Letter

Novel Imprinted *DLK1/GTL2* Domain on Human Chromosome 14 Contains Motifs that Mimic Those Implicated in *IGF2/H19* Regulation

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The evolution of genomic imprinting in mammals occurred more than 100 million years ago, and resulted in the formation of genes that are functionally haploid because of parent-of-origin–dependent expression. Despite ample evidence from studies in a number of species suggesting the presence of imprinted genes on human chromosome 14, their identity has remained elusive. Here we report the identification of two reciprocally imprinted genes, *GTL2* and *DLK1*, which together define a novel imprinting cluster on human chromosome 14q32. The maternally expressed *GTL2* (gene trap locus 2) gene encodes for a nontranslated RNA. *DLK1* (delta, *Drosophila*, homolog-like 1) is a paternally expressed gene that encodes for a transmembrane protein containing six epidermal growth factor (EGF) repeat motifs closely related to those present in the delta/notch/serrate family of signaling molecules. The paternal expression, chromosomal localization, and biological function of *DLK1* also make it a likely candidate gene for the callipyge phenotype in sheep. Many of the predicted structural and regulatory features of the *DLK1/GTL2* domain are highly analogous to those implicated in *IGF2/H19* imprint regulation, including two hemimethylated consensus binding sites for the vertebrate enhancer blocking protein, CTCF. These results provide evidence that a common mechanism and domain organization may be used for juxtapositioned, reciprocally imprinted genes.

Genomic imprinting refers to an epigenetic chromosomal modification that results in the preferential expression of a gene in a parent-of-origin–dependent manner. Genomic imprinting evolved in mammals >100 million years ago (Killian et al. 2000) possibly because of an interparental genetic conflict to control maternal-dependent growth of the offspring (Haig and Graham 1991). Imprinted genes have been linked to a number of human behavioral and developmental disorders, including Angelman, Prader-Willi, and Beckwith-Wiedemann syndromes, as well as a variety of pediatric and adult malignancies (for reviews, see Nicholls et al. 1998; Falls et al. 1999; Mann and Bartolomei 1999; Reik et al. 2000). Evidence also suggests that a number of unidentified imprinted genes underlie the etiology of other human disorders, including autism, schizophrenia, bipolar disease, and Crohn's disease (Morison and Reeve 1998; Isles and Wilkinson 2000). Therefore, the isolation and characterization of novel imprinted genes will provide further insight into their roles in these disorders as well as into the regulatory mechanisms fundamental to this intriguing phenomenon.

Abnormal phenotypes associated with uniparental disomy (UPD) have implied the presence of imprinted genes on a number of chromosomes (Ledbetter and Engel 1995). These include distinct clinical abnormalities associated with both maternal and paternal UPD of the long arm of human chromosome 14 (14q24.3–32). Maternal UPD (mUPD) of chromosome 14 is associated with low birth weight, short stature, small hands and feet, motor delay, and precocious puberty, whereas paternal UPD (pUPD) is not only observed less frequently, but it also leads to more severe musculoskeletal problems and mental retardation. Consistent with these observations in humans, genetic studies using Robertsonian or reciprocal translocations to generate UPD for mouse distal chromosome 12 result in early embryonic lethality, indicating the presence of an imprinted gene or genes in a region homologous with human chromosome 14 (Cattanach and Beechey 1997). The parent-of-origin inheritance of the *callipyge* gene, mapped to the distal portion of chromosome 18 in sheep, is also consistent with the presence of imprinted genes in this homologous region of the long arm of human chromosome 14 (Cockett et al. 1996; Freking et al. 1998; Lien et al. 1999).

Despite compelling evidence for the presence of maternally and paternally imprinted genes on human chromosome 14, their identity has remained elusive. We used a bioinformatics-based approach to select candidate regions of chromosome 14 for further expression and DNA methylation analysis. This led to the identification of two reciprocally imprinted genes on

human chromosome 14q32. *GTL2* is maternally expressed and appears to lack an open reading frame. In contrast, *DLK1* is paternally expressed, and encodes for a cell-surface transmembrane protein containing epidermal growth factor-like (EGF-like) repeats that are closely related to the EGF-like repeats of the invertebrate proteins delta and notch (Laborda et al. 1993; Artavanis-Tsakonas et al. 1995; Fleming 1998). Further analysis of the structural, spatial, and epigenetic characteristics of the *DLK1/GTL2* domain revealed a striking similarity to the *IGF2/H19* domain on human chromosome 11.

