animals (n=6) and analysis of the chorion, allantois, liver, lung and brain in all demonstrated biallelic expression of WT1. To confirm these results, multiple sequencing reactions were performed using RNA that was DNaseI treated 2X to ensure no false reports were obtained. These results indicate an absence of genomic imprinting at the bovine WT1 locus (Fig 2.6).

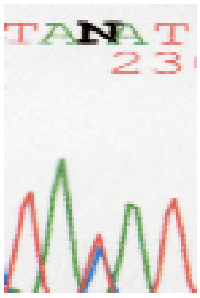
X chromosome inactivation specific transcript

Expression of the *XIST* locus was detected faintly in males while strong bands were detected in chorion, allantois, liver, lung and brain of all females (Fig 2.8). The C/T SNP was only detected in females (as expected) and was detected in the four used for analysis. The allelic expression analysis of the bovine *XIST4* within chorion (placenta) of hybrid females shows preferential paternal expression, whereas in all other tissues

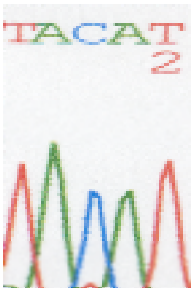
(A) Gaur genomic PCR



(B) Hybrid genomic PCR



(C) Chorion RT-PCR



(D) Liver RT-PCR

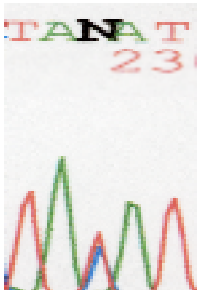


Figure 2.7. Sequence chromatograms of the *XIST* transcript. **(A)** Sequence chromatogram of *XIST 4* amplified from *Bos gaurus* genomic DNA. **(B)** Sequence chromatogram of hybrid fetus A *XIST* 4 amplified from genomic DNA. A C/T polymorphism exists between the *Bos gaurus* (C allele) and *Bos taurus* (T allele). **(C)** Sequence chromatogram of hybrid Fetus A chorion (shown) (female) amplified from cDNA. Paternal allele (C) is preferentially expressed in chorion. Biallelic expression occurs in allantois (not shown), liver (shown), lung and brain (not shown) (chorion specific maternal imprinting).

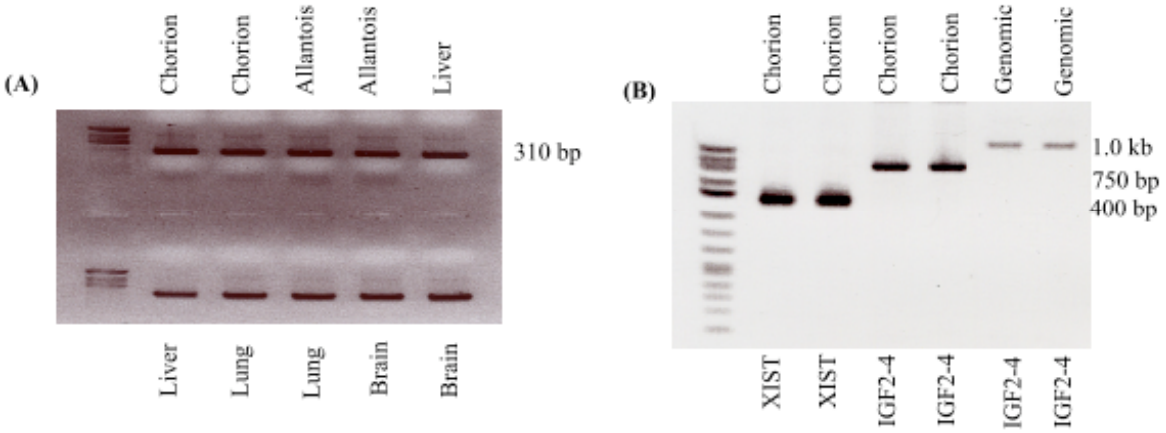


Fig 2.8. RT-PCR amplicons of the *XIST* transcript. **(A)** 2% agarose gel of *XIST4* amplified from chorion, allantois, liver, lung and brain. **(B)** Internal control for genomic contamination of Fetus A chorion resolved on an 8% Polyacrylamide Gel (PAGE). Lanes 1 and 2 are *XIST4* amplified from chorion, lanes 3 and 4 are *IGF2-4* amplified from the same cDNA sample of chorion and lanes 5 and 6 are *IGF2-4* amplified from genomic DNA. *IGF2-4* spans intron 6, thus genomic contamination in cDNA samples can be detected by presence of two bands.

examined (allantois, liver, lung and brain) biallelic expression was observed (Fig 2.7). These results indicate tissue specific maternal genomic imprinting of the *XIST* locus in the bovine.

Comparative sequence analysis of the *XIST* locus

Comparative sequence analysis of the entire region spanning the *XIST* and *TSIX* genes was performed using PipMaker dot plots (Fig 2.9). Since our previous findings indicated genomic imprinting at the *XIST* locus within the chorion, demonstrating concordance with the mouse, an attempt was made to assess the conservation that existed between mice and cattle at regions presumably involved in imprinting at the *XIST* locus. PipMaker plots indicate partial homology in the 5' region of the *XIST*

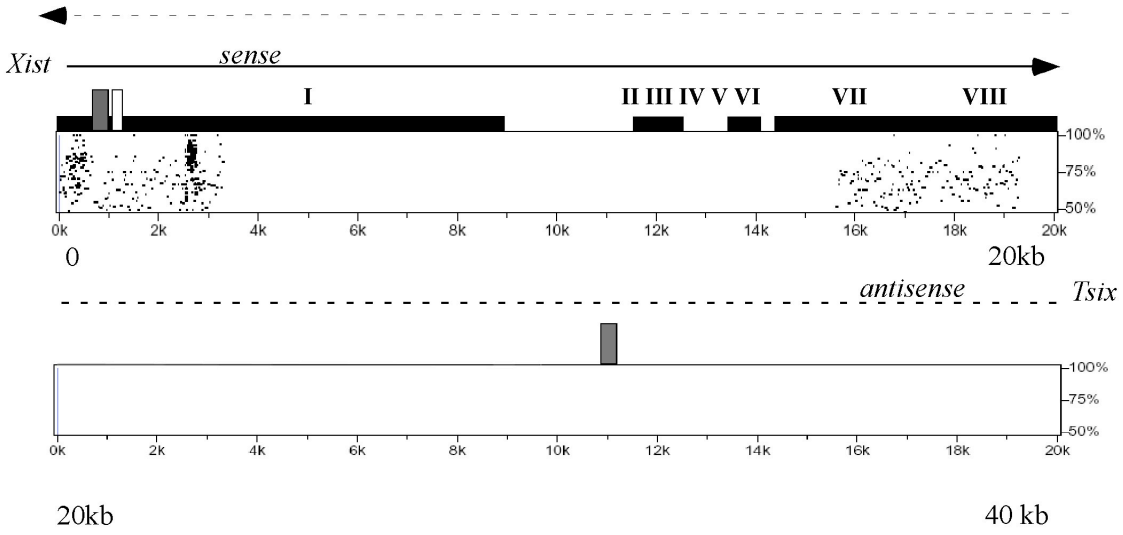
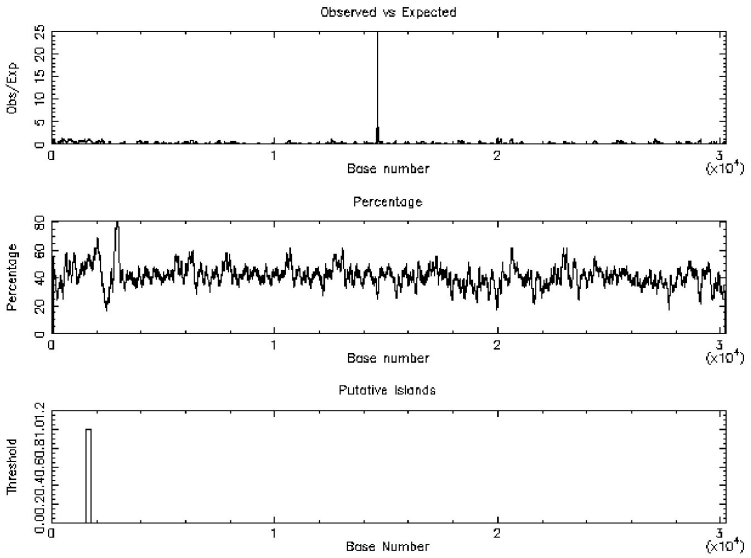


Figure 2.9. PipMaker dot plot schematic of the bovine and mouse *XIST/Xist* locus. PipMaker percent identity plot of the bovine *XIST* region and 3' sequence relative to the mouse *XIST* and *TSIX* genes. Nucleotides 0-40,000 are shown of the X chromosome sequence ranging from 116,296-156,296 in the 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 grey boxes.

between bovine and mice, but homology diminishes as the sequence proceeds into the 3' region and into the *TSIX* 5' region and promoter. Sequence comparison of the –45 minimal promoter region between the horse, human, rabbit, bovine and mouse indicates greater homology between bovine, horse, rabbit and human than between the bovine and mouse (unreported). These finding indicate relatively low levels of homology between the mouse and bovine *XIST* and virtually no homologous regions in the 3' region extending into the *TSIX* promoter.

Comparative analysis of the mouse and bovine was extended to regions presumably associated with the imprinting of the *XIST* locus in the preimplantation embryo and placenta. CpG island prediction of 40 kb of DNA extending from the *XIST* promoter into the adjacent *XIST* antisense, *TSIX*, revealed that only one island located in the 5' region of exon 1 is conserved between the mouse and bovine (Fig2.9). This is consistent with our earlier finding of relatively low or no homology in the 5' region of *TSIX*.

