Imprinted expression of the canine IGF2R, in the absence of an anti-sense transcript or promoter methylation

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SUMMARY Imprinted genes are epigenetically modified in a parent of origin-dependent manner, and as a consequence, are differentially expressed. Although the evolution of genomic imprinting is a subject of intense debate, imprinted genes have been studied primarily in mice and humans and in a small number of marsupial mammals. Comparative studies involving rodents and primates are of limited value, as they belong to the same superordinal group of eutherian mammals (Euarchontoglires). On the other hand, comparisons involving marsupials may not be informative, due to phylogenetic distance. Canis familiaris belongs to Laurasiatheria, a sister-group of Euarchontoglires, and should prove useful in comparative studies of imprinted genes. Using RT-PCR we demonstrate monoallelic expression of the canine IGF2R in several tissues, including uterus and umbilical cord. In the case of umbilical cord, we identify the expressed allele as maternally derived. The canine IGF2R is thus an imprinted gene. Using bisulfite sequencing, we show that the canine IGF2R resembles the imprinted mouse Igf2r in having a CpG island in intron 2 that is hemi-methylated. However, it differs from the mouse gene in that maintenance of the monoallelic expression of canine IGF2R does not require expression of an anti-sense transcript from the paternally derived allele, or methylation of the repressed IGF2R promoter. In these two important features, the imprinted canine gene resembles the imprinted opossum IGF2R. Our data suggest that these features were properties of the ancestral imprinted IGF2R and that the anti-sense transcript (Air) and promoter methylation observed in mouse are derived features of the mouse Igf2r locus.

INTRODUCTION

Genomic imprinting is a method of gene regulation whereby an epigenetic modification or “imprint” enables cells to identify a gene as maternally or paternally derived, and to either express or repress the gene in accordance with its parental origin. Imprinting has been observed in eutherian and marsupial mammals, but not in monotremes or non-mammalian vertebrates, and is thought to have evolved over 180 Ma, in a common ancestor of therian mammals (Killian et al. 2001). Imprinted genes are functionally haploid and thus have an increased vulnerability to mutations, loss of heterozygosity, and dysregulation of imprinting mechanisms. Despite this vulnerability, genomic imprinting survives in extant orders (Weidman and Jirtle 2007). Imprinting affects a small number of mammalian genes (<1%), but many of these have important roles in development (Reik and Walter 2001; Smith et al. 2006), and aberrant imprinting has been implicated in developmental and neurobehavioral disorders, and in many cancers (Reik and Walter 2001; Murphy and Jirtle 2003).

The factors that drove the evolution of imprinting are much discussed, yet imprinting has been studied in relatively few mammalian orders. To date, imprinted genes have been identified in rodents, primates (human), and artiodactyls, among placental mammals, and in a small number of marsupial species (Wilkins and Haig 2003). Comparative studies of imprinted genes have mainly focused on mice and humans and may be somewhat limited by the relative phylogenetic proximity of these species, as both are members of the same superordinal group, Euarchontoglires (Madsen et al. 2001; Murphy et al. 2001). On the other hand, comparisons of rodents and primates with marsupial mammals are not ideal either, because of the evolutionary distance between marsupial and eutherian mammals. Clearly our understanding of the evolution of imprinted genes would benefit from studies in mammals that are phylogenetically distant from rodents and primates, but closer to them than marsupial species. In this respect, the domestic dog (Canis familiaris) has the potential to be an extremely informative species, as the order Carnivora, to which the dog belongs, is a member of
Laurasiatheria, a sister-group to Euarchontoglires (Madsen et al. 2001; Murphy et al. 2001).

In addition to its phylogenetic position, the canine offers several advantageous features for studying the biological relevance of genomic imprinting. As companion animals, dogs share man’s environment and are medically investigated to a much greater extent than any other domesticated animal. They exhibit a wide range of diseases, including many of the complex diseases of humans to which imprinted genes are thought to contribute (cancer, diabetes, heart-disease and neurological disease) (Ostrander and Wayne 2005). Because of the unique population structure of modern dogs and the availability of extensive, high quality genome sequence (Kirkness et al. 2003; Lindblad-Toh et al. 2005), the canine may be an efficient system for whole-genome association studies of these diseases (Ostrander and Wayne 2005).