RESULTS

Identification of Novel Imprinted Genes

Using gene trap technology, Schuster-Gossler et al. (1996) identified a transgene-induced insertional mutation (*Gtl2*^{lacZ}) on mouse distal chromosome 12 that conferred proportionate dwarfism in a parent-of-origin–dependent manner. The gene subsequently identified from the site of transgene integration was *Gtl2* (gene trap locus 2) (Schuster-Gossler et al. 1998). Using BLAST analysis of the NCBI GenBank database, we found that human cDNA clone 23887 (AF052114) had significant homology with mouse *Gtl2*, and that it lacked a significant open reading frame. This clone and its associated UniGene cluster (Hs.112844) map to human chromosome 14q32, a region homologous with distal mouse chromosome 12. Alignment of clone 23887 sequence (henceforth referred to as human *GTL2*) with published human BAC sequence AL117190 revealed the presence of five exons in the mRNA sequence, with the transcription start site located at nucleotide 69187 (Fig. 1A).

To determine if *GTL2* is monoallelically expressed, we identified a single nucleotide polymorphism (SNP) in exon 5 (Fig. 1A), and analyzed allelic expression of *GTL2* in tissues from five human conceptuses heterozygous for the polymorphism. As shown in Figure 1B, *GTL2* was monoallelically expressed in fetal heart ($n = 1$), kidney ($n = 2$), liver ($n = 2$) and lung ($n = 2$). *GTL2* was also monoallelically expressed in fetal brain ($n = 4$, data not shown). Thus, *GTL2* was shown to be monoallelically expressed in 11 tissues from five different human conceptuses. The expressed allele was determined to be of maternal origin by genotyping matching maternal decidua tissue (Fig. 1B). Using an alternative experimental approach, Miyoshi et al. (2000) recently identified a maternally expressed human homolog of mouse *Gtl2*, which they called *MEG3*, and using Northern blot analysis showed that *MEG3* (*GTL2*) was an abundant transcript. The function of *GTL2* is presently unknown because neither the mouse nor human homologs contain a significant open reading frame.

Gtl2, being maternally expressed, was unlikely to be directly involved in generating the abnormal phenotype of the *Gtl2*^{lacZ} mouse. It was therefore likely that the dwarfism phenotype of the *Gtl2*^{lacZ} mouse resulted from disruption of a paternally expressed gene located in close proximity to *Gtl2*. BLAST analysis of a 500 kb region surrounding *GTL2* (BACs AL117190, AL132711, and AL163974), using both the nonredundant and human EST GenBank databases, identified *DLK1* (delta, *Drosophila* homolog-like 1) located 102 kb centromeric to *GTL2*.

A SNP was identified in exon five of *DLK1* (Fig. 2A), and it was used to analyze gene expression in seven heterozygous individuals. As shown in Figure 2B, *DLK1* is monoallelically expressed in fetal brain ($n = 7$), kidney ($n = 3$), liver ($n = 3$) and lung ($n = 2$). Monoallelic expression was also detected in other fetal tissues, including adrenal gland ($n = 2$), skeletal muscle ($n = 1$), gut ($n = 2$), heart ($n = 4$), spleen ($n = 1$) and placenta ($n = 2$, data not shown). Thus, *DLK1* was monoallelically expressed in 27 tissues from seven different human conceptuses. The parent-of-origin of the expressed allele was determined to be exclusively paternal following the genotyping of matched maternal decidua tissue (Fig. 2B). This is consistent with the recent finding that mouse *Dlk1* is also paternally expressed (Schmidt et al. 2000).

