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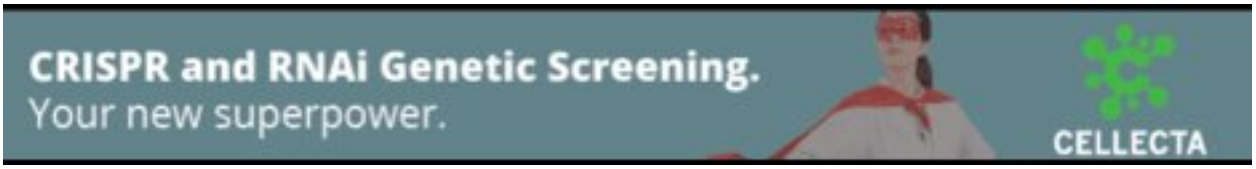

Letter

Comparative Sequence Analysis of the Imprinted *Dlk1–Gtl2* Locus in Three Mammalian Species Reveals Highly Conserved Genomic Elements and Refines Comparison with the *Igf2–H19* Region

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The *Dlk1–Gtl2* domain on mouse chromosome 12 contains reciprocally imprinted genes with the potential to contribute to our understanding of common features involved in imprinting control. We have sequenced this conserved region in the mouse and sheep and included the human sequence in a three species comparison. This analysis resulted in a precise conservation map and identification of highly conserved sequence elements, some of which we have shown previously to be differentially methylated in the mouse. Additionally, this analysis facilitated identification of a CpG-rich tandem repeat array located ~13–15 kb upstream of *Gtl2*. Furthermore, we have identified a third imprinted transcript that overlaps with the last *Dlk1* exon in the mouse. This transcript lacks a conserved open reading frame and is probably generated by cleavage of extended *Dlk1* transcripts. Because *Dlk1* and *Gtl2* share many of the imprinting properties of the well-characterized *Igf2–H19* domain, it has been proposed that the two regions may be regulated in the same way. Comparative genomic examination of the two domains indicates that although there are similarities, other features are very different, including the location of conserved CTCF-binding sites, and the level of conservation at regulatory regions.

[The sequence data described in this paper have been submitted to the GenBank data library under accession no. AJ320506.]

To date, >40 imprinted genes, characterized by preferential expression from only one of their parental alleles, have been identified in human and mouse. Although regulation of imprinted gene expression is under intensive investigation, little is known about common elements that may be responsible for the parental origin-specific silencing of one allele. During development, the imprint that differentially marks the parental alleles is likely to be set in the germline and after fertilization is stably transmitted during somatic cell division. For that reason, the regulation of imprinting involves heritable epigenetic modifications that affect chromatin structure and the ability of the DNA to interact with regulatory factors. DNA methylation is one modification known to have a key role in the regulation of imprinted genes and differential modifications to chromatin-associated proteins may also be involved. So far, however, it is not known whether there are common genomic features that distinguish imprinted domains from the majority of other genes that are expressed from both parental alleles. One approach to address this issue is to look for genomic features common to imprinted domains within species and to conduct comparative genomic analysis of imprinted regions between species. This approach becomes more feasible as more imprinted domains are being cloned and characterized and more mammalian genomic sequence is being generated.

Two pairs of reciprocally imprinted genes in the mouse, *Dlk1–Gtl2* on distal chromosome 12 (Schmidt et al. 2000; Takada et al. 2000) and *Igf2–H19* on distal chromosome 7, share a number of intriguing features. *Dlk1* and *Igf2* are both paternally expressed, whereas *Gtl2* and *H19* are maternally expressed and appear to encode untranslated RNAs. Both pairs of genes are located ~80 kb apart and share similar patterns of differential DNA methylation. In general, there is some evidence that *Dlk1* and *Gtl2* are co-expressed in the same tissues during development as are *Igf2* and *H19* (Takada et al. 2000). Furthermore, both pairs of genes exhibit the same reciprocal behavior in *Dnmt1* −/− mice (Schmidt et al. 2000).

There is compelling evidence that *Dlk1–Gtl2* and *Igf2–H19* are both involved in the regulation of prenatal growth. For *Igf2–H19*, this has been documented extensively (DeChiara et al. 1991; Ferguson-Smith et al. 1991; Leighton et al. 1995; Eggenschwiler et al. 1997; Sun et al. 1997). Evidence for the involvement of *Dlk1–Gtl2* in growth regulation is derived from different types of imprinting anomalies in mouse, sheep, and human. Mouse embryos harboring maternal or paternal uniparental disomy for chromosome 12 have growth defects and are inviable. (Georgiades et al. 2000, 2001). In the sheep, the Callipyge phenotype is a muscular hypertrophy that is

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subject to a parent-of-origin effect. The phenotype is only present in individuals that have a single mutated allele of the Callipyge (*Clpg*) locus inherited from the father. The *Clpg* locus has been mapped to a 400-kb long interval that includes *Dlk1* and *Gtl2* on ovine chromosome 18 (Berghmans et al. 2000). The exact nature of the mutation is not yet known. Further evidence for a role of this locus in growth regulation is derived from transgenic mice that carry a *lacZ* insertion in the upstream region of *Gtl2* (Schuster-Gossler et al. 1998). When the *lacZ* transgene is paternally inherited, the mice are growth retarded (Schuster-Gossler et al. 1996). The *lacZ* integration located in the intergenic *Dlk1–Gtl2* region, indicates the presence either of a third growth-regulating gene or of an important regulatory element whose function is disturbed by the *lacZ* integration on the paternal allele. In the human, *DLK1* and *GTL2* are located on chromosome 14q and have also been shown to be imprinted (Wylie et al. 2000). In agreement with the observed deregulation of growth in the described animal models, patients with maternal uniparental disomy for chromosome 14q exhibit growth retardation (Georgiades et al. 1998; Sutton and Schaffer 2000).

Here we have sequenced 112 kb encompassing the mouse *Dlk1–Gtl2* domain and conducted a three species comparison of the same regions in sheep and human with the aim of identifying conserved genomic features that may be functionally important in imprinting control. In addition, we used this information to compare the *Dlk1–Gtl2* domain with the well characterized *Igf2–H19* domain. This study indicates that although the two regions have similarities, there are also striking differences in their genomic properties. This analysis provides further insight into our understanding of the genomics of imprinting.