(A) Bovine *XIST* 0-30kb



Bovine *XIST* 30-45kb

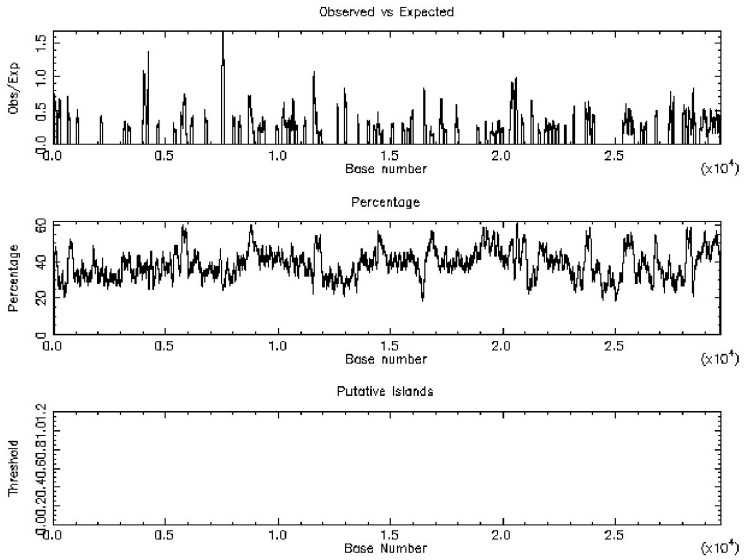
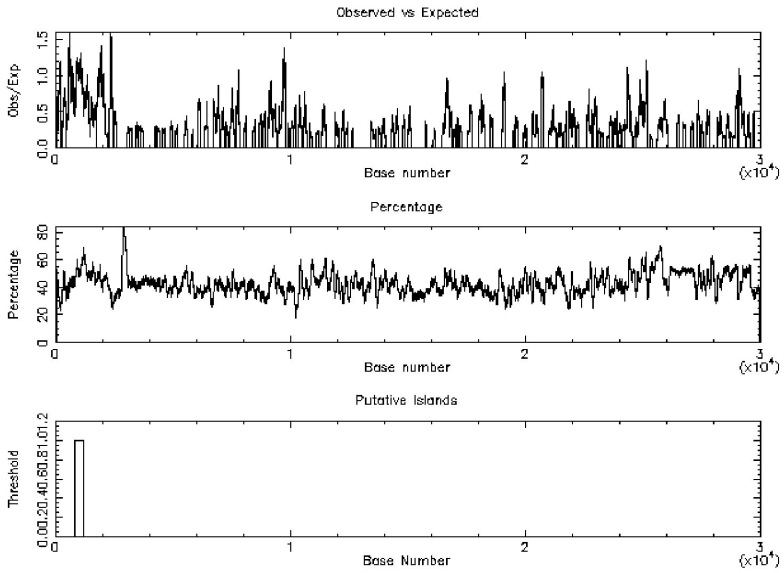


Figure 2.10. Schematic of CpG island prediction in exon 1 of the bovine and mouse *XIST/Xist* locus. CpG island prediction of the **(A)** Bovine and **(B)** Mouse *XIST/Xist* and 3' downstream regions. 45 kb of DNA were analyzed from start of exon 1 (0) into the Tsix promoter (45kb). Horizontal boxes: (Top) indicate observed/expected CG within regions, (Middle) percent CG in region and (Bottom) presence of CpG island (observed/expected > 0.60) denoted by vertical boxes. Primers were designed to flank the bovine CpG island region detected in this analysis.

(B) Mouse *Xist* 0-30kb



Mouse *Xist* 30kb-45kb

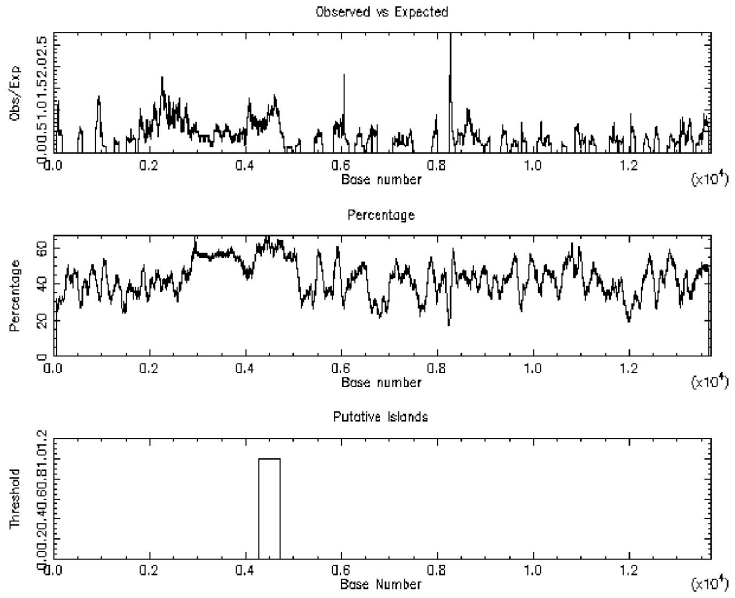


Fig 2.10 Continued

Analysis of the minimal promoter (-45 to –36) between the mouse, bovine, human, horse and rabbit demonstrated high conservation between the bovine, human, horse and rabbit, but little homology in the promoter region and no homology in the –45 minimal promoter region (Fig 2.11), which has been shown to be differentially methylated in mouse gametes and presumably associated with imprinting at this locus.

Mouse Xist GGCCACTCCTCTTCTGGTCTCTCCGCCTTC-A**GCGCCGCGG**-----ATCA
Bovine XIST GGCCACTCCTCTTCTATTCCCTCCGCCCTC-A**GTCCCCTCC**CTCTCACT
Human XIST GGCCACGCCTCTTATGCTCTCTCCGCCCTC-A**GCCCCCCCCT**T---CAGTT
Horse Xist GGCCACGCCTCTTCTACTTCCTCCACCCCCCA**GTCCCCTCC**CCCTCACT
Rabbit Xist GGCCACGCCTCTTGT--TCACCCCGCCCCC-A**ACCCCCCCCT**----CAATT

Figure 2.11. Comparative sequence analysis of the – 45 region of the *XIST/Xist* promoter between the mouse, bovine, human, horse and rabbit.

DNA methylation analysis of the bovine *XIST* DMR

In the bovine a CpG island was detected at + 1475 to +1681 in exon 1, as was determined by CpG island prediction software (www.ebi.ac.uk/emboss/cpgplot/) (Fig 2.10) and three primer sets (*XIST5Bis1*, *XIST5Bis2* and *XIST5Bis3*) were designed for amplification of bisulfite treated DNA encompassing this area. Samples from a female liver and chorion were chosen since this region had previously been identified as biallelic and monoallelic respectively in all females analyzed. Sequencing of the amplified regions however proved to be difficult; it became apparent after analyzing

multiple sequences from each of the three primer sets, that a secondary structure had formed from the bisulfite conversion and was inhibiting the sequencing reaction from proceeding past a similar region in all sequences. Difficulties sequencing the same region in mice have also been reported (McDonald et al. 1998). Their attempts to bisulfite sequence the region resulted in a failure to obtain a positive cloned transcript. This suggests a structure or motif created in this region after bisulfite modification that makes the sequence difficult to clone and sequence. However, after modifying the cycling parameters, 4, full-length sequences were obtained from *XIST5Bis3* allowing for analysis of methylation and use of the SNP to determine parental origin; however, this was only obtained from sequences resulting from liver samples. These results indicate differential methylation of the *XIST* DMR in exon 1 in female livers, in concordance with the mouse and human.

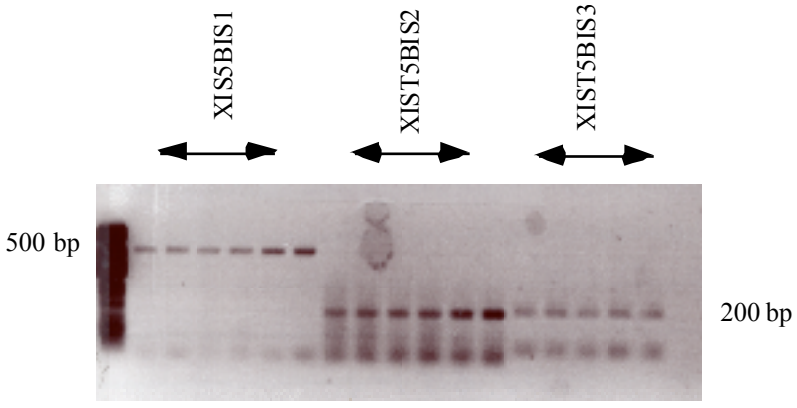


Figure 2.12. Amplification of the bovine *XIST* CpG island in exon 1. PCR amplification of bisulfite treated DNA flanking the *XIST* CpG island in exon 1. Three primer sets (*XIST5Bis1, XIST5Bis2 and XIST5Bis3*) gave correct band sizes and were subsequently cloned into a plasmid for sequencing.

DISCUSSION

Our results validate the use of *Bos gaurus* and *Bos taurus* interspecies hybrids for the analysis of allelic expression. Since the identification of genomic imprinting nearly 12 years ago (Bartolomei et al. 1991; Ferguson-Smith et al. 1991), *Mus musculus* X *Mus spretus* and *Peromyscus polionotus* X *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 (Hemberger et al. 1998; Jong et al. 1999; Mayer et al. 2000; Schmidt et al. 2000; Villar et al. 1995; Yevtodiyenko et al. 2002). Although *Mus musculus* X *Mus spretus* and *Peromyscus polionotus* X *Peromyscus maniculatus* exhibit parental specific phenotypes in their offspring (Dawson 1971; Hemberger et al. 1999; Vrana et al. 2000; Vrana et al. 1998; Zechner et al. 2002), no apparent abnormalities were detected in day 72 *Bos gaurus* X *Bos 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. Therefore, the *Bos gaurus/Bos taurus* hybrid is an ideal experimental model for allelic expression analysis of genes due to lack of phenotypic abnormalities in offspring and high incidence of SNPs in the hybrids. The use of these animals can be further expanded into systematic and comprehensive analysis of genomic imprinting and nuclear reprogramming in the bovine.

The *Igf2/IGF2* locus has been the most widely investigated imprinted gene in all mammals. It is located within the human and mouse imprinting cluster on chromosomes 11p15 and 7 respectively (Reik et al. 2003a). In the bovine, conservation of this region has been indicated by radiation hybrid mapping and is found on chromosome 29. Maternal imprinting 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 genomic imprinting has been demonstrated in most tissues analyzed except for the choroids plexus and leptomeninges in mice and the livers of adult humans and sheep (McLaren and Montgomery 1999; Pham et al. 1998). Allelic expression analysis of the bovine *IGF2* locus in prenatal day 72 chorion, allantois, liver, lung and brain demonstrated preferential paternal expression in all animals investigated (n=6). In contrast to findings in the mouse, where the *Igf2* locus in the choroids plexus and leptomeniniges is biallelic, allelic expression of the *IGF2* in prenatal bovine brain was determined to be preferentially paternal. It should be noted, however, that analysis in the fetal bovine brains consisted of whole brain and not specific regions and it is possible that biallelic expression of *IGF2* in the choroids plexus and leptomeninges went undetected due to the prevalence of other monoallelicly expressed brain tissues in the samples used for analysis. Therefore, further examination of this locus in postnatal livers and in specific regions of the brain will give further insight to the conservation that exists in the bovine with other species.

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 (Bidwell et al. 2001; Wylie et al. 2000). The bovine *GTL2* locus maps to chromosome 18 and has been demonstrated to have a similar organization of this region to the sheep suggesting high levels of organization 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 all animals investigated.