Methylation Analysis

Methylation appears to be a key component of the imprint regulation in eutherian mammals, and most known imprinted genes are associated with differentially methylated CpG-rich regions. GRAIL analysis of the upstream regions of *DLK1* and *GTL2* showed that the putative promoter regions of both genes contain sequences rich in CpG dinucleotides. To determine their methylation status, three independent areas of each region were analyzed using bisulphite sequencing of DNA isolated from fetal brain, kidney, liver, and pancreas (Fig. 3). The methylation profile of both putative promoter regions was indistinguishable in the four tissues analyzed, and a representative analysis is shown in Figure 3. All three areas of the CpG-rich region examined upstream of *GTL2* (G1, position 65,973–66,085; G2, position 66,667–66,793, and G3, position 67,780–67,926; GenBank accession no. AL117190) were found to be hemimethylated, consistent with the notion that allelic methylation differences contribute to the imprinted regulation of *GTL2* expression. In contrast, whereas ~150 bp of the upstream *DLK1* promoter region were hemimethylated (D1, position 140,543–140,687; accession no. AL132711), the two downstream regions were predominantly unmethylated (D2, position 141,101–141,205; and D3, position 141,459–141,594; accession no. AL132711).
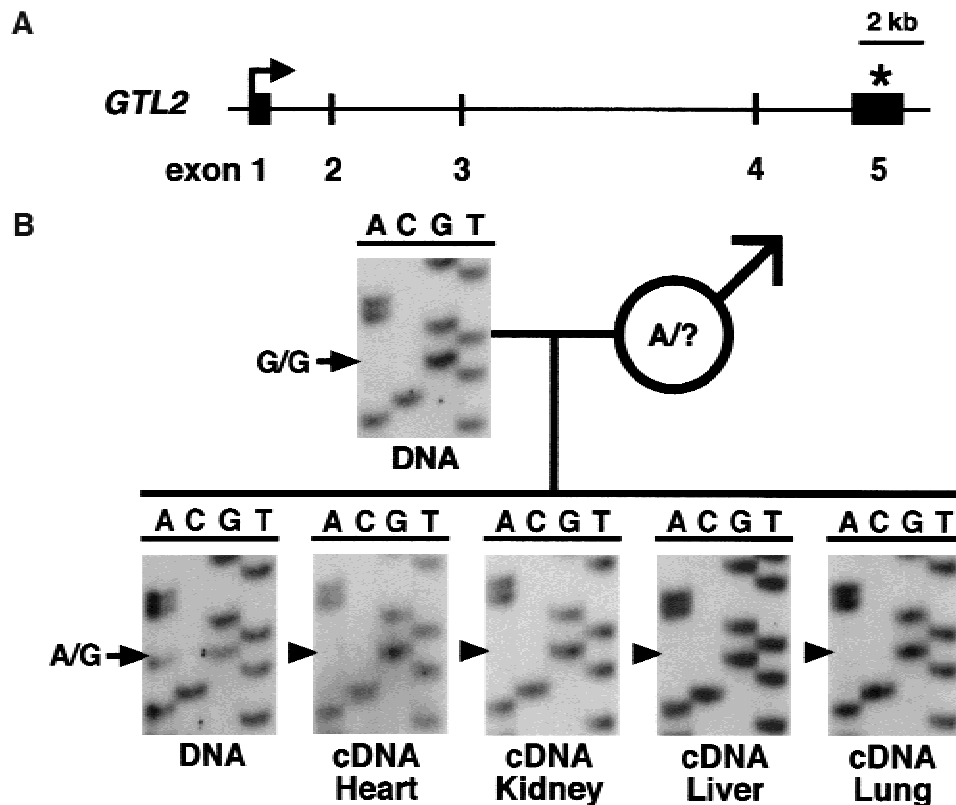
Figure 1 Predicted genomic structure and expression patterns for *GTL2*. (*A*) Predicted genomic structure of *GTL2*. Black boxes and horizontal lines denote exons and introns, respectively. (*) shows the location of a single nucleotide polymorphism in exon 5 (position 86,647 of accession no. AL117190). (*B*) Parent-of-origin–dependent monoallelic expression of *GTL2*. An 85-d gestation conceptus heterozygous for the A/G polymorphism in exon 5 of *GTL2* was used to determine the allelic expression of *GTL2*. The maternal genotype is G/G, demonstrating paternal inheritance of the A allele in the conceptus. Analysis of RNA isolated from the indicated tissues shows exclusive expression of the maternally derived G allele (i.e., A allele is unexpressed; arrowhead).

Comparison of the overall methylation profile of the *GTL2/DLK1* imprinted domain with that of other imprinted domains revealed a striking similarity to the imprinted *IGF2/H19* locus on chromosome 11. Like *GTL2*, a region ~2 kb upstream of *H19*, is also hemi-methylated and contains imprinting control elements important for the reciprocal imprinting of *H19* and *IGF2* (Thorvaldsen et al. 1998; Webber et al. 1998). One of the elements involved in this control was recently identified as the vertebrate enhancer blocking protein, CTCF, whose binding to DNA is inhibited by methylation (Bell and Felsenfeld 2000; Hark et al. 2000; Szabo et al. 2000). Differential methylation of CTCF binding sites has therefore been proposed to contribute to imprint regulation by the formation of chromatin boundaries on the unmethylated allele. This is postulated to prevent downstream enhancers from interacting with the promoter of the protein-encoding gene (Reik and Murrell 2000). Inspection of the sequence upstream of *GTL2* revealed two consensus CTCF binding sites (Fig. 4A), CTCF(a) and CTCF(b), located 1131 bp (position 68,056 of AL117190) and 840 bp (position 68,347 of AL117190), respectively, upstream of the predicted

GTL2 transcription start site. Analysis of 500 kb of flanking sequence (BACs AL117190, AL132711, and AL163974) confirmed these CTCF consensus-binding sites were unique to this region. Furthermore, bisulphite sequencing of fetal DNA at this locus confirmed the presence of hemimethylation within and surrounding both CTCF consensus sequences (Fig. 4B).