To initiate the study of genomic imprinting in this emerging model organism, we examined the canine ortholog of an intensely studied imprinted gene, IGF2R. IGF2R codes for the insulin-like growth factor 2 receptor (IGF2R), a multifunctional receptor involved in lysosome biogenesis and, in viviparous mammals, in regulation of fetal growth (Nolan and Lawlor 1999; Ghosh et al. 2003). The IGF2R is imprinted in rodents, artiodactyls, and opossum, with predominant expression of the maternally inherited allele (Barlow et al. 1991; Killian et al. 2000, 2001), but its expression is biallelic in the vast majority of humans (Killian et al. 2001; Monk et al. 2006). Although this gene is intensely studied, many questions remain as to the nature of the imprint and the mechanism by which monoallelic expression is achieved. In particular, imprinted expression in the mouse and opossum appears to involve different mechanisms (Killian et al. 2000; Weidman et al. 2006).

In mice, imprinting of Igf2r involves a differentially methylated CpG island in intron 2 known as DMR2. Methylation of this region takes place during oogenesis and appears to constitute the imprint associated with this gene (Stoger et al. 1993). In its unmethylated state, DMR2 functions as a promoter for a non-coding transcript termed Air, in antisense direction to Igf2r (Wutz et al. 2001). The Air transcript, or its expression, is necessary for methylation of the promoter of the paternally derived Igf2r and for repression of this allele (Zwart et al. 2001; Sleutels et al. 2002). The opossum Igf2r does not possess a DMR2 and its monoallelic expression does not require expression of an anti-sense transcript or promoter methylation (Killian et al. 2000; Weidman et al. 2006). These differences led to suggestions that monoallelic IGF2R expression is achieved by different means in the two species, following divergent evolution of the locus, or alternatively that both species share an imprinting mechanism whose fundamental features have not yet been defined (Killian et al. 2000). Examination of the imprint status of the canine IGF2R should help in elucidating evolution at the IGF2R locus.

In this report, we identify the canine IGF2R as an imprinted gene, by showing preferential expression of the maternally inherited allele and repression of the paternally derived allele. Somewhat surprisingly, we show that the imprinted canine IGF2R differs from the mouse ortholog and resembles the opossum IGF2R, in that monoallelic expression is observed in the absence of an anti-sense transcript and of promoter methylation. The most parsimonious explanation of our findings is that the ancestral imprinted IGF2R did not feature a paternally derived anti-sense transcript, and that the Air transcript associated with the mouse Igf2r locus is a derived feature.

MATERIALS AND METHODS

Tissues

Umbilical cord and parental hair were collected at birth and immediately submerged in RNAlater (Ambion/Applied Biosystems, Warrington, UK) in accordance with the manufacturer’s guidelines. Following incubation at 4 °C for 24 h, samples were subsequently stored at − 20 °C. Additional tissues (uterus, brain, heart, kidney, liver, lung, and skeletal muscle) were obtained during surgery, or during routine postmortem examinations of adult dogs, and processed in RNA later as described above, or snap frozen in liquid N₂ and stored at − 80 °C.

Nucleic acid preparation

Genomic DNA was extracted from frozen tissues by traditional phenol–chloroform extraction methods (Sambrook et al. 1989) or using the DNeasy Tissue Kit (Qiagen GmbH, Hilden, Germany). Total RNA was isolated using Tri-Reagent (Molecular Research Center Inc., Cincinnati, OH, USA) or RNA-STAT 60 (Tel-Test Inc., Friendswood, TX, USA) in accordance with the manufacturers’ instructions.

cDNA synthesis

RNA was DNase treated using the DNA-free kit (Ambion) according to the manufacturer’s guidelines. cDNA was synthesized at 42 °C with Superscript II RNase H− reverse transcriptase (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s guide, using random hexamer primers (Invitrogen). As a control for genomic DNA contamination, an identical reaction was carried out without the reverse transcriptase (-RT).