At the *WT1* locus, our results in the bovine are the only systematic allelic expression analysis done so far aside from humans. Analysis of all animals in the chorion, allantois, liver, lung and brain indicates that genomic imprinting does not exist in the bovine at this locus, in contrast to human data reporting it as an imprinted gene. In humans, *WT1* has been reported as both paternally and maternally as well as polymorphic in individuals within the human brain, lymphocytes, fibroblast tissue and preterm placental villi. The data is conflicting, but Jinno et al (1994) have demonstrated paternal expression of *WT1* in human brain and placenta, and Mitsuya et al (1997) have demonstrated maternal expression of fibroblast and lymphocytes, and in each scenario, the allelic expression was polymorphic; that is half of the patients in both assays displayed monoallelic expression, whereas the others displayed biallelic expression. It was further determined that this did not result from developmental stage switching of the imprint. In humans, the *WT1* gene maps at the proximal end of the imprinting cluster on chromosome 11p15 and the corresponding region in cattle maps to chromosome 29 and 15, as determined through radiation hybrid mapping analysis. Interestingly, the bovine *WT1* locus has been separated from upstream *IGF2/H19* and *KCNQ1* imprinting centers

(IC), which induce "spreading" of imprints to adjacent genes. In cattle, a chromosomal rearrangement has occurred, resulting in dissociation of the *WT1* gene (chromosome 15) from the imprinting cluster (chromosome 29). This is speculative, but might indicate that the dissociation from the region resulted in protection from the "spreading" mechanisms that are observed within this region. Further analysis of the locus is required in humans and mice to determine if it is under the control of the two IC regions or if it possesses its own regulatory elements that act independent of location, but it is plausible that the imprinting status in cattle is different to that in humans due to dissociation from adjacent imprinting centers.

Our findings of genomic imprinting at the *XIST* locus in cattle is especially exciting, 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, where as in males this locus is silent. The expression of the *Xist* gene, in conjunction with other elements 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 only observed in the mouse preimplantation embryo and polar trophectoderm. Analysis in the bovine reveals that the *Xist* is preferentially expressed in the chorion of all females tested, but is biallelic in the allantois, liver, lung and brain; demonstrating conservation of genomic imprinting with the mouse, but not the human. Furthermore, random expression the *Xist/XIST* locus in females has been associated with DNA methylation of the promoter

and 5' region of exon 1 in mice and humans. Through the use of bisulfite sequencing and PCR based techniques, we established that the bovine *XIST* is also methylated in the liver and chorion.

Identification of genomic imprinting at this locus in the bovine presented us with a unique opportunity to compare two regions between mice and bovine, 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 incapable of methylation due to the lack of any CpG dinucleotides. We compared this corresponding region to the bovine and human and found no conservation of this region (Fig 2.11), indicating that this site is unique to the mouse. Although this is not a proven site for imprinting in the mice, its binding activity to a nuclear protein in a methylation dependent manner has implicated it as one. Our results suggest that it is not the imprinting site or that is not at least in the bovine.

Others have suggested an alternate region, which they is capable of imprinting the *Xist* locus, and involves an antisense transcript to the *Xist*, termed *Tsix*, which encompasses the entire *Xist* locus into the promoter region in mice (Fig 2.10). In mice, *Tsix* has been shown to inhibit the maternal *Xist* allele in placental cells and of the future active X chromosome in embryonic stem cells (Migeon 2003; Migeon et al. 2001; Migeon et al. 2002). 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 (Fig 2.10). To ensure that the parameters were the same when identifying CpG islands in these regions, we analyzed the mouse *Tsix* promoters and obtained the same results as previously reported (Fig 2.10). In light of evidence indicating that all known imprinted genes are associated with CpG islands, we predict that the bovine *TSIX* in not differentially expressed. Furthermore, evidence from other reports suggest that the bovine *TSIX* does not span into the promoter region and this is supported by the homology observed in Fig 2.9 (Chureau et al. 2002). This would further suggest that *TSIX* in the bovine does not regulate the imprinting that was observed in the chorion. These findings suggest a different mechanism for the establishment of *XIST* imprinting in the placenta of the bovine and mouse. Moreover, we are confident that the imprinting observed at the *XIST* locus in our hybrid females is not a consequence of the interspecies cross, since preferential paternal X chromosome inactivation have already been demonstrated by Xue *et al* in intraspecies crosses of cattle (Xue et al. 2002). Furthermore, it is unlikely that the mouse and bovine *XIST* have evolved to show similar patterns of tissue specific expression of the *XIST*, but by different mechanisms. Therefore, the bovine *XIST* presents a unique experimental model for the identification and analysis of genomic imprinting at this locus.

CHAPTER III

EPIGENETIC AND GENOMIC IMPRINTING ANALYSIS IN NUCLEAR TRANSFER DERIVED *Bos gaurus/Bos taurus* HYBRID FETUSES

INTRODUCTION

In the sexually undetermined primordial germ cell, genomic imprinting and DNA methylation are erased and are reprogrammed during gametogenesis. Furthermore, after gametic marks are imparted onto the genome, additional demethylation occurs in an asymmetrical pattern between parental genomes (Surani 2001). During the period of epigenetic reprogramming in the early embryo, the totipotent epiblast and trophectoderm undergo differentiation and ultimately produce all the cell lineages in the body and placenta (Surani 2001). Presumably, proper epigenetic reprogramming and genomic imprinting at this time is necessary so that the fetus and placenta develops properly. Yet, the nuclear transfer (NT) process completely circumvents the events that normally reprogram the genome (Rideout et al. 2001).

It is apparent that the generation of cattle through nuclear transfer leads to embryonic and fetal death as well as high incidences of abnormalities in animals born to term. Improper epigenetic reprogramming of donor nuclei has been implicated and widely investigated in mice as a cause for this, either entirely or partially, but a systematic and comprehensive analysis has not been performed in bovine. Previously we identified conservation of genomic imprinting at the *GTL2* and *IGF2* loci in the

bovine with humans, mice and sheep and at the *XIST* locus with mice. In this experiment, we demonstrate the use of the interspecies hybrids as a model for elucidating the reprogramming capacity of imprinted genes and epigenetic modifications in the fetuses and placentas derived by nuclear transfer.

A day 72 female *Bos gaurus/Bos taurus* hybrid was used as the donor cell for nuclear transfer in this experiment and resulting fetuses were analyzed for fidelity of allelic expression of the imprinted *IGF2*, *GTL2* and *XIST* loci. In addition, methylation analyses in the chorion and livers of cloned fetuses were performed within a single copy promoter region, a differentially methylated region and a repeat element.

MATERIALS AND METHODS

Production of day 40 control *Bos gaurus/Bos taurus* hybrids

Heifers and mature (approximately 16 months to 3 years) Angus and Angus cross cows housed at the NC State Beef Center Complex in Raleigh, North Carolina were used to generate day 40 control hybrid fetuses. Cows were synchronized for estrous by serial injections of Prostaglandin F2alpha (Lutalyse) (2 shots 11 days apart with estrous detection 3 days post second shot). Upon detection of heat, heifers were transvaginally inseminated following the am/pm rule (a.m. detection of estrous, pm insemination/ p.m. detection of estrous, a.m. insemination). Heifers were then checked for establishment of pregnancy at day 28 through transrectal ultrasonography.

Isolation of fetal fibroblast from *Bos gaurus/Bos taurus* hybrids

Day 72 *Bos gaurus/Bos taurus* hybrid fetus D was used as the donor genotype for nuclear transfer in this experiment. The head and viscera were removed and the remaining tissue was minced with a sterile razor blade. The tissue was added to 10 ml of 0.05% trypsin (Gibco) supplemented with 0.9 mM potassium chloride, 0.9mM dextrose, 0.7mM sodium bicarbonate, 0.1mM EDTA (Sigma), and 20mM sodium chloride (EM Science). The tissue/trypsin solution was shaken at 37ºC for 15 minutes a total of three times. After incubation, the supernatant was collected, pooled, and pelleted. The cell pellet was resuspended in DMEM/F12 media (Gibco Laboratories Inc) supplemented with 10% fetal bovine serum (FBS) and 5% calf serum (CS) (Hyclone), 30mM sodium bicarbonate, 0.5mM pyruvic acid, and 2mM N-acetyl-L-cysteine (all from Sigma). In addition, 100 units (U) penicillin, 100 micrograms (ug), and 250 nannograms (ng) amphotericin (Gibco) were added to inhibit microbe growth. The cells were placed in the appropriate number of 10cm tissue culture plates (Corning) and placed in a 5% CO^2 incubator (Nuaire) at 38ºC, allowed to attach and grow to confluency and then passaged (1:2 or 3). The cells were then trypsinized and frozen in 50% FBS, 40% media, and 10% DMSO (Sigma), placed in -80ºC overnight and in liquid Nitrogen for long time storage and future use.

Production of day 40 cloned *Bos gaurus/Bos taurus* hybrids

Ooctye maturation

Oocytes were obtained from a commercial supplier (Ovagenix, San Angelo, TX) and matured in Medium 199 (M199; Gibco Laboratories Inc. Grand Islands, NY) supplemented with 10% FBS (Hyclone), 0.1 U/ml LH (Sioux Biochemical, Sioux City, IA), 0.1 U/ml FSH (Sioux Biochemical), and 1% penicillin-streptomycin (P/S; Sigma) for 20-22 hrs.

Preparation of donor cells

Bovine fetal fibroblast were seeded in a four well plate at 35% confluency and grown in Dulbecco modified Eagle medium-F12 (Gibco Laboratories Inc) containing 10% FCS at 37oC in air containing 5% CO^2 until contact inhibited. Cells were then trypsinized and resuspended in DMEMF-12 in preparation for reconstruction.

Nuclear transfer

Following maturation, cumulus cells were removed from the oocyte by vortexing in 0.1% hyaloranidase (Sigma) in Hepes-M199 (H-M199). Denuded oocytes were rinsed through three drops of manipulation media (H-M199 containing 10% FCS) and then incubated for 10 minutes in culture media (B-M199 supplemented with 10% FCS) containing 5 µg/ml Hoechst 3342 (Sigma). Oocytes were then placed in manipulation media containing 7.5 µg/ml cytochalasin B (Sigma) and enucleated by aspiration of the

first polar body and metaphase plate utilizing a 22-µm beveled glass pipette. Absence of the metaphase plate was visualized by exposure to ultraviolet florescent light.

Reconstruction was conducted in manipulation media. The cells were placed in a separate drop of manipulation media and groups of 15-20 cells were loaded in the pipette. A single cell was then placed in the perivitelline space of each enucleated oocyte. Following reconstruction, the oocytes were placed in a 1-mm fusion chamber and fused by two DC pulses of 220 V for 10 µsec in 275 mM mannitol (Sigma), 0.1 mM CaCl2 (Sigma), and 0.1 mM MgSO4 (Sigma). Following fusion, the oocytes were placed in culture media for 4 hrs prior to activation.

Oocyte activation

Reconstructed oocytes were activated by exposure to 5 µm ionomycin (Calbiochem, San Diego) for 4 minutes, rinsed three times in manipulation media and placed in culture media containing 2 mM 6-dimethylaminopurine (Sigma) for 4 h. Following activation embryos were placed in G1 culture media (Vitrolife) for four days then transferred to G2 culture media (Vitrolife) for an additional 2 days. On day six, compact morula were loaded into a tube containing pre-equilibrated G2 media and shipped to North Carolina State University for transfer on day 7.

Synchronization of recipients

Heifers and mature Angus and Angus cross cows (approximately 16 months to 3 years) housed at the NC State Beef Center Complex in Raleigh, North Carolina were

used as recipients for the cloned *Bos gaurs/Bos taurus* embryos. Cows were synchronized for estrous by serial injections of Prostaglandin F2alpha (Lutalyse) (2 shots 11 days apart with estrous detection 3 days post second shot). Cows were monitored for estrous twice daily and at the onset of estrus, cows were categorized as day 0 of estrus. At day 7 of estrus, cloned blastocysts were transvaginally transferred into the gravid horn.