Genomic and Epigenetic Comparison of the *DLK1/GTL2* and *IGF2/H19* Domains

Comparison of the spatial, structural, and epigenetic characteristics of both *DLK1/GTL2* and *IGF2/H19* domains shows a number of intriguing similarities (Fig. 5). *GTL2* contains five exons and is located 102 kb telomeric from *DLK1*, whereas *H19* also contains five exons and is located ~100 kb telomeric from *IGF2*. Like *H19*, *GTL2* contains multiple small open reading frames (ORFs) with the longest ORF potentially encoding for a protein consisting of 117 amino acids. However, there is no KOZAK consensus sequence around the initial ATG, and it lacks significant homology with known proteins. *DLK1* and *GTL2* are transcribed in the same orientation and are reciprocally imprinted, as is
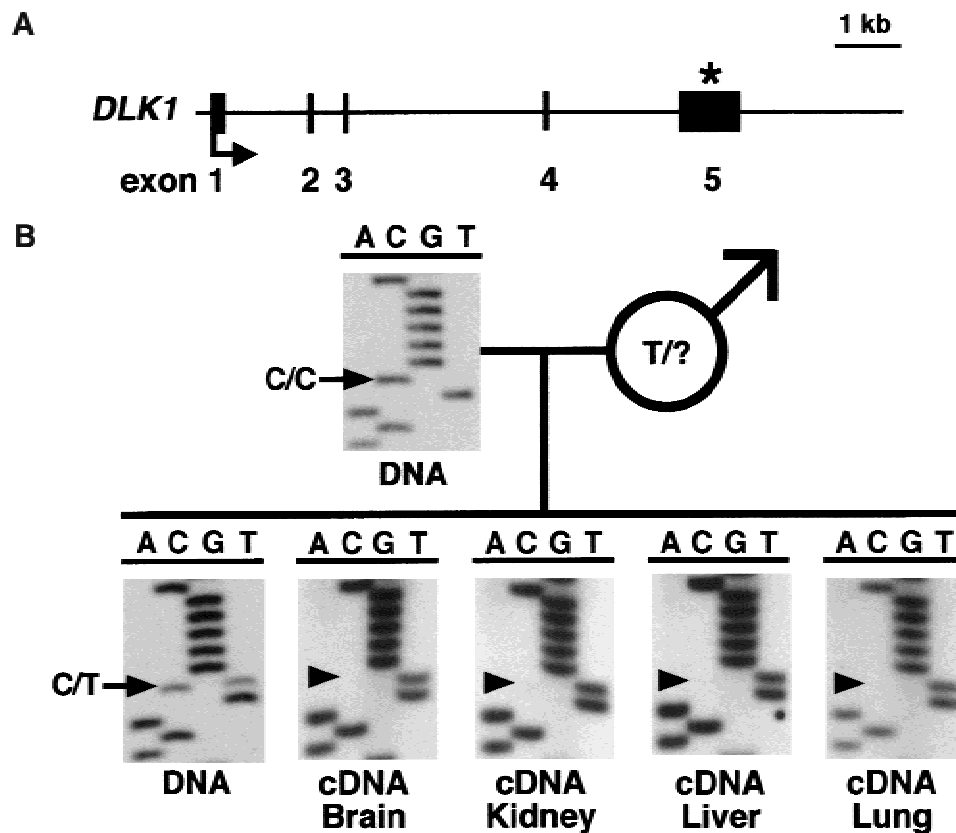
Figure 2 Predicted genomic structure and expression analysis of *DLK1*. (*A*) Predicted genomic structure of *DLK1*. Black boxes and horizontal lines denote exons and introns, respectively. (*) shows the location of a single nucleotide polymorphism in exon 5 (position 148,740 of accession no. AL132711). (*B*) Parent-of-origin–dependent monoallelic expression of *DLK1*. The allelic expression of *DLK1* was determined using a 108-d gestation conceptus heterozygous for the C/T polymorphism in exon 5. The maternal genotype is C/C, demonstrating paternal inheritance of the T allele in the conceptus. RNA analysis of the indicated tissues shows exclusive expression of the paternally derived T allele (i.e., C allele is unexpressed; arrowhead).

the case with *IGF2* and *H19*. The protein encoding genes, *IGF2* and *DLK1*, are paternally expressed, whereas the noncoding genes, *H19* and *GTL2*, are expressed only from the maternal allele. Upstream regions of both *H19* and *GTL2* contain hemimethylated CpG rich regions, within which seven CTCF consensus-binding sites are present upstream of *H19* and two CTCF binding sites are upstream of *GTL2*. *H19* expression is regulated by two enhancer elements, 8 and 8.7 kb downstream from the *H19* transcription start site (Yoo-Warren et al. 1988). Analysis of the downstream sequences of *GTL2* also revealed the presence of the same two enhancer elements: consensus sequence TGTTTGCAG (position 78,126 of AL117190) and TGTCTGCAG (position 79,856 of AL117190), 8.9 and 10.7 kb downstream, respectively, from the predicted *GTL2* transcription start site. The striking parallels between these two independent imprinted domains suggest that many of the features held in common between each region are key components required for the establishment, maintenance, or regulation of imprinting for domains with this type of organization.