RESULTS

The *Dlk1–Gtl2* Regions Are Highly Conserved in Human, Mouse, and Sheep

Using a *Gtl2* cDNA probe, genomic clones were isolated from a genomic bacterial artificial chromosome (BAC) library derived from the mouse strain 129/SvJ. One of these clones, Bac 103N10, harbored *Gtl2* as well as *Dlk1* and was therefore chosen for sequence analysis (see Methods). The obtained sequence (GenBank accession no. AJ320506) is 111805 bp long and can be regarded as high-quality sequence; on average, each nucleotide is covered by nine sequence reads, and the error rate is estimated as <0.005%.

An initial analysis of the new mouse sequence revealed that the entire *Dlk1* gene and also 6.5 kb of upstream region was covered by the genomic mouse sequence, whereas the *Gtl2* gene was missing the last two 3′ exons (exon 9 and exon 10). Therefore, the comparison between the mouse and human sequences encompasses the entire *Dlk1* gene and terminates in intron 8 of mouse *Gtl2*. Both genes are separated by an 80-kb-long intergenic region. The homologous human and sheep genomic sequences are 124 kb and 110 kb long (GenBank accession no. AL117190, AL132711, AF354168), see Methods. The organization of the studied regions in the three species is shown in Figure 1.

As suitable computer software for one multiple alignment that encompasses all three sequences is not freely available, we developed a new approach making use of the existing software. The genomic sequences were pairwise aligned using `PipMaker` software (Schwartz et al. 2000), generating three alignment pairs: Human–mouse, human–sheep and mouse–sheep. Interestingly, the closer phylogenetic relationship between mouse and human rather than human and sheep (Madsen et al. 2001; Murphy et al. 2001) is not reflected in the similarities of the sequences analyzed here. The average similarity in the local alignments is 58.9% identity in the human–sheep comparison, and 56.4% identity in the human–mouse comparison. Whereas 104,046 bp of the human sequence are spanned by local alignments in the human–sheep comparison, in the human–mouse alignment, the coverage is lower (65,685 bp). One reason for that might be a faster evolution of the mouse genome.

In all three sequences, the stretches of homologies were mainly interrupted by blocks of repetitive elements (Fig. 1), indicating that during evolution no significant insertions/deletions of unique sequences, for example of entire genes, have occurred. The content of repetitive elements is highest in the human and is the major cause for the expansion of the human genomic sequence. Furthermore, the positions of the two most prominent clusters of repetitive elements are conserved, located ~5–8 kb downstream of *Dlk1*, and ~20 kb upstream of *Gtl2* (Fig. 1).

Conservation of the *Dlk1* and *Gtl2* Genes

As expected for a protein-encoding gene, similarities of the *Dlk1* genomic sequences were most pronounced in the exons of this gene. The human and mouse cDNA sequences (GenBank accession no. U15979, U15980) are identical in 84.81% of all positions (84.9% of amino acids). The exons of the sheep *Dlk1* gene are identical to the human cDNA in 87.11% of all positions, and to the mouse cDNA sequence in 81.47% of all positions (82.0% and 80.8%, respectively, of amino acids). In contrast, the *Gtl2* genes show a general conservation in their physical organization (Fig. 1) but are less conserved at the sequence level. Taking the originally identified mouse cDNA sequence (GenBank accession no. Y13832) (Schuster-Gossler et al. 1998) as a reference sequence, the homologous human and sheep cDNA sequences are identical in 72.99% and 71.26%, respectively, of all positions. For mouse *Gtl2*, 10 exons have been identified (Miyoshi et al. 2000). The human and sheep *Gtl2* genes encompass 12 and 10 exons, respectively (Charlier et al. 2001). Homologs for human exons 2 and 6 have not been identified in sheep and mouse. The human *GTL2* cDNA sequence described previously by Miyoshi et al. (2000) commences in exon 1 and is conserved in the three species. The human exon 1 shows sequence similarity to the mouse and sheep exon 1 (74.47% and 81.25% identity, respectively). This exon 1 is also confirmed by various expressed sequence tags (ESTs). A previous report (Wylie et al. 2000) appears to misplace the start of transcription at human exon 4.

A Conserved Imprinted Transcript Downstream of *Dlk1*

In an early approach for the isolation of probes that cover potential CpG islands, *Hpa*II fragments derived from Bac103N10 DNA were randomly subcloned and sequenced. A 557-bp-long *Hpa*II fragment showed homology to four mouse sequences (GenBank accession no. AA437756, AW60763, AI551552, AW120464) in the EST section of the GenBank database. The *Hpa*II fragment was mapped to a position 2.2 kb downstream of mouse *Dlk1*. The presence of a polyadenylation signal 14 bp upstream of the poly A+ tail at the 3′ end of