Embryo transfer

Cloned embryos were shipped from Genetic Savings and Clone, College Station TX in a 39^{o}C heated incubator at day 6 of embryo culture and embryos arrived in North Carolina within 24 hours (day 7). The cloned embryos were maintained in the pre-equilibrated G2 media at 39^{o}C until time of transfer. Upon time of transfer, cloned embryos were moved to ViGro™ Holding (hepes buffered) media and washed 2X to remove residual G2 media. Embryos were maintained within loading media in a 39^{o}C incubator, until time of transfer, at which time two embryos were drawn into a 0.5 cc embryo transfer straw. Embryos were then transvaginally loaded into the gravid horn.

Isolation of control and nuclear transfer derived *Bos gaurus/Bos taurus* hybrid fetuses

At day 40 of gestation, recipient cows were slaughtered at a local abattoir, reproductive tracts were transported to the NC State Veterinary School on ice and tissues isolated. Weights and measurements were taken so as to monitor development of hybrid

animals. Chorion, allantois, liver and brain samples were isolated and flash frozen in liquid nitrogen to preserve RNA and DNA until further use.

RNA and DNA extraction

RNA was extracted from frozen samples utilizing the Ambion RNA aqueous kit and resuspended in 10 μg aliquots in DEPC H_2O and stored at – 80 ^oC. 2 μg of RNA for each sample was DNase I treated using the Ambion DNase I Kit and subsequently converted to cDNA through the Ambion First Strand Synthesis Kit for all RT-PCR reactions. DNA was extracted from frozen tissues using the Promega Wizard DNA Extraction Kit and resuspended at 20 ng/μl and stored at –20 ^oC until further use.

PCR-based sexing determination of day 40 control *Bos gaurus/Bos taurus* fetuses

PCR-based sex determination reactions (Y chromosome specific) were performed on all fetuses and was carried out in 50 μl reactions consisting of:: 30.75 μl dH2O, 5 μl 10X PCR buffer (Promega), 4 μl MgCl2 (25 mM), 1.25 μl dNTP (10mM), 2.0 μl Forward primer (3 μm), 2.0 μl Reverse primer (3 μM), 0.5 μl Taq (Promega) and 2.0 μl DNA (100 ng) and cycled on a Perkin Elmer Thermocycler at 94^{o}C (5min); 94^{o}c (30 sec), 54^{o}C (30 sec), 72^{o}c (2 min) [30 cycles]. Products were resolved on 2% EthBr agarose gels and analyzed for the presence (male) or absence (female) of approximately 200 bp. Internal positive and negative controls consisted of known male and female genomic DNA

Microsatellite analysis of cloned *Bos gaurus/Bos taurus* fetuses

Genotyping of clones and donor cells was performed at the DNA Technologies Lab, Department of Veterinary Pathobiology, Texas A&M University, College Station TX (Schnabel et al. 2000).

Analysis of allelic expression of the *GTL2, IGF2*, and *XIST* loci

RT-PCR of the *IGF2*, *GTL2*, and *XIST* loci was performed on chorion, allantois and liver (Table 3.1). Amplicons resolved on 2% EthBr agarose gels were gel extracted (Qiagen Gel Extraction Kit), resuspended in 50 μls of dH_20 and used directly as sequencing template. Sequencing primers consisted of forward primers used in the amplification of each of the four RT-PCR reactions. Twenty-five μl sequencing reactions using Big Dye Terminator mix were run on a Perkin Elmer Thermocycler at 94°C (30 sec), 50°C (30 sec), 60°C (4 min) [25 cycles]. Cleanup of sequencing reactions was performed in 800 μl Sephadex columns (Sigma). Sequences were then run on either an ABI 370 or 3700 and sequence chromatograms were visually analyzed for the presence or absence of both SNPs. RT-PCR and sequencing reactions were run in triplicates. To confirm the absence of genomic contamination in cDNA samples, an internal control was utilized through the *IGF2* amplicon, which spanned intron 6. Genomic contamination results in the presence of an additional 1kb band.

Table 3.1. Primer sequences for the amplification of *IGF2, GTL2,* and *XIST* loci.

Locus	Forward primer (5'-3')	Reverse primer (5'-3')
X- chromosome inactivation specific transcript (*XIST*)	GAACATTTTCCAGACCCCAAC	AAACCAGGTATCCACAGCCG
Gene trap locus 2 *(GTL2)*	CCCACCAGCAAACAAAGCAAC	CATCAAGGCAAAAAGCACATCG
Insulin like growth factor 2 (*IGF2*)	CAAGGCATCCAGCGATTAG	TTCAAGGGGGCTGATTGAG

Allelic quantification of the *XIST* locus

RT-PCR products of the *XIST4* from chorion of clones and controls was cloned into TOP4 sequencing vectors (Invitrogen) and were transformed into TOP10 (Invitrogen) chemically competent E. coli cells. Plasmids were purified and sequencing reactions run using M13 universal primers (5'-CTGGCCGTCGTTTTAC-3') (3.25 pM) in 25 □l sequencing reactions with Big Dye Terminator mix and were run on a Perkin Elmer Thermocycler at 94^{o}C (30 sec), 50^{o}C (30 sec), 60^{o}C (4 min) [25 cycles]. Sequences were examined individually for the presence or absence of the paternal (C) SNP located at position 220 of the sequence chromatogram. Results are expressed in percentages of total sequences.

Bisulfite treatment of genomic DNA

Genomic DNA was isolated (Promega Wizard DNA Isolation Kit) from the chorion and liver of control (n=3) and clones (n=3). The sodium bisulfite reaction was carried out with one μg of DNA from each sample using the CpG DNA Conversion Kit (IntergenCo). Sodium bisulfite catalyzes the deamination of cytosines to uracils (thymines), where as methylated cytosines (m5C) are protected. This technique allows for the rapid identification of m5C in genomic DNA. Genomic DNA was denatured through incubation of 3M NaOH at 37^{o}C. Denatured DNA was then incubated for 16-20 hrs at 50^{o}C in the presence of 3 M Sodium bisulfite and 0.5 mM hydroquinone. Carrier glycogen was added to bisulfite treated DNA and incubated at room temp for 5 min. DNA was then washed, centrifuged (13,000 rpm) and vortexed in successive (3X) volumes of 90 % and 70% ethanol. DNA was then resuspended in 50 μl TAE and incubated at 60^{o}C for 15 min. DNA sample was centrifuged at 13,000 rpm for 30 sec and the supernatant containing DNA transferred to a new tube and 1.5 μl of the superernatant was used in subsequent PCR reactions.

DNA methylation analysis of the *XIST* Differentially Methylated Region (DMR) in exon1

DNA methylation analysis of the *XIST* DMR in exon 1 was performed by digestion of 500 ng of genomic DNA with AciI and Bst UI restriction enzymes. 20 μl reaction consisting of 2 μl 10X buffer and 2 μl (10 units) of the restriction enzyme AciI

(TGN$^{C/G}$CGG) or Bst UI (CGCG) for approximately 24 hours. 1.5 µl of digested DNA was run in a 50 µl PCR reaction consisting of 5 µls 10X PCR buffer (Promega), 4 µls 25 mM MgCl2, 1.25 µls 10mM dNTPs, 2.5 µls 3 M forward primer, 2.5 µl 3 M reverse primer, 2 µls DNA and 1 µls Taq (Promega) using primers flanking the CpG island. Cycle parameters were: 94^{o}C (5min) (denaturation); 94^{o}C (30 sec), 60^{o}C (30 sec), 72^{o}C (3 min) [10 cycles]; 94^{o}C (30 sec), 60^{o}C (30 sec), 72^{o}C (3 min) [25 cycles]. Products were resolved on 2% EthBr agarose gels and analyzed for the presence or absence of bands. Negative controls consisted of undigested DNA and positive controls consisted of digestion with sperm DNA, which possesses no methlyation (Goto and Monk 1998; Monk 1995; Zuccotti and Monk 1995).

Bisulfite sequencing of the epidermal cytokeratin and satellite I regions

Bisulfite sequencing of the epidermal cytokeratin promoter (Table 3.2) was performed on chorion and liver obtained from control and cloned pregnancies. Hemi-nested amplification of the epidermal cytokeratin promoter was carried out in two 25 µl reactions consisting of : 15.375 µl H_2O, 2.5 µl 10X PCR buffer (Promega), 2 µl MgCl2 (25mM), .625 µl dNTP, 1.25 µl Forward primer (3 µM), 1.25 µl Reverse primer A (3 µM), 1.256 µl Taq (Promega) and 1.25 µl bisulfite treated DNA (100 ng) and cycled at 94^{o}C (10 min); 94^{o}C (30 sec), 55^{o}C (60 sec), 72^{o}C (30 sec) [35 cycles] and then two µls of this reaction was added to another PCR mix consisting of the same reagents, but with the heminested primer B and cycled under the same parameters. Bands were resolved on 2% EthBr agarose gels and were subsequently gel purified (Qiagen Gel Purification Kit).

Bisulfite sequencing of the satellite I region (Table 3.2) was performed on chorion and liver obtained from control and cloned pregnancies. Amplification of the region was carried out in 50 μl reaction consisting of: 30.75 μl dH_2O, 5 μl 10X PCR buffer (Promega), 4 μl MgCl2 (25 mM), 1.25 μl dNTP (10mM), 2.5 μl Forward primer (3 μm, 2.5 μl Reverse primer (3 μM), 0.5 μl Taq (Promega) and 2.0 μl DNA (100 ng) and cycled on a Perkin Elmer Thermocycler at 94°C (5min); 94°C (60 sec), 46°C (60 sec), 72°C (30 sec) [35 cycles]. Bands were resolved on 2% EthBr agarose gels and were subsequently gel purified (Qiagen Gel Purification Kit).

Products from each epidermal cytokeratin and satellite I amplification for liver and chorion of all animals generated were cloned into a sequencing vector (TOPO4, Invitrogen). Positive colonies containing plasmid and insert (cytokeratin and satellite I) were isolated and grown in 96 deep well plasmid culture plates (Invitrogen) overnight. Plasmids were purified using a 96 well plasmid isolation kit (Invitrogen). Approximately 20 plasmids were purified for each sequencing reaction for each sample and animal. Sequencing reactions were performed using M13 universal primers (5'-CTGGCCGTCGTTTTAC-3') (3.25 pM) in 25 μl sequencing reactions with Big Dye Terminator mix and were run on a Perkin Elmer Thermocycler at 94°C (30 sec), 50°C (30 sec), 60°C (4 min) [25 cycles]. Products were sequenced on an ABI 3700 sequencer. Sequences were analyzed on MacVector 6.0 Clustall query for presence or absence of methylated CpG dinucleotides.

Table 3.2. Primer sequences for the amplification of the epidermal cytokeratin promoter and satellite I repeat element.