DISCUSSION

In this report we describe the reciprocal imprinting of the juxtaposed *DLK1* and *GTL2* genes, which together constitute a novel imprinted domain on human chromosome 14. *DLK1* encodes for a cell-surface transmembrane protein that contains EGF-like repeat motifs, whereas the *GTL2* transcript lacks a significant open reading frame. Many imprinted genes have been shown to function in embryonic and fetal development. *DLK1* expression patterns likewise implicate its involvement in the development and differentiation of adipose, mesenchymal, neuroendocrine, and hematopoietic tissues (for review, see Laborda 2000). *DLK1* was first shown to be overexpressed in the adrenal medullar neuroendocrine tumor, pheochromocytoma, in which it was referred to as *pG2* (Helman et al. 1987). Subsequently, *DLK1* was identified as *PREF-1* (preadipocyte factor-1), a crucial negative regulator of adipocyte differentiation (Smas and Sul 1993), and as *ZOG* (zona glomerulosa-specific protein), a gene involved in the zonal differentiation of the adrenal gland (Okamoto et al. 1998; Raza et al. 1998). *DLK1* has also been
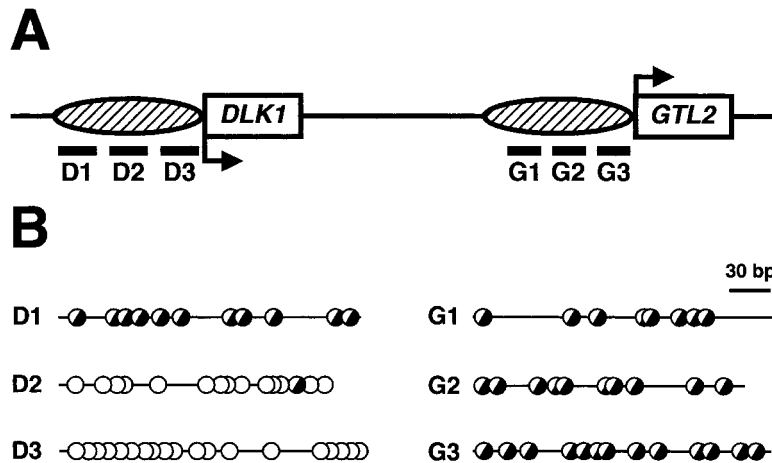
Figure 3 Methylation analysis of the *DLK1/GTL2* CpG-rich regions. (*A*) Schematic representation of the *DLK1/GTL2* genomic region. The transcription units of *DLK1* and *GTL2* are indicated by the boxes with arrows above and below showing the direction of transcription for the maternally and paternally expressed genes, respectively. The hatched ovals represent CpG-rich regions. *D1*, *D2*, and *D3* (positions 140,543–140,687, 141,101–141,205, and 141,459–141,594 of accession no. AL132711, respectively) and *G1*, *G2*, and *G3* (positions 65,973–66,085, 66,667–66,793, and 67,780–67,926 of accession no. AL117190, respectively) correspond to sequences within the CpG-rich regions upstream of *DLK1* and *GTL2*, respectively, that were analyzed by bisulphite sequencing. (*B*) Summary of methylation data. The data shown represent the methylation status of CpG dinucleotides from fetal brain, kidney, liver, and pancreas, which were indistinguishable from one another. All CpG dinucleotides present in these regions are shown. The open and half-filled circles represent unmethylated and hemimethylated CpGs, respectively.

implicated in pancreatic islet cell differentiation, and is critically involved in regulating the cellularity of developing thymocytes (Carlsson et al. 1997; Kaneta et al. 2000). A soluble variant of *DLK1* named *FA1* (fetal antigen 1) has also been isolated from amniotic fluid (Fay et al. 1988). Despite this confusing nomenclature, these findings indicate that *DLK1* plays an important role in normal cellular differentiation and carcinogenesis.

DLK1 encodes for a cell-surface transmembrane protein containing six tandem EGF-like repeats that are closely related to the EGF-like repeats of the invertebrate proteins delta, serrate, notch, and lin-12 (Laborda et al. 1993; Artavanis-Tsakonas et al. 1995; Fleming 1998). Mutational analysis of these EGF-like repeats indicates that they function to stabilize or modify ligand/receptor interactions (Fleming 1998). Despite the homology of the *DLK1* EGF-like repeats with those in the delta/notch proteins, it is unclear whether DLK1 is involved directly or indirectly with delta/notch signaling. Unlike other delta family ligands, DLK1 does not have a DSL (delta, serrate, lin12) motif proposed to be crucial for notch signaling. Nevertheless, it does activate expression of HES-1, the downstream target of notch signaling (Kaneta et al. 2000).