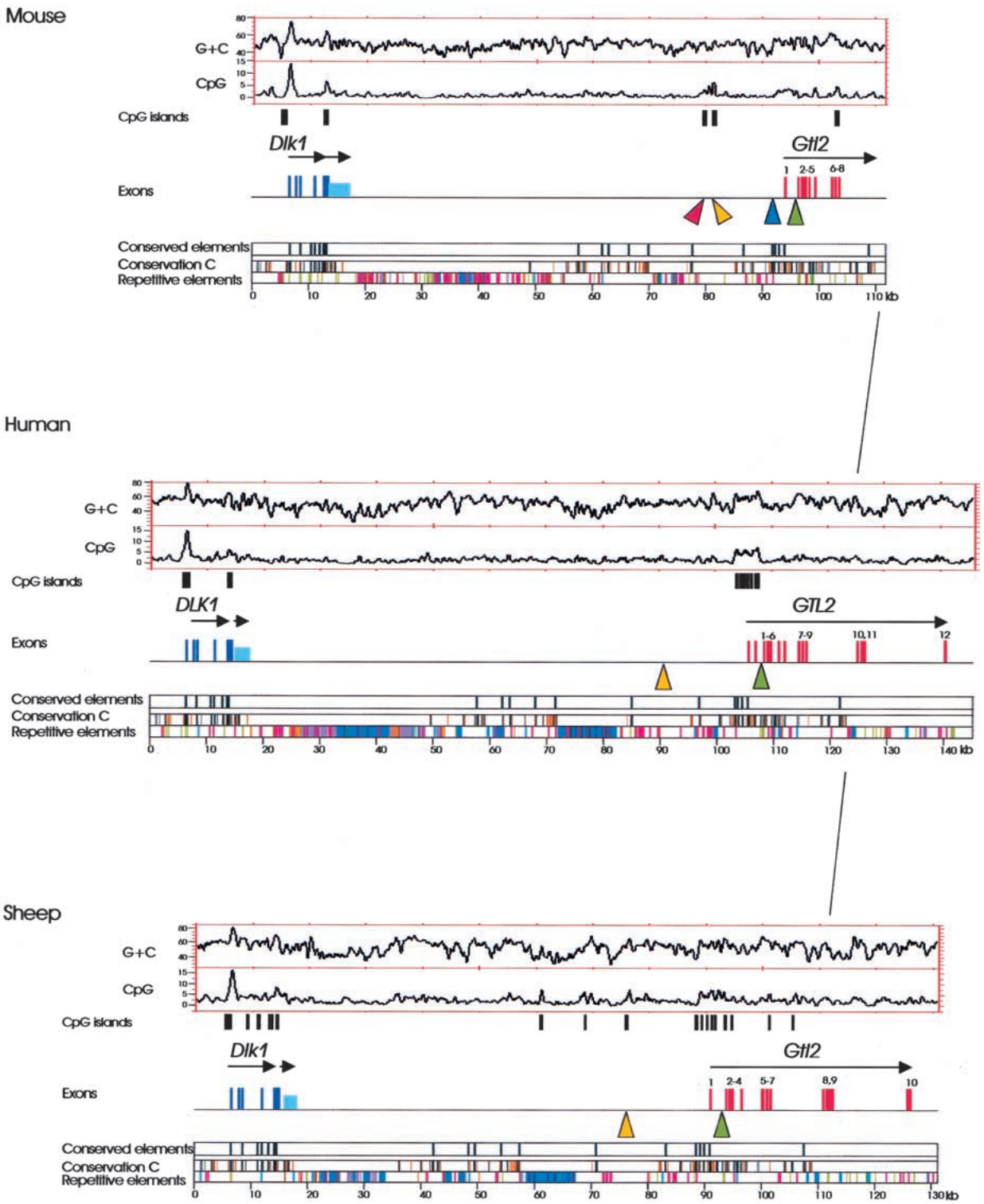


Figure 1 Conserved organization of the *Dlk1–Gtl2* regions in mouse, human, and sheep. Above colored panels that describe sequence conservation and distribution of repetitive elements in the analyzed sequences, the structures of *Dlk1* (dark blue), the transcript downstream of *Dlk1* (light blue), and *Gtl2* (red) are indicated by bars that represent exons. Arrows above the depicted exons indicate the orientation of gene transcription. (Conservation C) Conserved elements that are present in the mouse–human, sheep–human, mouse–sheep alignments. Elements that are identical in at least 40% of all positions (>40 bp in length, aligned without gap) are shown in orange, elements that are identical in at least 70% of all positions are shown in black (>40 bp, aligned without gap). (Conserved elements) Elements that are aligned without gaps, identical in at least 70% of all positions, and at least 100 bp long. (Repetitive elements) LINE elements (blue); SINE elements (red); LTR elements (purple); DNA elements (orange); small RNAs (grey); satellites, simple repeats, low complexity DNA (green). (Orange triangles) Conserved CpG-rich repeats; (red triangle) nonconserved CpG-rich repeat; (blue triangle) *lacZ* integration site; (green triangles) conserved putative CTCF-binding sites. The overall G+C and CpG densities (window size 500 bp, values given as percentages) are presented as plots. CpG islands identified by the CpG plot software (http://www.ebi.ac.uk/index.html) are shown as black bars below the plots.

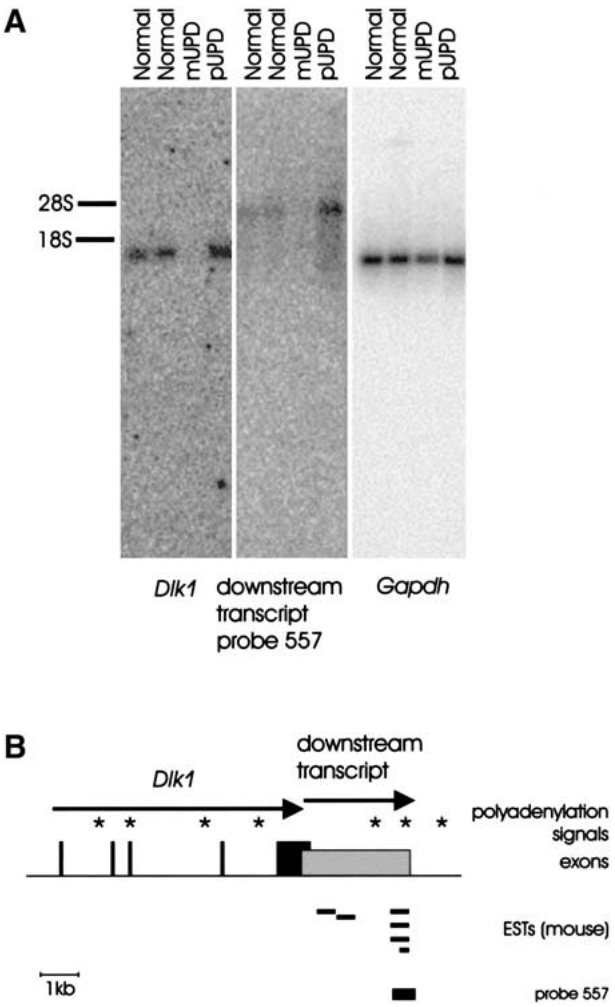


Figure 2 Imprinted expression and physical structure of a transcript downstream of *Dlk1* in mouse. (*A*) Northern blot hybridization of enriched poly A+ RNA hybridized with probes specific for *Dlk1*, the transcript downstream of *Dlk1*, and *Gapdh*, respectively. The probe specific for *Dlk1* spanned exons 1 and 2, the expression of the *Dlk1* downstream transcript was tested using a genomic *Hpa*II fragment overlapping with the 3– end of the transcript. Hybridization of *Gapdh* transcripts proved similar amounts of RNAs in all lanes. The positions of the 18S and 28S rRNA bands are indicated. (*B*) *Dlk1* exons are shown as black bars. The *Dlk1* downstream transcript overlaps with the last exon of *Dlk* and is shown as a grey box, starting at the most upstream 5′ end predicted by the 5′RACE experiment. Mouse ESTs that indicated the presence of a transcript downstream of *Dlk1* are shown as horizontal bars. Asterisks represent polyadenylation signals. The position of the *Hpa*II fragment used as a probe for the detection of transcripts downstream of *Dlk1* is indicated. The schematic is drawn to scale.

the ESTs indicated that these sequences were derived from mRNAs and that the orientation of transcription is the same as for *Dlk1* and *Gtl2*. Northern blot hybridization to poly A+ RNA identified a transcript ~2.5–3 kb in size (Fig. 2). This transcript was present in pUPD12 embryos and placentae but absent in mUPD12 mRNA, indicating expression solely from the paternal allele. Because the hybridization signal did not colocalize with signals obtained with probes that were specific for exon 1, 2, or 5 of *Dlk1*, we initially assumed that this transcript is not *Dlk1*.