PCR amplification

Routine PCR amplifications used Platinum Taq DNA Polymerase (Invitrogen) or the Expand Long Template PCR System with Buffer 3 (Roche Diagnostics GmbH, Mannheim, Germany). For amplification of the GC-rich region toward the S′ end of the IGF2R cDNA, the Advantage² cDNA PCR Kit and Polymerase Mix (Clontech Laboratories, Palo Alto, CA, USA) was used. PCR amplifications used variations of the following touchdown protocol: 94 °C for 3 min, 94 °C for 20 sec, 72 °C for 15 sec (annealing temperature decreased by 1 °C/cycle in the next six cycles), 72 °C for 150 sec; followed by 30 cycles of 94 °C for 20 sec, 65 °C for 15 sec,
72°C for 150 sec, with a final extension of 72°C for 5 min. In some reactions, the touchdown annealing range was 70–60°C, and an annealing temperature of 60°C was used in subsequent cycles. For allelic expression studies involving exon 48 polymorphisms, the total number of cycles was 38. Allelic expression studies in intron 1 and intron 2 used a total of 50 cycles. Oligonucleotide primers were purchased from Sigma-Genosys (Haverhill, Suffolk, UK). Primer sequences used in this study are available on request.

**Purification and sequencing of amplicons**

PCR products were purified using the JetQuick PCR Purification Spin Kit (Genomed GmbH, Lohne, Germany) in accordance with the manufacturer’s guidelines, and sequenced commercially (Macrogen Inc., Seoul, Korea; MWG-biotech, Ebersberg, Germany) using internal primers (sequences available on request).

**Canine IGF2R cDNA sequence**

A search of the NCBI EST database revealed two canine ESTs (BQ009182 and BQ839605), with similarity to the 3′end of the mouse and human IGF2R transcripts. The sequences of these ESTs, in conjunction with cross-species IGF2R primers (Killian et al. 2001), and the canine genome sequence publicly available at the Broad Institute (www.broad.mit.edu/mammals/dog/) and the UCSC genome browser (http://genome.ucsc.edu), were used to amplify overlapping fragments of the canine IGF2R from canine liver cDNA or from genomic DNA (gene-specific primer sequences available on request). Amplified fragments were sequenced in both directions and 9032 bp of canine IGF2R mRNA sequence (AY965264) was confirmed in this way.

**DNA methylation analysis**

CpG islands were identified using the UCSC Genome Browser (http://genome.ucsc.edu, May 2005 assembly) was used for identification of the IGF2R exon–intron structure. For prediction of IGF2R signal peptide, the prediction program at www.ebi.ac.uk and BLAST at www.ncbi.nlm.nih.gov/ was used. Sequences were compared using the ClustalW program at www.ebi.ac.uk and BLAST at www.ncbi.nlm.nih.gov/. CpG islands were identified at http://www.uscnorris.com/. For identification of tandem repeats, we used http://tandem.bu.edu/trf/trf.html as described in Benson (1999).

**RESULTS**

**The canine IGF2R is an imprinted gene**

The canine IGF2R is located on dog chromosome 1 and, similar to its orthologs in other mammals, consists of 48 exons (Liu et al. 1995; Killian and Jirtle 1999). It gives rise to an mRNA (AY965264) of at least 9 kb that encodes a protein of 2497 amino acids, the IGF2R. The predicted mature canine IGF2R is very similar to the receptors in opossum, mouse, human, and bovine (72%, 83%, 85%, and 80% amino acid identity, respectively). In order to determine the allelic expression status of the canine IGF2R, we re-sequenced exon 48 in a number of individuals and identified a G/T SNP and an AG INDEL in the 3′UTR (nt 7768 and 8593, respectively, of AY965264; sequenced in reverse in Fig. 1). We identified individuals heterozygous for at least one of these polymorphisms and isolated RNA from these animals. Following cDNA preparation, the regions surrounding the polymorphisms were amplified by PCR and the amplicons sequenced. To ensure that amplification was specific for cDNA and did not represent contaminating genomic DNA, we used a primer pair that spanned intron 47, and included control reactions in which reverse transcriptase was omitted (-RT).