Locus	Forward primer (5'- 3')	Reverse primer (5' –3')
Epidermal cytokeratin promoter	GTGGAYGGTAAGTTATTTAAAA	A: CCTCTTTCTACCAAACAAACCA B: ACAAACCAAAAACTAATAATACC
Satellite I	AATACCTCTAATTTCAAACT	TTTGTGAATGTAGTTAATA

Statistical analysis

Mean comparisons of values obtained from bisulfite sequencing and allelic expression analysis was determined using an unpaired t test using significance level of $P< 0.05$.

RESULTS

Production of day 40 control *Bos gaurus/Bos taurus* fetuses

Bos gaurus and Bos taurus hybrid fetuses and placentas were obtained at day 40 of gestation (Fig 3.2). Samples derived from the placentas (chorion and allantois) and fetuses (liver) were isolated. A total of three, day 40 hybrid fetuses were obtained (1 female and two male). Table 3.6 summarizes weights and measurements obtained from each of the hybrid fetuses. Additionally, Y chromosome specific PCR reactions (sexing reactions) were performed on the three control fetuses (Fig 3.3).

Table 3.3. Weights and measurements of the *Bos gaurus/Bos taurus* day 40 hybrid control fetuses.

Fetus	Fetus weight (g)	Placental weight (g)	Allantoic fluid (mls)	Cotyledon number	Heart weight (g)	Liver weight (g)	Sex
G	1.17	X	92	25	.027	.09	Male
H	1.28	X	64	16	.028	.10	Female
I	.091	10.211	71	4	.029	.11	Male

***X indicates measurement was not obtained.**

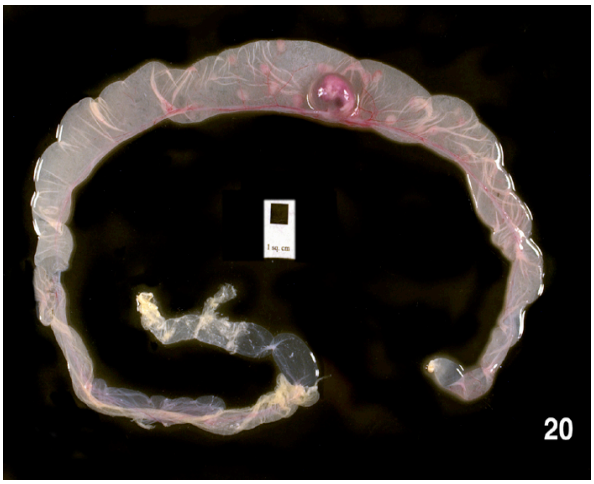


Figure 3.1. *Bos gaurus/Bos taurus* day 40 control fetus and placenta (Fetus G).

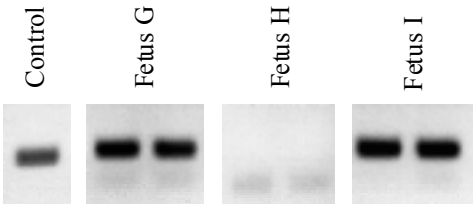


Figure 3.2. PCR-based sex identification of *Bos gaurus/Bos taurus* day 40 control fetus. Y specific PCR reactions of the three day 40 *Bos gaurus/Bos taurus* hybrid fetuses (Fetus G, H, I) and control fetus of known sex (male).

Production of day 40 nuclear transfer derived *Bos gaurus/Bos taurus* hybrids

Two-hundred oocytes were fused with *Bos gaurus/Bos taurus* lung fetal fibroblast cells derived from Fetus D, and lead to the generation of thirty-two grade one blastocysts. At day 28 of gestation, recipient cows were checked for pregnancy and three recipients (#35, 8010 and 27) were determined pregnant (Table 3.4). At day 40 of gestation, cloned fetal and placental tissues were isolated (Fig 3.1). Table 3.5 summarizes weights and measurements obtained from each of the pregnancies.

Table 3.4. Grade and developmental stage of cloned *Bos gaurus/Bos taurus* hybrid blastocysts.

Embryo Number	Stage (Development)	Embryo grade	Recipient #	Pregnant at day 28	Pregnant at day 40
1, 2	**MB**	**1**	**8029**	**Yes**	**Yes**
3, 4	XB	1	8012	No	No
5, 6	EB	1	8010	No	No
7, 8	**EB**	**1**	**35**	**Yes**	**Yes**
9, 10	MB	1	8005	No	No
11, 12	EB	1	8013	No	No
13, 14	**EB**	**1, -1**	**27**	**Yes**	**Yes**
15, 16	MB	1	8040	No	No
17, 18	MB	-1	41	No	No
19, 20	XB	1	9012	No	No
21, 22	MB	1	28	No	No
23-27	MB n=1 XB n=4	1	32	No	No
28-32	EB n=1 MB n=1 XB n=1 XB n=2	1	9033	No	No

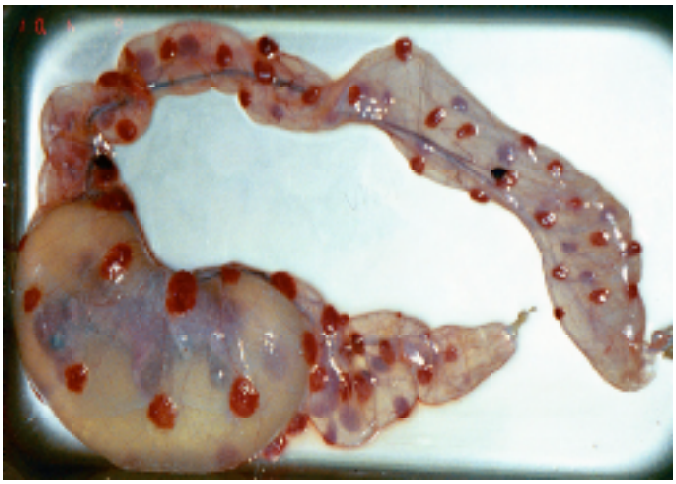
(EB= Early blastocyst, MB= Mid blastocytst and XB= Expanded blastocytst)

Table 3.5. Weights and measurements of the three cloned *Bos gaurus/Bos taurus* fetuses (1A, 1B, 2A, 2B, 3) obtained at day 40 of gestation.

Fetus	Fetus weight (g)	Placental weight (g)	Allantoic fluid (mls)	Cotyledon number	Heart weight (g)	Liver weight (g)	Sex
1A	.822	17.88	87	0	.030	.030	Female
1B	.290				.013	.017	
2A	.843	21.237	58.5	0	.014	.014	Female
2B	.822				.016	.015	
3	1.067	16.929	91	0	.012	.010	Female

A and B (1A, 1B, 2A and 2B) indicate twin pregnancies.

(A)



(B)

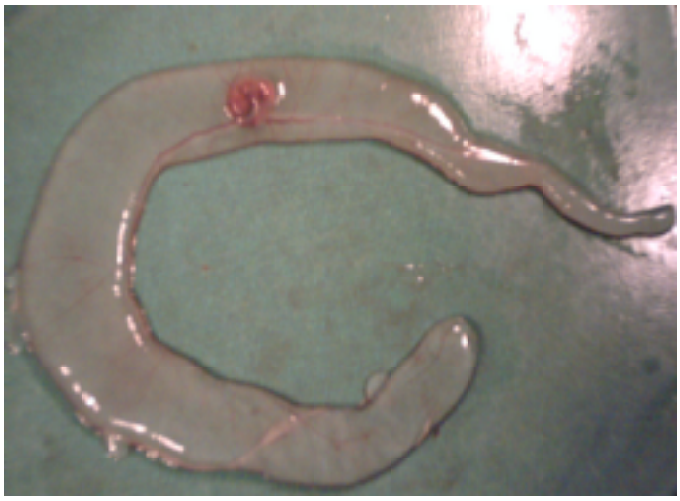


Figure 3.3. *Bos gaurus/Bos taurus* day 72 donor fetus and placenta and day 40 nuclear transfer derived fetus and placenta. **(A)** Day 72 Fetus D (donor cell line: lung fibroblast). **(B)** Day 40 cloned *Bos gaurus/Bos taurus* hybrid fetus and placenta (Clone 3).

Table 3.6. Genotype analysis of *Bos gaurus/Bos taurus* day 40 nuclear transfer derived fetuses and donor cell line.

Locus	Fetus 1A	Fetus 1B	Fetus 2A	Fetus 2B	Fetus 3	Donor cell line
BM1225	236	236	236	236	236	236
	267	267	267	267	267	267
BM1706	243	243	243	243	243	243
	251	251	251	250	251	251
BM17132	88	88	88	88	88	88
	88	88	88	88	88	88
BM1905	169	169	169	169	169	169
	177	177	177	177	177	177
BM2113	127	127	127	127	127	127
	138	137	138	138	137	137

Genotyping of day 40 cloned *Bos gaurus/Bos taurus* hybrids

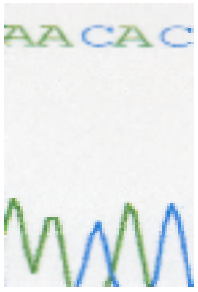
Microsatellite analysis at six loci (BM1225, BM1706, BM17132, BM1905, BM2113) from the five cloned fetuses indicates all are identical at loci examined and match the genotype of the donor cell line derived from Fetus D (Table 3.7). These results indicate all animals generated are clones.

Allelic expression profiles of the *IGF2, GTL2* and *XIST* loci

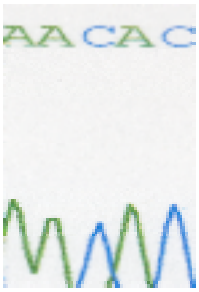
IGF2

Amplification using the *IGF2-3* primer set from day 40 control and cloned chorion, allantois and liver indicated expression in all tissues sampled. The C/A SNP, previously identified in day 72 hybrid controls, was also detected in the *IGF2-3* amplified from genomic DNA of the three day 40 control hybrids. Allelic expression analysis of *IGF2-3* in control liver, chorion and allantois showed preferential expression of the paternal allele (allele C). When analysis of *IGF2-3* was extended to the liver, chorion and allantois of the five cloned fetuses, preferential paternal expression of the locus was also observed. These results indicate fidelity of imprinting at the *IGF2* locus in bovine clones (Fig 3.3 and Fig 3.4).

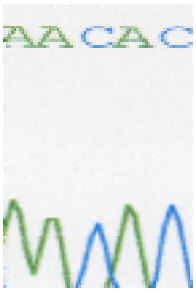
(A) Fetus G liver RT-PCR



(B) Clone 1A liver RT-PCR



(C) Fetus G chorion RT-PCR



(D) Clone 1 chorion RT-PCR

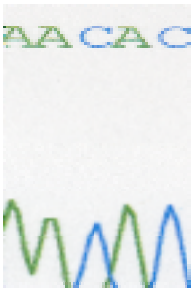


Figure 3.4. Sequence chromatograms of the *IGF2* locus amplified from control and nuclear transfer derived fetus and placenta. Sequence chromatograms of *Bos gauru/Bos taurus* control day 40 hybrid and day 40 clone *IGF2* transcripts. **(A)** *IGF2-3* amplified from Fetus G liver cDNA, **(B)** Clone 1A liver, **(C)** Fetus G chorion and **(D)** Clone 1 chorion. This pattern of expression was observed for all controls and clones in the chorion, allantois and liver (not shown).