To reconstruct the physical organization of the new transcript, RT-PCRs were performed. In 5′ RACE experiments, three different-sized products were amplified. The longest product placed the assumed 5′ end of the transcript in the last exon of *Dlk1*, 217 bp upstream of the *Dlk1* poly A+ tail (Fig. 2). From these analyses, we deduced a 2933-bp-long cDNA sequence (nucleotides 13452–16384, GenBank accession no. AJ320506) from the genomic sequence, consistent with the transcript size on Northern blots. The successful amplification of RT-PCR products using 5′ primers specific for all *Dlk1* exons and 3′ primers specific for the downstream transcript, indicated the existence of spliced transcripts that cover both *Dlk1* and the expressed region downstream of *Dlk1*. A probe for the entire *Dlk1* exon 5, however, did not detect the downstream transcript on Northern blots, indicating that transcripts consisting of this small portion of *Dlk1* extending into the downstream region may be less abundant. We suggest that the downstream transcript may be a cleavage product derived from extended *Dlk1* transcripts. This may be similar to the post-transcriptional processing of *IGF2* RNAs in human (Scheper et al. 1995).

Similar transcripts exist in the *Dlk1* downstream regions of human and sheep (Charlier et al. 2001). Sequencing of the inserts of two human IMAGE cDNA clones (IMAGE ID 1753255, 4345285) enabled us to reconstruct a human 2892-bp-long cDNA sequence that starts 200 bp downstream of *DLK1* (nucleotides 149945–152837, GenBank accession no. AL132711.4). We assume that the 5′ end of the deduced cDNA sequence is incomplete and that the 5′ end may be in the last exon of *DLK1*, similar to the situation in the mouse. Searches on potential protein-encoding open reading frames (ORFs) in the human and mouse cDNA sequences and also in the homologous genomic sheep sequence revealed the absence of a conserved ORF.

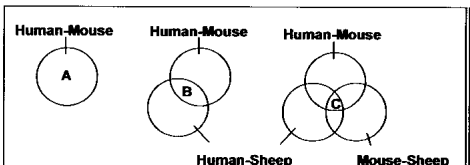
Apart from the *Dlk1* downstream transcript, there is no strong evidence from sequence analyses for additional genes in the intergenic region between *Dlk1* and *Gtl2*.

Identification of 20 Highly Conserved Elements Shared by Mouse, Human, and Sheep

To identify conserved elements in this region, the alignments of the three sequence pairs, human–mouse, human–sheep, mouse–sheep were compared. Three hundred eighty-eight sequence elements that were aligned without any gaps, were at least 40 bp long and showing sequence conservation of at least 40% identity in the mouse–human alignment, were selected for further analysis. The developed scheme, shown in Figure 3, involves a progressive increase in the stringency of conservation, and conserved elements were identified that are present in all three alignments. The 149 elements that show at least 40% identity (>40 bp length) in all three alignments were used to generate a general picture of the sequence conservation in the *Dlk1–Gtl2* region (Conservation C, Fig. 1).

Twenty identified elements of at least 100 bp in length were aligned without any gaps and were identical in at least 70% of all positions in all three alignments (Fig. 3B). The reliability of the alignment and selection procedure was proven using a different software (http://www-gsd.lbl.gov/vista/) for the alignments. This placed all but two elements (nucleotides 69863–69967 and 77558–77659, GenBank accession no. AJ320506) in regions that were highly conserved in all three species (data not shown). These two elements were present in two of the three species.

A



level of conservation	A Human - Mouse	B Human - Mouse x Human - Sheep	C Human - Mouse x Human - Sheep x Mouse - Sheep
level 1 ≥ 40% identity, ≥ 40 bp length	388 (100%)	183 (47.2%)	149 (38.4%)
level 2 ≥ 70% identity, ≥ 40 bp length	183 (100%)	120 (65.6%)	90 (49.2%)
level 3 ≥ 70% identity, ≥ 100 bp length	41 (100%)	25 (61.0%)	20 (48.8%)

B

position	length	G+C content	comment
6562-6779	218	72,0%	*Dlk* exon1
8538-8679	141	56,7%	*Dlk* exon3
10466-10597	132	50,8%	
11121-11279	159	56,6%	*Dlk* exon 4
12060-12216	157	44,6%	
12645-12761	117	60,7%	*Dlk* exon 5
12771-13279	509	63,7%	*Dlk* exon 5
13281-13415	136	50,7%	*Dlk* exon 5
57646-57804	159	50,3%	
61826-61938	113	45,1%	overlap with Line element
62809-62989	181	48,6%	similarities to human chr. 3,7,8,14
66344-66478	135	48,9%	
69863-69967	105	50,5%	
77558-77659	102	52,0%	
86494-86657	164	50,6%	
91609-91814	206	43,2%	overlap with Mir element
92288-92406	119	55,5%	*lacZ* integration site
92908-93042	135	48,2%	
93853-94065	213	53,1%	*Gtl2* exon 1
108755-108875	121	43,0%	

Figure 3 Identification of highly conserved elements. (*A*) The strategy for the identification of conserved elements is described by the scheme above the table. The table shows the numbers of conserved elements identified on different levels of sequence conservation. The inclusion of the human–sheep, and mouse–sheep alignments results in a reduction in numbers of identified elements. This reduction is described by the proportions of identified elements related to the values given for the human–mouse alignment only. (*B*) Positions of the 20 highly conserved elements (>100 bp, >70% identity) in the mouse sequence (GenBank accession no. AJ320506) that were identified in all three alignments. Additionally, the lengths, G+C content, and special features of the individual elements are shown.