The stoichiometry between the components of the notch signaling pathway is crucial in determining cell fate (Artavanis-Tsakonas et al. 1995; Fleming 1998), and imprinting may be a novel mechanism for maintaining these important stoichiometric relationships. The biallelic expression of *DLK1* that results from pUPD would increase *DLK1* expression relative to that of other pathway components with which it interacts. This may explain the severity and potential lethality of pUPD for chromosome 14. In contrast, the lack of *DLK1* expression resulting from mUPD may be less severe because of possible redundancy in the signaling pathways in which *DLK1* participates. The phenotypes associated with mUPD of chromosome 14 are unlikely to result from *GTL2* overexpression because this transcript lacks a significant open reading frame. Nevertheless, it is possible that mUPD abnormalities could result from the disruption of the imprint regulatory functions of *GTL2*. This is supported by the finding of hemimethylated CTCF binding sites immediately upstream of *GTL2* that are analogous to those required for the reciprocal imprinting of *IGF2* and *H19.*

CTCF is a vertebrate enhancer blocking protein that binds to unmethylated DNA, and is proposed to contribute to imprint regulation of neighboring genes by the formation of chromatin boundaries (Bell and Felsenfeld 2000; Hark et al. 2000; Szabo et al. 2000). The chromatin boundary purportedly blocks access of downstream enhancers to the promoter region of an upstream gene. In the case of the *IGF2/H19* imprinted domain, the enhancer elements are proposed to be diverted to the *H19* promoter on the unmethylated, CTCF-bound maternal allele, resulting in *H19* expression (Bell and Felsenfeld 2000; Hark et al. 2000; Reik and Murrell 2000; Szabo et al. 2000). Interestingly, two CTCF consensus binding sites are also present immediately upstream of *GTL2*, in a region we have shown to be hemimethylated. The hemimethylation observed within and surrounding the CTCF consensus sequences is consistent with there being allele-specific differential methylation at this locus. However, further analysis is required to confirm this and its parent-of-origin. The significance of the number of CTCF binding sites is presently unclear, but the presence of seven sites in the *IGF2/H19* domain would provide greater insurance against domain disruption because of genetic or epigenetic mutations than the two sites contained within the *DLK1/GTL2* domain.

The spatial, structural, and epigenetic characteristics of the *DLK1/GTL2* domain are similar to those of the *IGF2/H19* domain on chromosome 11. The mater-
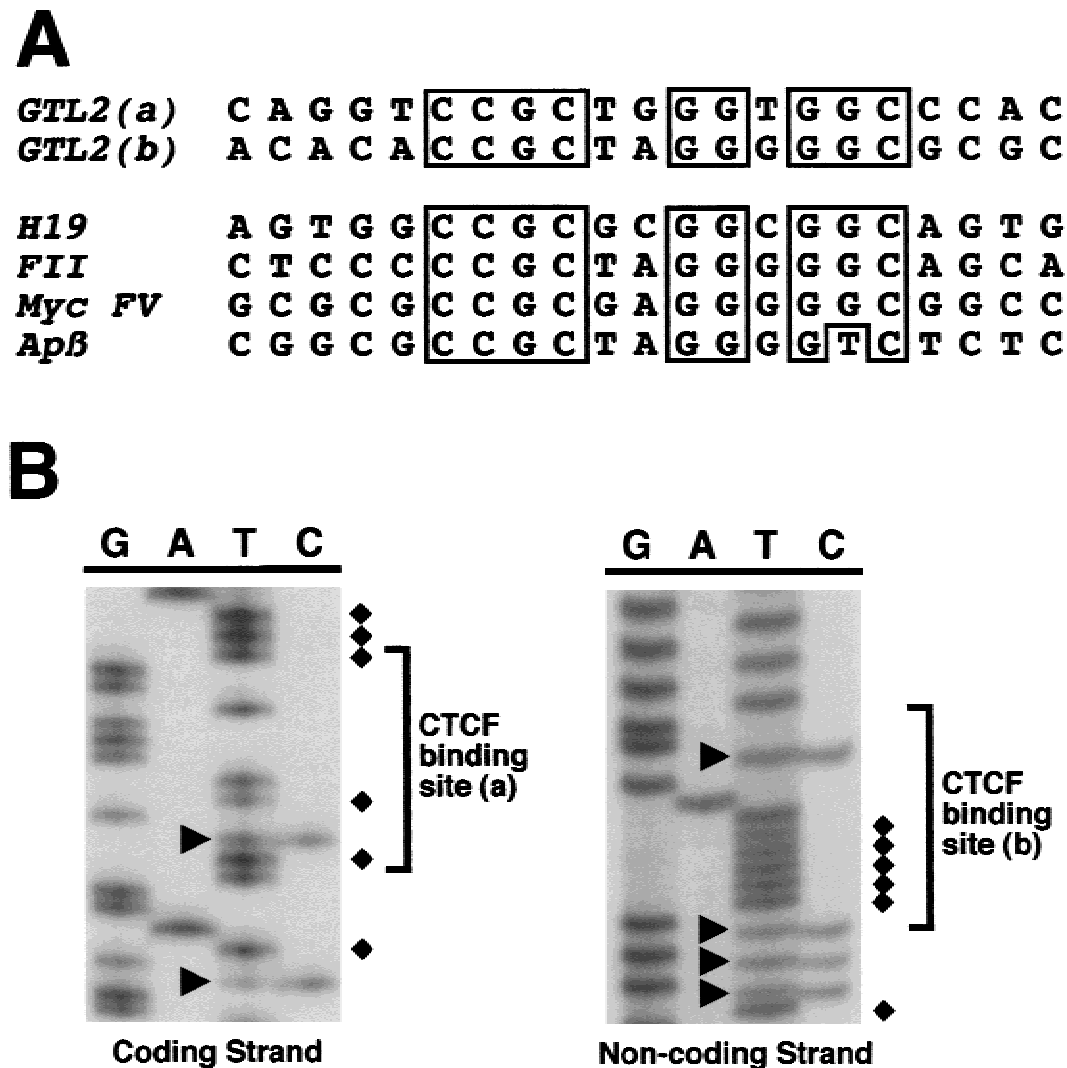
Figure 4 Identification and methylation analysis of putative CTCF binding sites. (*A*) Alignment of the putative *GTL2* CTCF binding sites with known CTCF binding sites. *GTL2(a)/GTL2(b)*, human *GTL2* promoter; *H19*, human *H19* upstream region; *FII*, chicken β*globin* insulator; *Myc FV*, chicken *c-myc* promoter; *Ap*β, human amyloid β protein promoter. The *GTL2(b)* consensus sequence is present on the noncoding strand, and is therefore inverted for the purposes of sequence alignment. (*B*) Methylation analysis of the putative *GTL2* CTCF binding sites. Genomic DNA isolated from fetal liver was bisulphite treated and amplified across putative CTCF binding sites (a) and (b). Diamonds denote the positions of unmethylated cytosines not present in CpG dinucleotides; they have been fully converted by bisulphite treatment. The presence of bands in both the *T* and *C* lanes (arrowheads) shows hemimethylation of cytosine residues within the CpG dinucleotides contained within and adjacent to CTCF binding sites (a) and (b).