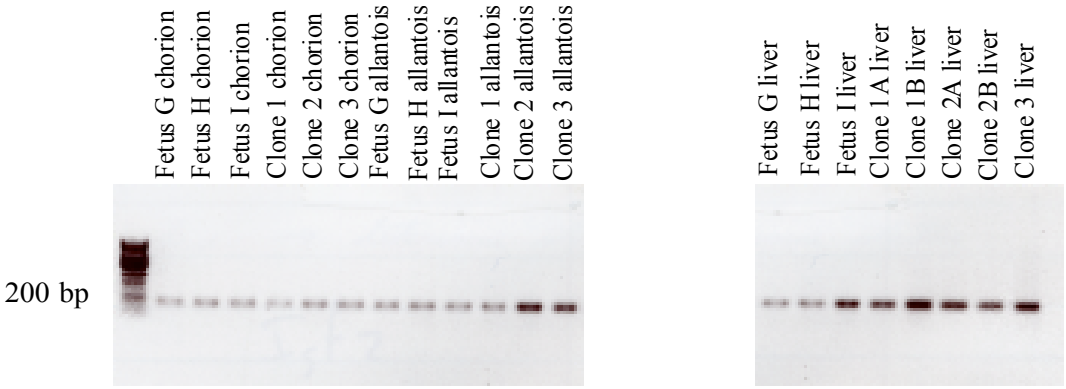
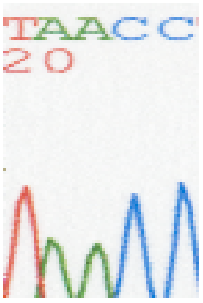


Figure 3.5. RT-PCR transcript of the *IGF2* locus amplified from control and nuclear transfer derived fetus and placenta. 2% agarose gel of *IGF2-3* amplified from chorion, allantois and liver from all clone and control fetuses.

(A) Fetus G liver RT-PCR

(B) Clone 1A liver RT-PCR

(C) Fetus G chorion RT-PCR



(D) Clone 1 chorion RT-PCR

Fig 3.6. Sequence chromatograms of the *GTL2* locus amplified from control and nuclear transfer derived fetus and placenta. **(A)** Fetus G liver, **(B)** Clone 1A liver, **(C)** Fetus G chorion and **(D)** Clone 1 chorion. *GTL2* transcripts from three control liver and chorion exhibited preferential maternal expression as well as all samples sequenced obtained from chorion and livers of controls.

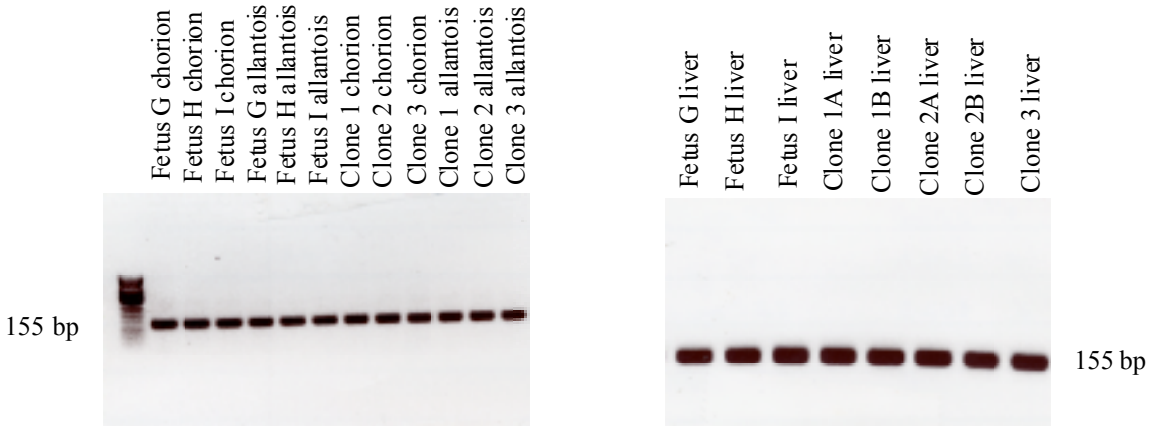


Fig 3.7. RT-PCR amplicons of the *GTL2* locus amplified from control and nuclear transfer derived fetus and placenta.

GTL2

Amplification of *GTL2*, using primers previously used to determine genomic imprinting in day 72 hybrid fetuses, was extended to day 40 controls and cloned hybrid chorion, allantois and liver. The C/A SNP was first identified to exist in day 40 controls and allelic expression analysis of chromatograms revealed that the maternal allele was preferentially expressed in chorion, allantois and liver in both controls and clones indicating maintenance of imprinting at the *GTL2* locus in cloned day 40 hybrids.

XIST

Expression of the *XIST* was not detected in day 40 hybrid males (Fetus G, I), but strong bands were detected in the hybrid female control (Fetus H) and in all clones (Clone 1, 2, 3). The C/T SNP was also detected in Fetus H, as previously detected in all female day 72 hybrids, and allelic expression analysis indicated biallelic expression of the *XIST* in liver and allantois, and monoallelic expression (paternal) in the chorion of Fetus H. In the clones (1A, 1B, 2A, 2B and 3), biallelic expression was detected in the liver and allantois, however, biallelic expression was detected in the chorion of clone 2 and 3, whereas monoallelic expression (paternal) was detected in clone 1 (Fig 3.7).

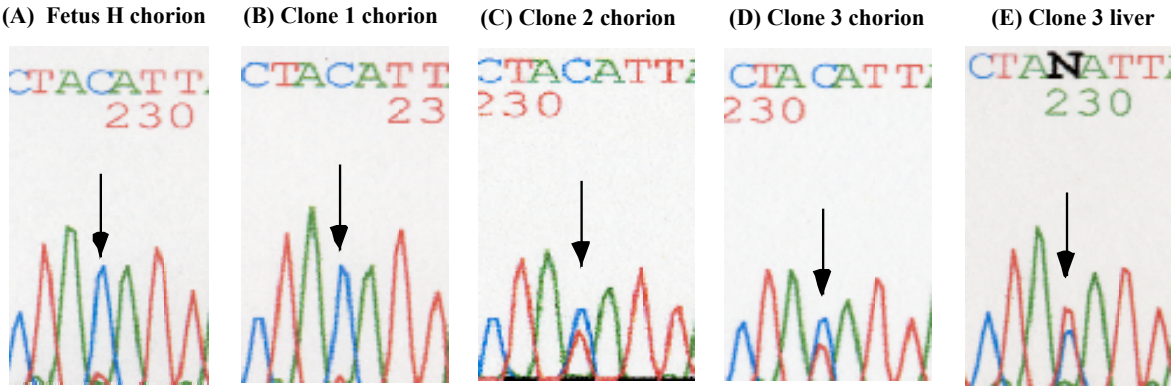


Figure 3.8. Sequence chromatograms of the *XIST* locus amplified from control and nuclear transfer derived fetus and placenta. **(A)** Fetus H chorion, **(B)** Clone 1 chorion, **(C)** Clone 2 chorion, **(D)** Clone 3 chorion and **(E)** Clone 1A liver. Chromatograms indicate monoallelic expression of the *XIST4* in chorion in Fetus H and Clone 1, however biallelic expression is observed in Clone 2 and 3.

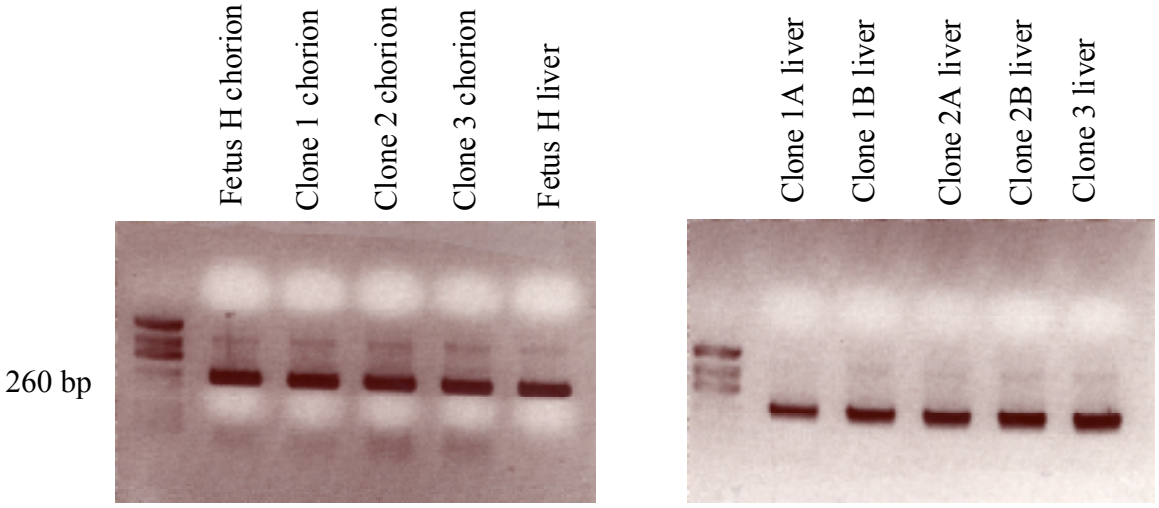


Figure 3.9. RT-PCR amplicons of the *XIST* locus. 2% agarose gel of the *XIST4* amplicon from Fetus H chorion and liver and Clones 1-3 chorion and liver.

In an attempt to quantify the levels of *XIST4* parental expression from the chorion of Clones 1-3, RT-PCR products were cloned into TOPO4 sequencing vectors and multiple plasmids were sequenced (45-83 on average) to more accurately determine the ratio of paternal to maternal transcripts present. In sequences obtained from Clone 1, 83.7 % were paternally derived (36/43), in Clone 2, 65.9 % were paternally derived (29/44) and in Clone 3 71.4 % were paternally derived (40/56), whereas in controls, 94 % (78/83), 97 % (36/37) and 95% (40/42) of the sequences were paternally derived in fetuses A, B and H respectively. These results indicate abnormal monoallelic vs. biallelic expression of the *XIST* locus deriving from increased expression of the maternal allele in the chorion of cloned fetuses 1-3. Overall, the expression at the *XIST* locus was significantly different ($P<0.02$) with the paternal expression of clones equaling 73.6 ± 5.2 (Mean ± S.E.) versus the controls whose paternal expression was 95 ± 0.8 (Mean ± S.E.) (Fig 3.9).