Initial experiments examined cDNAs from adult uterus and neonatal umbilical cord (uterus, n = 4; umbilical cord, n = 9), and in both tissues and all individuals, expression of IGF2R was monoallelic (as shown by representative individuals in Fig. 1), strongly suggesting that the canine IGF2R is an imprinted gene.
To determine the parental origin of the expressed allele, we genotyped the parent(s) of heterozygous pups who had contributed umbilical cord samples. Two independent parent/offspring combinations were informative, in that at least one parent was homozygous for the polymorphism for which the pup was heterozygous. The genotype of the parents clearly identifies the expressed allele in umbilical cord as maternally derived (shown for two offspring/parent combinations in Fig. 1B). Thus the canine IGF2R is an imprinted gene, with expression and repression, respectively, of maternally and paternally derived alleles. This allelic expression pattern is similar to that seen with the imprinted IGF2Rs of opossum, rodents, and artiodactyls (Barlow et al. 1991; Mills et al. 1998; Killian et al. 2000, 2001), and contrasts with the situation in humans where biallelic IGF2R expression is apparent in the vast majority of individuals (Killian et al. 2001; Monk et al. 2006).

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For some imprinted genes, the degree of allele repression varies according to tissue type, or shows temporal variation with repression restricted to certain developmental stages. For example, both alleles of murine Igf2r are expressed early in development, before implantation, and the paternal allele is subsequently repressed in all somatic tissues except for the brain, where the Igf2r expression remains biallelic (Wang et al. 1994; Lerchner and Barlow 1997; Hu et al. 1999). Biallelic Igf2r expression has also been demonstrated in primary cultures of mouse neurons, whereas expression in primary cultures of glial cells and fibroblasts is monoallelic (Yamasaki et al. 2005). To see whether the extent of monoallelic expression of the canine IGF2R varies with tissue type, we examined expression in heterozygous adult individuals for which a range of tissues was available. Although there was some variation in the expression patterns from individual to individual, expression in the brain and the kidney \((n = 2)\) was biallelic, whereas expression in other tissues \((n = 2 \text{ or } 3)\) was predominantly or exclusively monoallelic (Fig. 2). We did not have genomic DNA from the parents of these individuals and were thus unable to confirm the parental origin of the expressed allele. However, for a given individual, the same allele was predominant in each tissue, and we assume that this represents the maternally derived allele.
Having identified the canine IGF2R as an imprinted gene, we investigated the mechanisms involved in achieving monoallelic expression, to see whether the canine IGF2R resembles the mouse gene or the opossum ortholog. Intron 2 of the canine IGF2R contains a 2923 bp CpG island (chromosome 1, 52228773–52231695, May 2005 assembly; %GC 572.6; CpGobs/CpGexp = 0.91). The canine CpG island shows no sequence similarity to mouse DMR2 or to the CpG islands in intron 2 of human, sheep, or bovine IGF2R. In particular, the canine CpG island does not contain a sequence similar to a putative imprinting control element demonstrated in mouse Igf2r (Birger et al. 1999). However, the canine CpG island contains several tandem repeats (Table 1). Tandem repeat arrays are significantly enriched in CpG islands associated with many imprinted genes (Hutter et al. 2006) and are present in mouse DMR2 (Smrzka et al. 1995), although their significance is not yet clear. To see whether the canine intron 2 CpG island is methylated, we performed bisulfite DNA sequencing of a portion of the CpG island (beginning 1805 bp from the beginning of the CpG island). We found hemimethylation of all the CpGs in this region (Fig. 3). Methylation was observed in all tissues examined, and in all individuals (uterus, n = 7; umbilical cord, n = 2; kidney, n = 8; brain, n = 3; liver, n = 2; lung, n = 2; blood, n = 1; skin, n = 1; heart, n = 1). Although our analysis does not allow us to specifically associate the observed methylation with the maternally derived allele, it seems likely that the canine IGF2R has a DMR2 similar to that seen in mice and artiodactyls (Zwart et al. 2001; Young et al. 2003; Long and Cai 2007), and that this could constitute the canine IGF2R imprint.

**No evidence for a putative canine AIR transcript**

In addition to constituting the putative Igf2r imprint, murine DMR2 is required for monoallelic expression of Igf2r: in its unmethylated state, it generates a non-coding anti-sense transcript (Air) that is necessary for repression of the paternally derived Igf2r (Wutz et al. 2001; Zwart et al. 2001; Sleutels et al. 2002). The structural similarities between the canine and mouse intron 2 CpG islands suggested that the canine CpG
island could also function as a promoter for an anti-sense transcript. It was important therefore to test for the presence of a putative canine AIR transcript in tissues where canine IGF2R is monoallelically expressed.