nally expressed, noncoding *GTL2* and *H19* transcripts are both positioned ~100 kb from their corresponding protein-encoding genes, *DLK1* and *IGF2*, respectively. The 100 kb separation between these genes may be because of a distance requirement for the formation of a chromatin boundary element or the regulation of gene transcription by downstream enhancer elements. The two enhancer elements involved in the imprinted expression of *IGF2* and *H19* are located ~8 kb downstream from the *H19* transcription start site (Yoo-Warren et al. 1988). Similar enhancer consensus sequences are located ~8 kb downstream from the predicted *GTL2* transcription start site. As with *IGF2* and

H19, *DLK1* and *GTL2* are transcribed in the same orientation and are reciprocally imprinted. The relevance of the protein-encoding genes, *IGF2* and *DLK1*, being paternally expressed, and the noncoding genes, *H19* and *GTL2*, being maternally expressed, is presently unknown. However, it may reflect a unique ability of female gametes to impose a restricted pattern of expression on protein-encoding genes when organized in this type of domain.

The paternal expression of *DLK1* coupled with the paucity of genes flanking *GTL2* make *DLK1* a likely candidate gene for the parent-of-origin dependent phenotypes observed in several species. The dwarfism
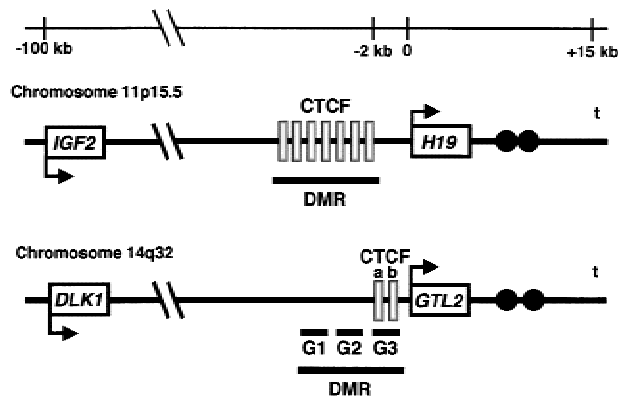
Figure 5 Comparison of the imprinted *IGF2/H19* domain on chromosome 11p15.5 with the *DLK1/GTL2* domain on chromosome 14q32. Physical distances are indicated at the top of the diagram. CTCF binding sites are indicated as shaded vertical rectangles, and black circles indicate the positions of enhancer elements. Differentially methylated regions (DMR) are indicated. *G1*, *G2*, and *G3* are shown as black bars and correspond to the three areas analyzed for differential methylation (Figs. 3*A,B*). The position of the telomere (t) is shown relative to each imprinted domain. The transcription units for each gene are shown as boxes, and the direction of transcription for maternally and paternally expressed genes is denoted by arrows above and below the boxes.