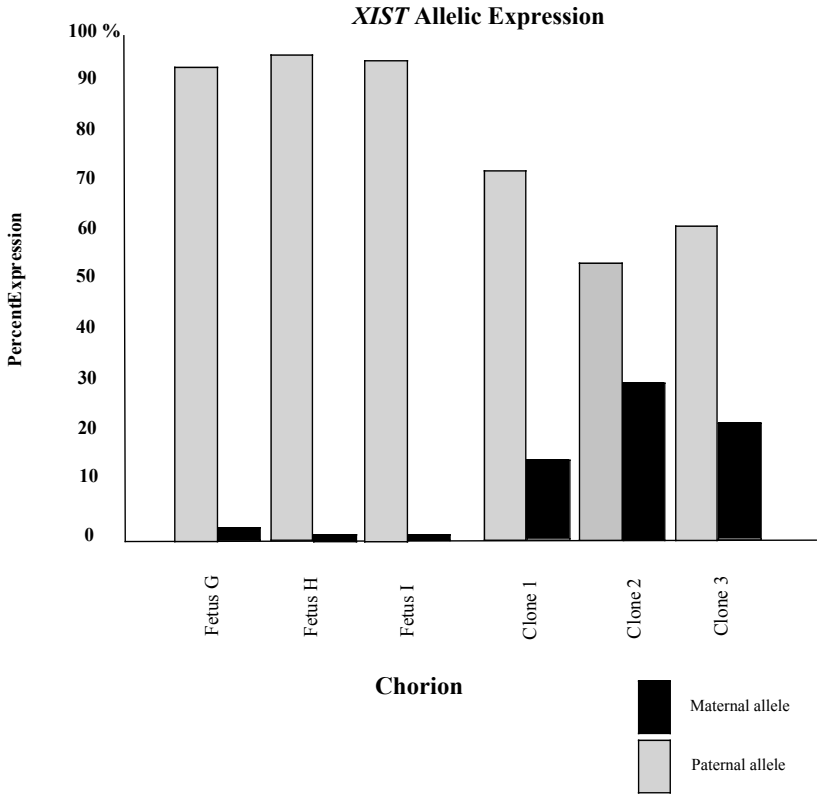


Figure 3.10. Parental expression analysis of the *XIST* locus in day 40 control and nuclear transfer derived fetus and placenta.

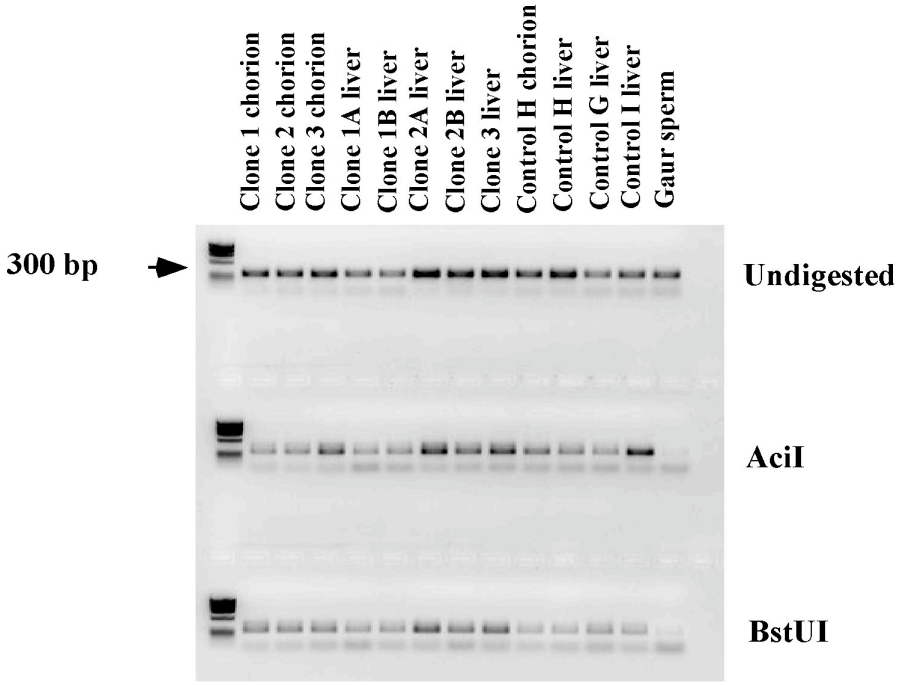


Figure 3.11. Amplification of the CpG island in exon 1 of the bovine *XIST* locus after digestion with AciI and BstUI. 2 % agarose gel of *XIST* 5 PCR products amplified from undigested genomic DNA, Aci I digested, and Bst UI digested. Note that all produce products except for hypomethylated Gaur sperm (AciI & Bst UI).

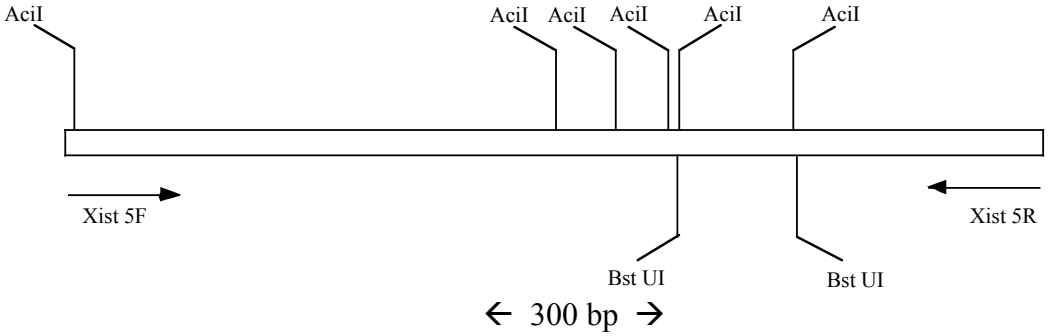


Figure 3.12. Schematic of the CpG island in exon 1 of the bovine *XIST* locus representing AciI and BstUI restriction sites.

Methylation analysis of the *XIST* DMR in exon 1

To determine the methylation status of the *XIST* DRM in exon 1, the digestion of the genomic DNA with the restriction enzymes AciI and BstUI was expanded to include the animals derived from nuclear transfer. Genomic DNA isolated from the chorion and livers of cloned animals was digested and used as template for a PCR reaction spanning the *XIST* DMR in exon 1 (Fig 3.11). Results indicate that the methylation status of these animals is in concordance with controls (Fig 3.10). This indicates that there is not a loss of methylation in this region in any of the clones or tissues. Digestion with AciI and Bst UI encompasses eight of the eleven CpG dinucleotides present in this region.

Methylation analysis of the epidermal cytokeratin promoter and satellite I repeat element

In order to understand the effects of nuclear reprogramming, it was essential to assess imprinted gene expression in conjunction with DNA methylation analysis. Since the DNA sequence of the bovine DMR regions regulating imprinting at the *IGF2* and *GTL2* are not available, the DNA methylation analysis of the epidermal cytokeratin promote*r*, which is methylated in a tissue specific manner and the bovine Satellite I repeat element, which is a relic of retrotransposons and is heavily methylated, were incorporated into the experiment. Bisulfite sequencing of the cytokeratin promoter in control livers and chorion indicated the region was hypermethylated in the control liver: Fetus G 73%, Fetus H 75 % and Fetus I 73 %, with overall methylation at 73.7 % ± .7

(Mean ± S.E.). In the chorion of control fetuses the promoter was hypomethylated: Fetus G 5 %, Fetus H 18 % and Fetus I 11.5 % with overall methylation at 11.8 % ± 4.0. Analysis of the clones revealed that the methylation of the liver was in relative agreement with the controls: Clone 1A 52 %, Clone 1B 79 %, Clone 2A 52 %, Clone 2B 56 %, Clone 3 56 %, with overall methylation at 63.8 % ± 6.2; however, in contrast hypermethylation was observed in the chorion of clones: Clone 1 24 %, Clone 2 50 % and Clone 3 and overall was 37 % ± 13.0, although this value was not significantly different (P= 0.10). These results indicate normal methylation patterns at the cytokeratin promoter within the liver, but slight variation (hypermethylation) in the chorion of clones (Fig 3.12).

When analysis was extended to the Satellite I region, similar results were observed. Analysis indicated hypermethylation of control livers: Fetus G 50 %, Fetus H 65 % and Fetus I: 62.5 %, with overall at methylation 56.0 % ±3.5 (Mean ± S.E.) and hypomethylation in control chorion: Fetus G 8 %, Fetus H 11 % and Fetus I 18 % with overall methlyation at 12.9 % ± 2.8. Similarly, hypermethylation of clone livers was observed: Clone 1A 54.5 %, Clone 1B 68.4 %, Clone 2A 63 %, Clone 2B 75.6 and Clone 3 51.0 %, with overall methylation at 65.4 % ± 4.5, whereas in contrast to controls, analysis of the chorion in clones indicated hypermethylation at these regions: Clone 1 33.9 %, Clone 2 48.3 % and Clone 3 64.6 % with overall methylation at 49.9 % ± 8.9, and these values are statistically significant (P = 0.01). These results indicate improper reprogramming of the Satellite I repeat element of the clones within the chorion (Fig 3.13).

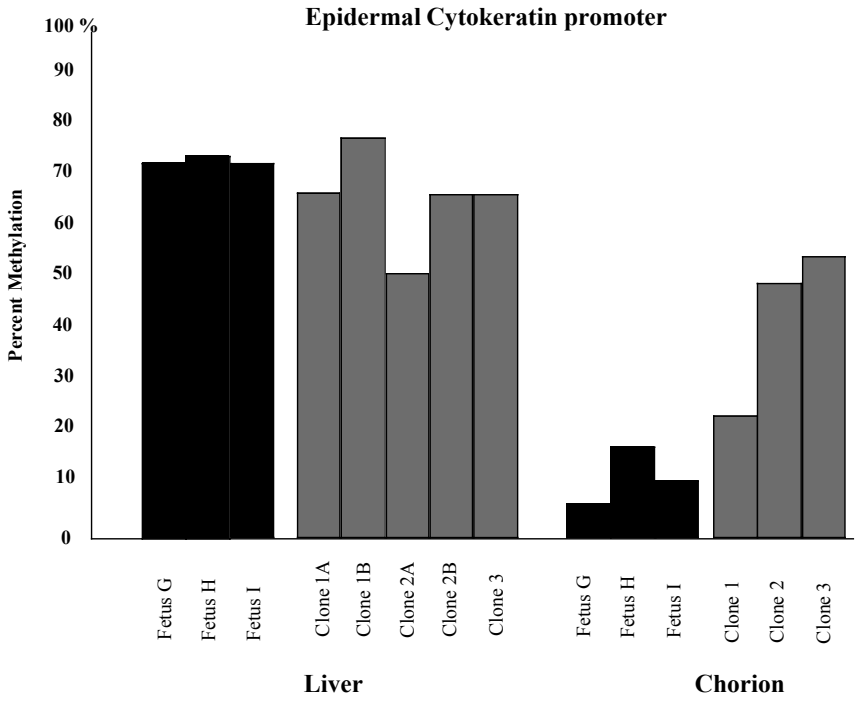


Figure 3.13. Percent methylation analysis of the bovine epidermal cytokeratin promoter.