Six of these 20 elements represented exons of *Dlk1*, two overlapping with the differentially methylated region in intron 4/exon 5 (Takada et al. 2000) (Fig. 3B; conserved elements in Fig. 1). In contrast, among the *Gtl2* exons, only exon 1, which is also embedded in a differentially methylated region, overlaps with a highly conserved element. Between the three species, the transcript downstream of *Dlk1* exhibited similar lack of conservation as the *Gtl2* exons downstream of exon 1. Ten highly conserved elements are present in the intergenic region between *Dlk1* and *Gtl2*. Three of these elements are clustered in a region up to 2.5 kb upstream of the first *Gtl2* exon, whereas highly conserved elements are not present immediately upstream of *Dlk1*. Precise localization of the 3′ sequence of the *lacZ* integration site described by Schuster-Gossler and colleagues (Schuster-Gossler et al. 1998) (see Introduction) localized the 3′ breakpoint of the integration within one of the conserved elements 1.7 kb upstream of the first *Gtl2* exon. The consequences of this insertion for local gene regulation remain to be determined.

Two elements showed similarity with highly repetitive elements, one overlaps with a LINE element, the second with a *Mir* element. A third element appeared to be a slightly repetitive element, showing sequence homologies to genomic sequences on human chromosomes 3, 7, 8, and a second locus on human chromosome 14.

CpG-Rich Repeats Upstream of *Gtl2*

CpG islands that are important for the regulation of imprinted gene expression are expected to be conserved in mouse, human, and sheep. The G+C and CpG distributions in the analyzed sequences are shown in Figure 1. The overall regional G+C contents (49.37% in mouse, 51.37% in human, 53.70% in sheep) differ slightly and might reflect species-specific genome-wide differences in the G+C content (Gautier 2000).

In this region, the average CpG content is 1.52% in mouse, 2.09% in human, and 2.81% in sheep. The average CpG/GC ratios are 0.28 in mouse, 0.33 in human, and 0.44 in sheep. These differences are also reflected in the number and distribution of CpG islands. Whereas the mouse sequence has five CpG islands (CpG/GC ratio > 0.6, length > 200 bp, G+C content > 50%) (http://www.ebi.ac.uk/index.html), the human sequence has eight, and the sheep sequence has 18 CpG islands (Fig. 1). All three species possess a strong CpG island in the promoter region of *Dlk1* and a less pronounced CpG island in *Dlk1* exon 5. CpG islands were identified in human and sheep at the transcriptional start site of *Gtl2*. In the mouse, this region can be regarded as a CpG-rich region, but is by definition not a CpG island. In the mouse, additional CpG islands were identified 12.3 and 14.1 kb upstream of *Gtl2* exon 1 (nucleotides 81341–81686 and 79721–79937, GenBank accession no. AJ320506). A CpG island in a similar position is present in the sheep but is absent in the human. Absence of sequence homology in the alignment pairs showed that this region is not conserved in all three species. More detailed analysis of this region, however, revealed the presence of direct repeats in head to tail order in all three species in positions overlapping with the CpG island 12.3 kb upstream of *Gtl2* in mouse and the CpG island in sheep (orange triangles in Fig. 1). In the mouse, the region between nucleotides 81291–81504 spans seven 24-bp-long repeated motifs (Figs. 1 and 4). In sheep and human, the repeat motifs are 18 bp long and are repeated 16 and nine times, respectively. The similarity of these motifs in human and sheep indicates that both arrays have the same phylogenetic origin (Fig. 4). In all three species, the repeats contain numerous CpG dinucleotides. The reduced length of this structure in the human compared with the sheep and the fact that in the human motif one CpG is replaced by a TpG are the reasons why pronounced CpG richness is not visible in the human CpG plot (Fig. 1). Interestingly, the central part of the mouse motif shows some similarities to the sheep and human motifs (Fig. 4), indicating that all three motifs may be derived from the same ancestral motif.

In the mouse, a second CpG-rich repeat array is present 590 bp upstream of the first repeat array at nucleotides 80151–80701 (GenBank accession no. AJ320506). This array encompasses 11 42-bp-long motifs (Fig. 4B). A similar array is not present in human and sheep.

Comparison of the *Dlk1–Gtl2* and *Igf2–H19* Loci

Dlk1–Gtl2 and *Igf2–H19* share similarities in their reciprocal imprinting, aspects of their regulation, and their patterns of