phenotype observed in the *Gtl2^lacZ^* mouse was inherited paternally indicating the disruption of a paternally expressed gene (Schuster-Gossler et al. 1996). However, the gene isolated from the site of transgene integration, *Gtl2*, is maternally expressed. Therefore, the *Gtl2^lacZ^* dwarfism phenotype is more consistent with aberrations in the expression of paternally expressed *Dlk1*. In sheep, paternal transmission of mutations in the *callipyge* (*CLPG*) gene results in improved feed efficiency, animal leanness, and muscular hypertrophy (Cockett et al. 1996; Freking et al. 1998). Although *CLPG* has not yet been identified, this locus has been tightly linked to sheep distal chromosome 18, which is homologous with regions in mice (distal 12) and humans (distal 14q) known to harbor imprinted genes. Using a comparative mapping approach, Fahrenkrug et al. (2000) have recently mapped the *CLPG* locus to an interval containing *DLK1* and *GTL2*. The paternal expression of *DLK1*, combined with its chromosomal location and role as a negative regulator of adipocyte differentiation, is consistent with the postulate that mutations affecting the ovine ortholog of *DLK1* are responsible for the *CLPG* phenotype in sheep.

In conclusion, *DLK1* and *GTL2* comprise the first known imprinted domain on human chromosome 14. The unprecedented association of imprinting with the delta/notch signaling pathways involved in cellular differentiation and cell fate decisions further shows the essential importance of imprinting to fetal development. We have also provided the first example of two independent imprinted regional domains that appear to share an analogous regulatory structure. The conservation of many of the predicted structural and regulatory motifs between the *DLK1/GTL2* and *IGF2/H19* domains suggests that this common domain organization may be required to establish and maintain other imprinted domains. We propose that this organization, consisting of two reciprocally imprinted genes, one coding and the other noncoding, with intervening CTCF binding sites and enhancers downstream from the noncoding gene, be referred to as the "juxtapositioned reciprocally imprinted gene" (JRIG) domain motif.

METHODS

Expression Analysis

Human tissues were obtained from the NIH-supported Birth Defects Research Laboratory at the University of Washington. Total RNA was extracted from 50–100 mg of frozen human tissue using RNA STAT-60 following the manufacturer's protocol (Tel-test, Inc.). Using Superscript II and oligo dT primers according to the manufacturer's protocol (Life Technologies), 2 µg total RNA was reverse transcribed. Of the cDNA obtained, 1 µL was used as template for PCR amplification. Primers were selected to span intron-exon boundaries to prevent amplification of contaminating genomic DNA. Amplification products were purified from agarose gels using GenElute spin columns (Sigma), and were sequenced using radiolabeled terminator cycle sequencing (USB Corp.). *GTL2* cDNA was amplified (35 cycles at 95°C for 30 sec, 65°C for 30 sec, and 72°C for 1 min) with the PCR primers *GTL2* F4 (5′-GCT ACT GAA TCA CCA AAG GCA C-3′) and *GTL2* R2 (5′-GAG GCA TAT ATT TGA GTT ACA CAT ACC CCT TAG TCC-3′) and sequenced using the primer *GTL2* F6 (5′-GTG TGT ACC TTG GTT GGT GAC TC-3′). *DLK1* cDNA was amplified (35 cycles at 95°C for 30 sec, 65°C for 30 sec, and 72°C for 1 min) with the PCR primers *DLK1* F9 (5′-AAC AAC GGG ACC TGC GTG AGC-3′) and *DLK1* R7 (5′-GCT TGC ACA GAC ACT CGT AGC TCA CC-3′) and sequenced using the primer *DLK1* F6 (5′-CCA ACC CAT GCG AGA ACG-3′).

Methylation Analysis Using Bisulphite Sequencing

Genomic DNAs were treated with sodium bisulphite to convert all unmethylated cytosines to uracils, leaving methylated cytosines intact. Sodium bisulphite treatment was performed using the CpGenome DNA modification kit according to the manufacturer's protocol (Intergen). Certain regions of the genome can be resistant to bisulphite treatment because of inefficient denaturation. To alleviate this problem, genomic DNA was first digested with the methylation insensitive restriction enzyme, *Taq*I, before proceeding with the bisulphite treatment protocol. Bisulphite treated DNA was amplified using two rounds of nested PCR (35 cycles at 95°C for 30 sec, 55°C for 30 sec, and 72°C for 30 sec), and sequenced as described above. Primer sets were selected to amplify DNA fragments containing both methylated CpG and nonmethylated CpG dinucleotides. These primer sequences are available on request from the authors. One hundred ng of bisulphite treated DNA was empirically determined to avoid stochastic PCR amplification bias because of a limiting number of starting DNA molecules, and therefore was used as template in the first round of PCR amplifications.

ACKNOWLEDGMENTS

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Novel Imprinted *DLK1*/*GTL2* Domain on Human Chromosome 14 Contains Motifs that Mimic Those Implicated in *IGF2*/*H19* Regulation

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