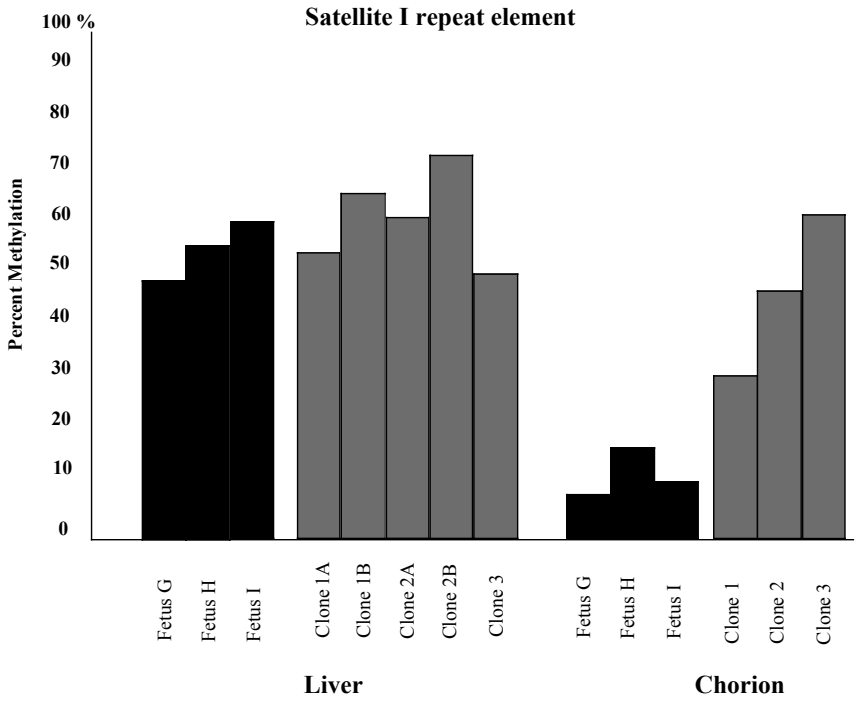


Figure 3.14. Percent methylation analysis of the bovine satellite I repeat element.

DISCUSSION

Here we demonstrate the ability to generate day 40 hybrid *Bos gaurus/Bos taurus* clones from somatic cell nuclear transfer of day 72 hybrid fetuses and analyses of genomic imprinting and DNA methylation. These results are the first report of a systematic and comprehensive analysis of genomic imprinting and DNA methylation in cloned bovine fetal and placental tissue and the first use of an interspecies model to address these questions in the bovine.

Observations of the three cloned pregnancies established indicated that no abnormalities of the fetuses were apparent except the reduced size of Clone 1B, whereas Clones 1A, 2A, 2B and 3 were in concordance with control animals. However, analysis

of the placenta of each clone indicated differences in cotyledon number between the three clones and controls (Clones 1-3 = 0, 0, 0 cotyledons, Controls G-I = 25, 16, 5 cotyledons). These finding are similar to Hill et al. (2000) in that clones between day 35 and 55 have reduced placentome number and reduction in chorio-allantoic fusion. However, it should be noted though that we made no attempt to characterize pathological conditions in the pregnancies, but only made gross physical observations between animals. Furthermore, after observing differences in cloned vs. control pregnancies it was realized that proper control animals consisting of *in vitro* produced embryos, such as through *in vitro* fertilization that are cultured in media for an equivalent amount of time as clones, would have been better for comparison of phenotypes, since the nature of reduced cotyledon number in clones cannot be determined without knowledge of the effect of culture on the pregnancies.

Allelic expression analysis of the imprinted genes identified in the previous experiment indicated fidelity of expression of the *IGF2* and *GTL2* in the somatic tissue (liver) and placenta (chorion) of Clones 1-3. In contrast we observed abnormal biallelic expression at the imprinted *XIST* locus in the placenta (chorion) of Clones 1-3, but proper biallelic expression of the locus in the soma (liver). This demonstrates the first report of loss of imprinting (LOI) at a locus in an animal derived from somatic cell nuclear transfer. Analysis of chromatograms obtained from sequencing the *XIST* in the chorion indicated biallelic expression in Clone 2 and Clone 3 (Fig 3.7). These results were confirmed after multiple sequences were obtained from multiple sets of RNA preps and were additionally confirmed to be free from genomic contamination through the

sequencing of the *IGF2* locus from the same cDNA sample (and observing monoallelic expression at this locus). Further examination of the *XIST* locus through the cloning of RT-PCR products and subsequent sequencing of multiple plasmids revealed that all three clones had skewed allelic expression of the *XIST* locus, with the paternal transcript predominating, but with higher levels of maternal expression than expected (Clone 1 16.3 % maternal, Clone 2 34.1 % maternal and Clone 3 28.6 % maternal) in comparison to controls (Fetus H 6% maternal, Fetus A 3% maternal and Fetus B 5% maternal), which exhibited maternal expression as well, although overall the levels of expression were significantly different ($P<0.02$) between clones and controls. Observation of abnormal *XIST* expression in the chorion of clones can be further supported by observations from Xue et al (2002), which demonstrated aberrant patterns of X chromosome inactivation in the placenta of cloned cattle. In this experiment, analysis of 10 genes located on the X chromosome, which undergo inactivation, were observed for allelic expression in live and deceased clones, and results demonstrated that some deceased clones exhibited biallelic expression of genes. They expanded their analysis to the *XIST* DRM in exon 1 and found correlation between hypomethylation of the region and biallelic expression. However, when we analyzed the *XIST* DMR in exon 1 of cloned hybrids, through digestion with AciI/Bst UI and subsequent PCR across the region (*XIST5*), no apparent loss of methylation was observed. This can be explained in part by the lack of information we have about the biallelic expression of the *XIST* locus in our clones. In the chorion of control animals, presumably, the paternal *XIST* is preferentially expressed within each individual cell. In our assay, we are unable to

determine if the biallelic expression of *XIST* is resulting from both alleles being expressed in an individual cell or if parental expression is random in a subset of cells. These two situations are theoretically possible, but ascertaining which one is occurring is difficult. Therefore, detection of biallelic expression of *XIST* was observed in the chorion of clones and is abnormal, but identifying which situation it arose from our assay is not possible.

Results of the methylation status of the epidermal cytokeratin and satellite I region further support disregulation of reprogramming in the chorion of Clones. In the liver of Clones 1-3 and Control fetuses G-I, DNA methylation analysis of the epidermal cytokeratin promoter and satellite I region were in agreement between clones and controls respectively (clones: 63.8 and 65.4 % vs. Controls: 73.7 and 56.0 %). Analysis of the chorion revealed that the methylation of clones was greater at the epidermal cytokeratin promoter (Controls: 11.8 % and Clones: 37.0 %), but was not significantly different. However, hypermethylation was observed at the Satellite I region in the chorion (Controls 12.9 5 vs. Clones 49.8 %) and this value was significantly different ($P< 0.02$). In contrast to Xue et al. (2002), our results suggest that hypermethylation of the DNA occurs in the placenta, as shown by hypermethylation of the multi-copy satellite I (>100,000 copies), and is possibly associated with the biallelic expression that is observed at the *XIST* locus, although only speculation. Our observations are further supported by DNA methylation analysis of cloned bovine embryos. Kang et al. (2002) has demonstrated hypermethylation of the bovine trophectoderm (cells giving rise to chorion) in day 7 NT derived embryos and Dean et al. (2001) has demonstrated

incomplete demethylation of cloned bovine genomes during early embryonic development (Fig 1.2).

These results suggest that nuclear reprogramming of the cells giving rise to the placenta (trophectoderm) are improperly reprogrammed during early embryonic development, and potentially induce the placental abnormalities that are prevalent in cloned animals. This is not to say there are other areas in which improper reprogramming may occur, but DNA methylation and genomic imprinting have been shown to be crucial for the development in the early embryo when these lineages are established.

CHAPTER IV

SUMMARY AND FUTURE WORK

Due to the high incidences of abnormalities and the inefficiency of generating calves, the development of a model system in cattle to investigate potential problems is warranted. In nuclear transfer, where genomic imprinting has been implicated as the cause for these problems, use of an interspecies model is ideal since the parental inheritance of alleles can be easily ascertained. Here we have reported the use of a *Bos gaurus/Bos taurus* interspecies bovine model, which has facilitated the identification of three imprinted genes in the bovine and has allowed for their analysis in cloned fetuses.

Interspecies models are commonly used for the identification of imprinted genes in the mouse, in spite of abnormalities that are created from crosses in some species. The use of the Gaur crossed with domestic cattle has been used extensively for linkage analysis and frequent mating between the two species have also been reported for commercial purposes. The use of *Bos gaurus/Bos taurus* hybrids for the identification of imprinted genes as well as for the analysis of cloned fetuses was ideal since imprinting was shown to be normal and fetuses possessed no abnormalities.

Information obtained from use of these animals revealed conservation of genomic imprinting at the *IGF2* and *GTL2* loci with humans, mice and sheep and the *XIST* locus with mice. Additionally, it was demonstrated that the *WT1* locus is not imprinted in cattle, in contrast to reports in humans. To date, this is the largest

identification of imprinted genes in the bovine and further use of the *Bos gaurus/Bos taurus* interspecies model will undoubtedly identify more.

Additionally, this model has facilitated the first report of abnormal imprinting at a locus from somatic cell nuclear transfer. Abnormal biallelic expression of the *XIST* locus in the chorion of clones demonstrates the inability of cloning to properly reprogram certain loci. These results are supported by other observations of improper X chromosome inactivation in the bovine, which is regulated by the *XIST* locus. Further research will look into what specifically caused this abnormal allelic expression.

In future experiments, a more wide scale analysis of known imprinted genes can be obtained through identification of SNPs in regions that were not analyzed, such as the 5' and 3' untranslated regions (UTR). Also, as more sequence in the bovine is made available, differentially methylated regions will be identified and a comparative analysis performed with mice and humans so as to determine conserved necessary for establishing imprints. The analysis of imprinting in the bovine can also be expanded into the identification of novel imprinted genes. Gynogenetic and androgenetic embryos have facilitated the identification of imprinted genes in mice and can be expanded into the bovine where these are readily made and can be confirmed with the interspecies model. Further analysis of the cloned hybrid will facilitate the identification of other improperly reprogrammed genes. The model can also be used to adjust current nuclear transfer protocols and analyze the effects on imprinted genes.

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VITA

Scott Victor Dindot was born on May 30, 1975 in San Antonio, TX to Victor and Mary Dindot. He was raised in central Texas with his two sisters, Lisa and Kathleen. In 1993 Scott graduated from Lampasas High School and attended Howard Payne University in Brownwood, TX where he played football and ran track. In 1994, he transferred to Texas A&M in College Station, TX where he later graduated with a Bachelor of Science in molecular and cell biology in August 1999. In the fall of 1999, he began his studies as a doctoral student at Texas A&M under the supervision of Dr. Jorge Piedrahita where he began investigating the abnormalities that are associated with cloned cattle. Scott was married to Lisa Swanberg on January 1, 2000 and has two children, Payton and Tyler, born on April 8, 2001 and January 31, 2003. He received his doctoral degree in genetics in August 2003.

Permanent address: 301 Ovilla Rd. Waxahachie, TX 75167