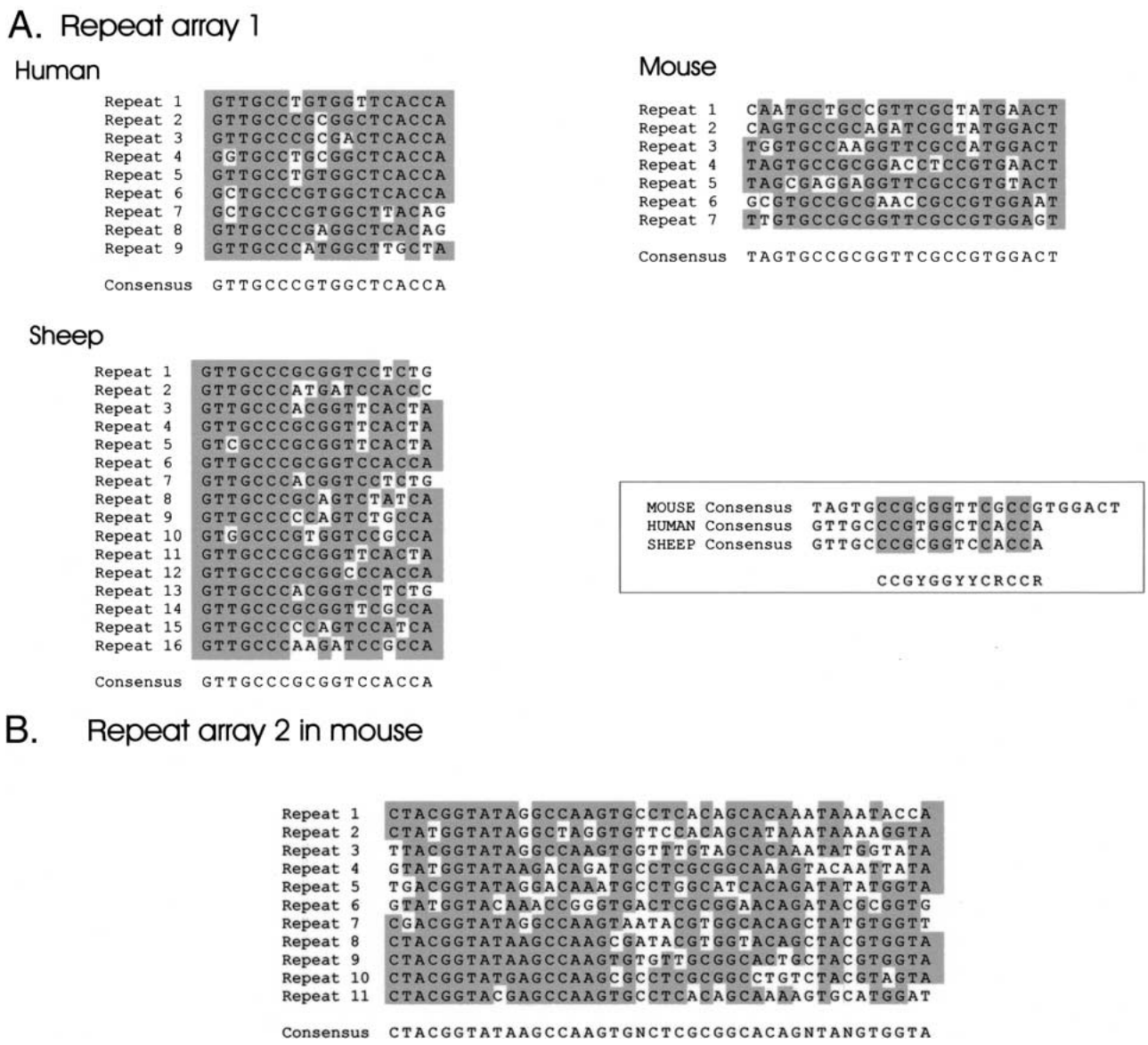


Figure 4 Repeated sequence motifs in the *Gtl2* upstream region in mouse, human, and sheep. (*A*) Repeat array 1 is located 12.5–15 kb upstream of *Gtl2* in mouse, human, and sheep. Shown are the repeated motifs and the derived consensus sequences. Positions that fit the consensus sequences are shown on a grey background. The similarity of the mouse, human, and sheep consensus sequences is shown in the box. (*B*) Repeat array 2 in the mouse is located 13.3 kb upstream of *Gtl2* in the mouse. Positions that are identical to the consensus sequence are shaded.

differential methylation. There has been speculation that the two domains may have common imprinting control elements (Schmidt et al. 2000; Takada et al. 2000; Wylie et al. 2000). Initial BLAST and FASTA searches for similarities to known regulatory elements in the *Igf2–H19* region, such as the enhancer elements downstream of *H19* and a muscle-specific repressor element 40 kb downstream of *Igf2* (Ainscough et al. 2000), were unfruitful for the available sequence. Furthermore, searches using the sequences of the 20 conserved elements from the *Dlk1–Gtl2* region did not reveal any similarities to the *Igf2–H19* region. We then compared the *Dlk1–Gtl2* and *Igf2–H19* regions on the basis of features including the distribution of repetitive elements, G + C content, and the distribution of CpG islands. For this we selected the genomic sequences of the human and mouse *Igf2* and *H19* regions (Onyango et al. 2000), encompassing the entire *Igf2* and *H19* genes and 2 and 8 kb, respectively, of the *Igf2* upstream regions, and in both cases, 11 kb of the *H19* downstream regions. The analyzed human sequence is 138 kb long, the mouse sequence spans 101 kb.

For *Igf2* and *H19*, the G + C content is 51.37% in the mouse, 59.50% in the human, and is higher than in the *Dlk1–Gtl2* region (49.37% in the mouse, 51.37% in the human). Like the *Dlk1*-related CpG islands, the CpG islands at the *Igf2* transcription start sites are the most pronounced.

Repetitive Elements in the *Dlk1–Gtl2* and *Igf2–H19* Regions

It has been proposed that LINE1 elements might have a role in X inactivation (Lyon 1998; Smit 1999; Bailey et al. 2000). To address whether this might be also the case for imprinted domains, we have analyzed the content of repetitive elements in the *Dlk1–Gtl2* and also in the *Igf2–H19* regions. In contrast to repetitive elements in mouse and human, little is known about the properties of these elements in the sheep genome. We therefore focused on the repetitive elements in the human and mouse sequences of both domains (Table 1).

In general, the overall content of interspersed repeats (IR) is higher in the *Dlk1–Gtl2* region than in the *Igf2–H19* region in both species (Table 1). In both regions, however, the IR content is lower than the published average values for mouse and human sequences with similar G + C content (Smit 1999). A consistent enrichment of LINE1 elements in the analyzed imprinted domains compared with the published average values for autosomal sequences was not observed. In contrast to other subclasses of repetitive elements, a low proportion of SINE elements seems to be persistently related to the relatively low IR content in the *Dlk1–Gtl2* and *Igf2–H19* regions.

Conserved Putative CTCF-Binding Sites in the *H19* and *Gtl2* Regions Are Not at Corresponding Positions

CTCF-binding sites in the upstream region of *H19* in mouse and human contribute to the function of this region as methylation-sensitive insulator elements by affecting interactions between *Igf2* and the shared enhancers downstream of *H19* on the maternal allele (Bell and Felsenfeld 2000; Hark et al. 2000; Kanduri et al. 2000; Szabo et al. 2000). We looked for conserved putative CTCF-binding sites in the *Dlk1–Gtl2* regions in the three species. Among several different known motifs for CTCF-binding sites to date, only one motif (consensus sequence: CCGCNNGGNGNC; Wylie et al. 2000) is accessible to CpG methylation. A number of putative CTCF sites were identified in the *Dlk1–Gtl2* regions in all three species (two in mouse, five in human, 12 in sheep), but only one of these was conserved in all three species (green triangles in Fig. 1, nucleotide 96071 in GenBank accession no. AJ320506, nucleotide 68347 in GenBank accession no. AL117190.4, nucleotide 140153 in GenBank accession no. AF354168). This putative CTCF-binding site is located in a homologous position in the first *Gtl2* intron in mouse and sheep, and in the second intron in the human (Fig. 1).

DISCUSSION

Whereas previous sequence comparisons in imprinted regions were restricted to the comparison of the mouse and the human sequences (Engemann et al. 2000; Paulsen et al. 2000; Okamura et al. 2000; Onyango et al. 2000), we were able to include the sequence of third mammalian species, the genomic sheep sequence, in our analyses of the *Dlk1–Gtl2* region on mouse chromosome 12. Compared with the human–mouse comparison alone, a three-species comparison can result in a more precise identification of conserved regions (Dubchak et al. 2000). As our chosen selection procedure excludes gaps in the alignment of the conserved elements, the 20 elements identified should be regarded as cores of highly conserved regions rather than as isolated conserved stretches of high homology. These elements were clustered in *Dlk1* and upstream of *Gtl2*. The inclusion of the sheep sequence also facilitated the identification of short tandem repeats 13–15 kb upstream of *Gtl2* in all three species, although this region is not well conserved at the level of the DNA sequence.