In mouse, transcription of Air begins in DMR2 and continues for 108 kb in an anti-sense direction to Igf2r, terminating in the last intron of the nearest upstream gene, the Mas1 oncogene (Wutz et al. 1997; Lyle et al. 2000). In the dog, MAS1 and IGF2R are neighboring genes on chromosome 1. However, the intergenic region in the canine contains a predicted novel gene (http://genome.ucsc.edu/Genescan_1_10.79) that could complicate expression studies in this region, and we therefore focused our search for a putative canine AIR in the region where it would overlap with IGF2R. Our strategy was to identify polymorphisms in this region that would enable us to detect expression from the paternal allele, in tissues such as uterus and umbilical cord, where IGF2R is monoallelically expressed. We identified three SNPs in intron 2 of the canine IGF2R (C/T SNPs at nt 335 and 365, and a G/T SNP at nt 883, respectively, of Accession No. EF437958).

Table 1. Tandem repeats in canine IGF2R intron 2 CpG island

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<tr>
<th>Repeat sequence</th>
<th>Motif length (nt)</th>
<th>Number of repetitions</th>
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<td>2 GGTCTGGCCGGGCGGGTTCAAGGGCTGGCCACGGGTTCTGCAAGGACCCGGCGCGGCTGGCGCTGT 29 2.1</td>
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<tr>
<td>3 GGCCTGGCCGGGCGGGTTCTGCAAGGACCCGGCGCGGCTGGCGCTGT 22 2.8</td>
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<tr>
<td>4 TGGTCTGGCCGGGCGGGTTCAAGGGCTGGCCACGGGTTCTGCAAGGACCCGGCGCGGCTGGCGCTGT 28 3.6</td>
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<tr>
<td>5 GGTCTGGCCGGGCGGGTTCAAGGGCTGGCCACGGGTTCTGCAAGGACCCGGCGCGGCTGGCGCTGT 55 2.4</td>
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<tr>
<td>12 GGCCTGGCCGGGCGGGTTCAAGGGCTGGCCACGGGTTCTGCAAGGACCCGGCGCGGCTGGCGCTGT 14 2.6</td>
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Table 1. Tandem repeats in canine IGF2R intron 2 CpG island


Intron 2 CpG island is hemi-methylated. Genomic DNA isolated from the indicated tissues and individuals was subjected to bisulfite sequencing. PCR amplicons were generated from sequence located within the intron 2 CpG island. Lanes are loaded in the order indicated, with cytosines in the CpG dinucleotide context marked by arrowheads. Cytosines that are methylated in gDNA are unconverted by bisulfite treatment and appear in “C” lane, while unmethylated cytosines are converted and appear in “T” lane (Cs are migrating slightly faster than the corresponding Ts) (U. Cord, umbilical cord).
RNA from individuals heterozygous at one or more of these sites was DNase treated and used to generate cDNA. We used random hexamers to prime reverse transcription (so that both sense and potential anti-sense transcripts would be represented in the cDNA) and included -RT controls to confirm the absence of genomic DNA. PCR amplification of the uterus and umbilical cord-derived cDNAs (uterus, \( n = 4 \); umbilical cord, \( n = 9 \)), followed by sequencing of amplicons, demonstrated the presence of a single expressed allele in each tissue and individual (shown for a representative individual, heterozygous at two positions, in Fig. 4A). Genotyping of parent(s) of two heterozygous individuals (from independent litters) confirmed that the expressed allele in the umbilical cord was maternal in origin (shown for one individual, in Fig. 4A). This identifies the transcript as \( IGF2R \) pre-mRNA. No transcription was detected from the paternally inherited allele. We also looked further upstream in the predicted overlap region for evidence of a transcript from the paternally inherited allele, and identified four polymorphisms in \( IGF2R \) intron 1 (C/T SNPs at nt 206, 1026, and 1306, and an A/C SNP at nt 1162 of Accession No. EF437959). Three individuals (from two independent litters) were each heterozygous for two of these SNPs. In the three individuals, PCR amplification of random hexamer-primed umbilical