We were able to identify a third transcript in the *Dlk1–Gtl2* region in mouse and human. This transcript resides in the downstream region of *Dlk1* and is also present in the sheep (Charlier et al. 2001). Like *Dlk1*, this transcript is imprinted being silent on the maternal allele. The 5′ end of this transcript is in *Dlk1* exon 5, and it is likely that it represents a

Table 1. Contents of Interspersed Repeats

	G+C content	Sine	Line	Line1	LTR elements	DNA transposon-like elements	Unclassified elements	Total interspersed repeats
mouse								
Dlk1-Gtl2	49.4%	9.6%	3.2%	3.0%	1.6%	0.3%	0.0%	14.7%
Igf2-H19	51.4%	1.8%	0.9%	0.7%	2.0%	0.1%	0.0%	4.8%
genome	46–50%	14.4%	n.d.	3.4%	9.9%	n.d.	n.d	28.9%
	50–54%	16.5%	n.d.	1.4%	3.6%	n.d.	n.d.	20.3%
human								
DLK1-GTL2	51.4%	13.5%	12.6%	11.6%	7.4%	2.3%	0.0%	35.8%
IGF2-H19	59.5%	3.6%	4.4%	3.4%	3.1%	1.2%	0.0%	12.3%
autosomes	50–54%	25.5%*	n.d.	4.8%	4.7%	1.8%	n.d.	41%
	>54%	20.1%*	n.d.	3.0%	2.9%	1.2%	n.d.	31%
X chromosome	50–54%	24.9%*	n.d.	6.1%	2.7%	1.1%	n.d.	38%
	>54%	14.8%*	n.d.	5.3%	2.8%	1.0%	n.d.	26%
sheep								
Dlk1-Gtl2	53.7%	5.5%	12.0%	20.5%	20.5%	20.5%	20.5%	20.5%

Shown are summaries for the interspersed repeats that were identified using `RepeatMasker` (http://ftp.genome.washington.edu/index.html) for the analyzed sequences. The average values for human autosomal and X chromosome linked sequences and mouse genome-wide sequences were taken from Smit (1999). Separate values for mouse autosomes and X chromosome were not available. Because the content of interspersed repeats is related to the G+C content, the values are given for sequences that show G+C contents similar to the imprinted regions analyzed here.
*Sum of the values for *Alu* and Mir elements; n.d., not defined.

cleavage product of *Dlk1* transcripts. We cannot exclude, however, that expression of the transcript downstream of *Dlk1* is independent from *Dlk1* transcription and is initiated by a so-far-unknown promoter in the last *Dlk1* exon. We have no further indications for additional genes in the intergenic *Dlk1–Gtl2* region. This is in contrast to the *Igf2–H19* region where an additional transcript has been described (Onyango et al. 2000).

As expected for an imprinted region, the DMRs in intron 4/exon 5 of *Dlk1* and at the transcriptional start site of *Gtl2* are highly conserved. In addition, we identified CpG-rich short direct repeats ~12.5 –15 kb upstream of *Gtl2*. The similarity of the repeat cores in mouse, human, and sheep indicates that these repeats may be derived from the same ancestral motif. This indicates that either the repeat structure or the motif itself might be important for regulation in this domain. It has been hypothesized that short tandem repeat arrays might have a function in the regulation of imprinting (Neumann et al. 1995), however, the positions of such elements in imprinted regions are rarely conserved in mouse and human (Engemann et al. 2000; Paulsen et al. 2000). Nevertheless, CpG-rich tandem repeats have been identified upstream of *Magel2* in human and mouse (Boccaccio et al. 1999). Interestingly, the imprinted *Impact* gene in the mouse possesses a CpG island that is characterized by tandem repeats, whereas in the nonimprinted human *IMPACT* gene such repeats are not present (Okamura et al. 2000). Furthermore, the CTCF-binding sites upstream of *H19* are arranged in a repeated structure in both, mouse and human. In the mouse, however, the CTCF-binding sites are not short direct tandem repeats, therefore it is not very likely that they are functionally the same as the described repeats upstream of *Gtl2*. The G-rich short tandem repeats upstream of the mouse *H19* gene may be similar, but their function is still unclear and an analog is absent upstream of human H19.

Because *Dlk1–Gtl2* and *Igf2–H19* share many imprinting properties, it has been suggested that imprinting in both regions may be regulated by common elements. Interestingly, the distribution and "shape" of CpG islands are similar in both regions: *Igf2* and *Dlk1* have pronounced unmethylated CpG islands in their promoter regions and additional CpG islands in their last exons that are differentially methylated in both genes (Sasaki et al. 1992; Feil et al. 1994; Takada et al. 2000). Conversely, the *H19* and *Gtl2* promoters are associated with "weaker" CpG islands (Sasaki et al. 1992; Ferguson-Smith et al. 1993; Takada et al. 2000; this study). Analysis of general features, however, revealed that both regions differ in the content of interspersed repeats and their G + C contents. We have identified a number of features of the *Dlk1–Gtl2* region that do not have any sequence analogs in the *Igf2–H19* region. This includes the different positions of conserved CTCF-binding sites, and a conserved CpG-rich repeat structure 13–15 kb upstream of *Gtl2*. This indicates that the regulation of imprinted gene expression may be different in both regions. Our findings do not exclude the possibility that some regulatory aspects, such as those that are required for reciprocal imprinting, are shared. It is also possible that common transcription factors are involved, but that their precise action may differ, as is indicated by the different positions of the (putative) CTCF-binding sites in *H19* and *Gtl2*. Further analysis of the functional roles of these conserved and related features will contribute to our understanding of gene regulation at imprinted loci and the genomic evolution of imprinted domains.

METHODS

BAC Clone Isolation and DNA Sequencing

The BAC clone 103N10 was isolated from a genomic library (BAC ES (I), mouse strain 129/SvJ; Incyte Genomics Inc.) using a probe specific for exon 3 of *Gtl2*. Subsequently, the BAC DNA was sequenced at MWG Biotech (Milton Keynes). The assembled 213,094-bp-long sequence is covered in average by 9.06 sequence reads. The expected accuracy was estimated to be at least 99.995%. The first 100 kb of sequence belonged to a different locus indicating the BAC clone 103N10 was chimaeric. The breakpoint between both fragments was determined by sequencing a 14-kb-long *Bam*HI fragment that contained *Dlk1* and additional 7 kb of the true *Dlk1* upstream region. This clone (kindly provided by Dr. J. Laborda, Universidad de Castilla-La Mancha, Albacete, Spain) was originally isolated in an independent screen from a cosmid library. The breakpoint in the chimaeric BAC sequence was localized 6576 bp upstream of the start site of transcription of *Dlk1* and was chosen as the start site of the published sequence (GenBank accession no. AJ320506).