![Fig. 4. Allelic expression in the putative \( IGF2R/AIR \) overlap region. (A) Pup 522 is heterozygous for two polymorphisms in intron 2 of the \( IGF2R \) (gDNA, arrowheads; C/T SNPs at 335 and 365 of Accession No: EF437958). Amplification of random hexamer-primed umbilical cord cDNA indicates expression of only one allele. The genotype of the dam, homozygous for both SNPs, identifies the expressed allele as maternal in origin. (B) Pup 524 is heterozygous for two polymorphisms in intron 1 (gDNA, arrowheads; A/C SNP and C/T SNP at 1162 and 1306, respectively, of Accession No: EF437959, both sequenced in reverse). Amplification of umbilical cord cDNA indicates expression of one allele only. The genotype of the dam identifies the expressed allele as maternal in origin. Thus, in two putative \( IGF2R/AIR \) overlap regions there is no evidence of a transcript derived from the paternally inherited allele (gDNA, genomic DNA).}
cord cDNA detected transcripts from one allele only (shown for a representative individual in Fig. 4B). The genotypes of the parents of the individual in Fig. 4B showed that the transcript was derived from the maternally inherited allele. Again, there was no evidence of transcription from the paternally derived allele.

A potential explanation for the absence of a putative canine AIR transcript is a lack of sensitivity of our assay. We consider this unlikely, however. AIR has been detected in mouse (embryos and adult tissues) by RT-PCR approaches that use 35–40 cycles (Hu et al. 1999; Sotomaru et al. 2002; Pauzer et al. 2005; Yamasaki et al. 2005; Seidl et al. 2006). In order to maximize the detection of a putative low-abundance canine AIR transcript, we used 50 cycles in our assays (routine detection of mature canine IGF2R transcripts involved 35–40 cycles). In mouse, the levels of Air relative to mature Igf2r vary between tissues, from 17% in adult kidney to 67% in adult lung (Seidl et al. 2006). Our RT-PCR assays routinely detected canine IGF2R pre-mRNA, as well as the more-abundant mature IGF2R, and would have detected canine AIR, if its relative levels were comparable with those of mouse Air. Another possibility is that a putative canine AIR is spliced in such a way that our PCR primers do not facilitate its amplification. Again we consider this unlikely: the majority of murine AIR transcripts are unspliced (Seidl et al. 2006) and, if a putative canine AIR had a similar splicing pattern to that of the mouse, we would have detected it. We conclude therefore that canine AIR transcripts are absent in the uterus and the umbilical cord, where IGF2R is monoallelically expressed, and are therefore not required for maintenance of monoallelic expression. In this property, the imprinted canine IGF2R differs from the mouse Igf2r, and resembles the opossum ortholog.

**Hypomethylation of promoter-associated CpG island of canine IGF2R**

All mammalian IGF2Rs described to date have a CpG island associated with the promoter and first exon (Stoger et al. 1993; Riesewijk et al. 1996; Hu et al. 2006; Weidman et al. 2006). In mouse, this region is differentially methylated (methylated on the repressed allele, unmethylated on the transcribed allele) and for this reason is referred to as DMR1. Methylation at DMR1 occurs post-fertilization, following silencing of the paternally derived allele (Stoger et al. 1993), and is dependent on Air expression (Wutz et al. 2001; Sleutels et al. 2002). In opossum, however, the promoter-associated CpG island remains unmethylated on both alleles even though the paternally derived allele is repressed (Weidman et al. 2006). In view of the absence of a canine AIR transcript in tissues where IGF2R is monoallelically expressed, we examined the methylation status of a portion of the 2363 bp CpG island at the canine IGF2R promoter/exon 1 region (chr 1, 52202808 to 52205170, May 2005 assembly; %GC = 61.6%, ObsCpG/ExpCpG = 1.012). The sequence examined began 1131 bp within the CpG island, and encompassed sequence immediately downstream of the transcription start site and spanning the translational start codon. We found a marked hypomethylation at all CpGs examined: for 17 sites quantified, the average methylation was <6.4% (Fig. 5).

Hypomethylation was apparent in all tissues and individuals examined, including the uterus (n = 6) and the umbilical cord (n = 6), where expression of IGF2R is monoallelic, as well as in the brain (n = 3) and the kidney (n = 7), where expression is biallelic. Thus, maintenance of monoallelic IGF2R expression in the dog does not require methylation of the repressed promoter, and in this respect, the canine IGF2R resembles the imprinted opossum IGF2R rather than the murine ortholog.