Sequences Taken from the GenBank Database

The human sequence was obtained by assembly of nucleotides 135001–184740 from GenBank accession no. AL132711.3 and nucleotides 10426–84685 from GenBank accession no. AL117190.4. Therefore, in Figure 1, nucleotide 1 in the human sequence is nucleotide 135001 (AL132711.3). The analyzed region in the sheep spans nucleotides 47001–157000 in the published genomic sequence from GenBank accession no. AF354168. Likewise, in Figure 1, nucleotide 1 in the sheep sequence is nucleotide 47001 (AF354168). The genomic mouse sequence of the *Igf2* region was assembled using the genomic sequences of the *Igf2* and *H19* genes (nucleotides 1–27823 of GenBank accession no. U71085, nucleotides 1–19154 of GenBank accession no. AF049091) and an unfinished sequence for the intergenic region (Onyango et al. 2000) (reverse complement of nucleotides 57576–111598, downloaded from http://bio.cse.psu.edu/). The human genomic *IGF2–H19* sequence was downloaded from http://bio.cse.psu.edu/ (Onyango et al. 2000) (reverse complement of nucleotides 39001–177000).

The human, mouse, and bovine *Dlk1* cDNAs have the GenBank accession no. U15979, U15980, AB009278, and AF181462. The structure of the mouse *Gtl2* gene was derived from alignment of the cDNA sequence (GenBank accession no. Y13182) to the genomic sequence. For the human *Gtl2* gene, two different cDNA sequences have been characterized (GenBank accession no. AB032607, AF052114). The human *Gtl2* exons 2, 6, 7, and 8 are represented by ESTs (GenBank accession no. AW163035, H58895, AV701976, W44755). The structure of sheep *Gtl2* was established by alignment to bovine ESTs (GenBank accession no. AV594305, AV596262, BF076011, AV609668, BF601485).

Computational Characterization of the Genomic Sequences

Pairwise alignments were generated using the `PipMaker` software at Pennsylvania State University (Schwartz et al. 2000) (http://bio.cse.psu.edu/). The overall similarities of the sequence pairs were calculated using the obtained local alignments. The "concise" outputs contain lists of sequence matches in the analyzed sequence pairs. These lists were compared to identify conserved elements that were present in all three alignment pairs.

Interspersed repeats, small RNAs, satellites, simple repeats, and DNA elements of low complexity were detected using the `RepeatMasker` software at the University of Washington (http://ftp.genome.washington.edu/index.html). Ad-

ditionally, tandem repeats were detected using the `Compare` (window size 21, stringency 14) and `Dotplot` programs of the Wisconsin package, version 10.0 (Genetics Computer Group).

CpG islands were identified using the CpG plot software at the European Bioinformatics Institute (http://www.ebi.ac.uk/index.html), choosing the following settings: Window size 200, step 1, Obs/Exp 0.6, MinPC 50, Length 200. CpG and G+C plots were generated using the `window` (window size 500, shift increment 50) and `statplot` programs of the Wisconsin package, version 10.0 (Genetics Computer Group). Putative CTCF-binding sites were identified using the "findpatterns" option of the Wisconsin package, version 10.0 (Genetics Computer Group).

Northern Blot Analysis

Total RNA was prepared from UPD12 embryos (eight) at 15.5 dpc according to standard protocols (Chomzcynski and Sacchi 1987). Poly A+ RNA was enriched using Oligo(dT)25 Dynabeads (Dynal Ltd.) according to the manufacturer's protocol. Separation by agarose gel electrophoresis and Northern blot transfer were performed according to standard protocols (Sambrook et al. 1989). The subsequent hybridizations were performed using the following probes. *Dlk1* downstream transcript: genomic *Hpa*II fragment 557 (nucleotides 15899–16456, GenBank accession no. AJ320506); *Dlk1*: 680-bp-long *Pst*I fragment excised from IMAGE clone 604466; *Gapdh*: PCR product from genomic DNA (primers: 5′-ACAGTCCATGCCATCACTGCCACTC-3′, 5′-CCAGCCCCAGCATCAAAGGTGG-3′). These probes were radioactively labeled using the Megaprime DNA Labeling system (Amersham Pharmacia). The subsequent hybridization was performed according to Sambrook et al. (1989) with the following modifications: in 50% formamide, 5× SSPE, 0.5% SDS, 5% Bailey's Irish Cream Liquor, 50 µg/mL heat denatured salmon sperm DNA at 42°C overnight, the filters were subsequently washed to 65°C in 0.1× SSC, 0.1% SDS.

RT-PCRs and 5′RACE

RT-PCRs for the analysis of expression of the *Dlk1* downstream transcript were performed using two different sets of primers. Set 1: 5′-GTAGTGGCTGTGTGCCAGGC-3′ and 5′-TGGCTAGGTGTTTGGGGATC-3′; set 2: 5′-CAGCCCCCACCAAGGTTTGC -3′ and 5′-GGAAGCTAGAAAGAGCGCCC-3′ (1.5 mM $MgCl_2$, 80 µM dNTPs, 0.03 U/µL BIOTAQTM DNA Polymerase (BioLine), 1× PCR buffer (BioLine), 60°C annealing temperature, 35 cycles). For the identification of expanded *Dlk1* transcripts, the following primers were used: 5′-AACCCCCTGCGCCAACAATG-3′and 5′-GCTGGGTTAGGACTAGGTCCCGAC-3′ (1.5 mM $MgCl_2$, 80 µM dNTPs, 0.03 U/µL BIOTAQTM DNA Polymerase (BioLine), 1× PCR buffer (BioLine), 45 sec 95°C; 35 cycles: 30 sec 95°C, 30 sec 60°C, 3 min 72°C; 5 min 72°C). The 5′RACE PCR was performed on randomly primed cDNAs that had linkers ligated to their 5′ ends using the Marathon-Ready cDNA Kit (mouse 15.5 dpc) (Clontech) according to the manufacturer's protocol. For the nested PCR, the following specific primers were used: (1) Primer: 5′-GGTTGGAGGTGGGGGAATCTCGCC-3′; (2) Primer: 5′-GCTGGGTTAGGACTAGGTCCCG AC-3′.

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