**DISCUSSION**

In this study we demonstrate the existence of genomic imprinting in a representative of the order Carnivora, a mammalian order in which this epigenetic phenomenon has not previously been described. We show that the canine IGF2R is an imprinted gene, with the predominant expression of a single allele in most tissues. The expressed allele is maternally derived, as had been previously observed with IGF2R in opossum, mouse, and artiodactyls (Barlow et al. 1991; Killian et al. 2000). This contrasts with the situation in humans, where the majority of individuals express both IGF2R alleles (Killian et al. 2001; Monk et al. 2006). Our data support the hypothesis that IGF2R imprinting arose in a common ancestor of eutherian and marsupial mammals and was lost in the lineage leading to primates (Killian et al. 2001; Wilkins and Haig 2003). In addition to extending the known phylogenetic range of genomic imprinting, our demonstration of imprinting in Canis familiaris is timely, in that the dog is fast becoming a model system for the study of complex human diseases (Ostrander and Wayne 2005) and may therefore be useful in understanding how imprinted genes contribute to these diseases. In addition, the unique population structure of modern dogs offers significant potential for understanding how imprinted genes contribute to evolutionary processes: dogs have been subjected to strong selective pressures during the generation of the numerous “breeds” that currently exist, and exhibit more morphological diversity than any other mammal, as well as a wide range of behaviors (Lindblad-Toh et al. 2005; Ostrander and Wayne 2005). This diversity must have its origin in growth and differentiation patterns facilitated by the underlying genome and epigenome and, given the roles of imprinted genes in growth and behavior (Weidman and Jirtle 2007), they may have contributed to the generation of canine diversity. Finally, as we show here, the dog can offer insights into the evolution of imprinted genes.
Although the evolution of genomic imprinting is the subject of much speculation, imprinting has been studied in relatively few mammalian orders, with demonstrations of imprinted genes limited to rodents, primates (human), and artiodactyls, among placental mammals, and to a small number of marsupial species. This restricted analysis means that little is known of the evolution of imprinted genes. We chose to study the canine IGF2R because of well-characterized differences in imprinting between orthologs of this gene in mouse and opossum (Killian et al. 2000; Weidman et al. 2006). These differences suggested that the monoallelic expression of IGF2R was achieved by different means in the two species, following divergent evolution of the locus, or alternatively that both species share an as-yet uncharacterized imprinting mechanism (Killian et al. 2000). The phylogenetic position of the dog (a eutherian mammal) but in a different superordinal group to primates and rodents (Madsen et al. 2001; Murphy et al. 2001) suggested that characterization of the canine IGF2R could help resolve ancestral features of this imprinted gene from derived features. Although we found some similarities between the imprinted canine and mouse IGF2Rs, our major finding is that the canine IGF2R differs from the mouse Igf2r in two important features, and resembles the opossum ortholog.

In mice, imprinting of Igf2r involves a differentially methylated CpG island in intron 2 (DMR2). Methylation of this region appears to constitute the imprint associated with this gene, as it takes place during oogenesis (Stoger et al. 1993) and is required for monoallelic expression of the gene (Wutz and Barlow 1998). The canine IGF2R also contains a CpG island in intron 2, although it bears no sequence similarity to intron 2 CpG islands in mouse, human, sheep, or bovine IGF2Rs. We showed that the canine intron 2 CpG island is hemi-methylated, although we were unable to determine the parental origin of the methylation, due to the absence of appropriate polymorphisms. Nevertheless, from studies of mouse, human, and bovine IGF2Rs, it seems likely that it is oocyte derived and constitutes a canine DMR2 (Young et al. 2003, 2001; Long and Cai 2007). However, the association of DMR2 with imprinting at the IGF2R cluster is not straightforward, as shown by cross-species studies. The imprinted IGF2R in sheep and cow contains a DMR2 (Young et al. 2001, 2003; Long and Cai 2007), but there is no DMR2 in the imprinted opossum IGF2R (Killian et al. 2000); within Euarchonta, the non-imprinted IGF2Rs of tree shrew and ringtail lemur lack a CpG island in intron 2 (Killian et al. 2001) whereas human IGF2R contains a DMR2, although the human gene is not imprinted in the majority of
individuals (Killian et al. 2001; Monk et al. 2006). Thus, DMR2 does not appear to be either necessary or sufficient for imprinting IGF2R (Vu et al. 2006), and the functional significance of a DMR2 in the canine IGF2R is unclear.

For imprinted genes, inheritance of an imprint typically results in the expression of one allele, in a parent of origin-dependent manner, and repression of the other. Having identified the canine IGF2R as an imprinted gene with a probable DMR2, it was reasonable to expect that its monoallelic expression would correlate with the expression of an anti-sense transcript (a putative canine AIR generated from the intron 2 CpG island) and with methylation of the repressed promoter. Our demonstration of the monoallelic expression of canine IGF2R in the absence of an anti-sense transcript originating in intron 2, or of methylation of the promoter/exon1 CpG island, was therefore somewhat unexpected. We cannot formally exclude the possibility that an AIR transcript is required in canine embryos for the initiation of monoallelic IGF2R expression (although we suggest below that this is unlikely). However, it is clear that the maintenance of the imprinted expression of canine IGF2R does not require the expression of an anti-sense transcript originating in intron 2. Thus, in two important features (promoter hypomethylation and absence of anti-sense transcription), imprinting of the canine IGF2R differs from the mouse gene and resembles the phylogenetically distant opossum IGF2R (Weidman et al. 2006), suggesting that these features represent properties of the ancestral imprinted IGF2R.

Our study raises questions about the function and evolutionary origin of anti-sense transcription at the IGF2R locus. The involvement of murine AIR in the monoallelic expression of Igf2r, and in methylation of the repressed Igf2r promoter, has been well established by genetic experiments involving the targeted deletion of DMR2 or truncation of the AIR transcript (Zwart et al. 2001; Sleutels et al. 2002). However, the molecular mechanisms by which AIR contributes to monoallelic expression are not understood. Specifically it is not known whether AIR is needed to induce silencing, or acts at a later stage to maintain the silent state (Seidl et al. 2006). Expression studies describe the onset of monoallelic Igf2r expression in mouse embryos at E6.5 (Lerchner and Barlow 1997), while the earliest report of AIR expression is at E9.5 (Sotomaru et al. 2002; Seidl et al. 2006). These data are more consistent with a role for AIR in maintaining, rather than inducing, Igf2r silencing. The imprinted opossum IGF2R further dissociates AIR expression from initiation of IGF2R repression, as the gene does not contain an intron 2 CpG island that could function as a promoter for an AIR transcript (Killian et al. 2000). Furthermore, in the very small minority of human individuals where monoallelic expression of IGF2R was observed, no anti-sense transcripts corresponding to murine AIR were observed (Oudejans et al. 2001; Monk et al. 2006), in spite of the existence of a DMR2 in the human IGF2R (Smrzka et al. 1995).

We suggest therefore that the most parsimonious interpretation of all these expression data (mouse, human, opossum, and now dog) is that the ancestral imprinted IGF2R did not require an anti-sense transcript originating in intron 2 for either initiation or maintenance of monoallelic IGF2R expression. We further propose that the promoter of the ancestral imprinted IGF2R did not require methylation for maintenance of repression. In this scenario, murine AIR is a derived feature of the locus required for maintaining the repressed state in this species. These hypotheses can be tested experimentally by extending the study of IGF2R to additional mammalian orders. If supported, they are consistent with the idea that all species that possess an imprinted IGF2R may share a common mechanism for initiation of monoallelic expression (Killian et al. 2000). The nature of this mechanism remains to be identified. Post-translational modification of histone tails can contribute to the establishment of monoallelic expression patterns, including those observed with imprinted genes (Rougelle and Heard 2002). Allele-specific histone modifications have been detected in the mouse Igf2r promoter and in mouse DMR2 but were not detected in the human gene (Yang et al. 2003; Vu et al. 2004; Yamasaki et al. 2005). It is possible therefore that histone modifications are involved in the initiation of imprinted IGF2R expression. Characterization of the histone modifications associated with the imprinted canine and opossum IGF2Rs is thus warranted.

CONCLUSION

In summary, our demonstration of genomic imprinting in the canine and our observation that molecular aspects of the imprinted canine IGF2R resemble the phylogenetically distant opossum IGF2R rather than the murine Igf2r illustrate the value of the dog as an informative species for comparative studies of imprinted genes.

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REFERENCES


