Delta-like and gtl2 are reciprocally expressed, differentially methylated linked imprinted genes on mouse chromosome 12

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The distal portion of mouse chromosome 12 is imprinted. To date, however, Gtl2 is the only imprinted gene identified on chromosome 12. Gtl2 encodes multiple alternatively spliced transcripts with no apparent open reading frame. Using conceptuses with maternal or paternal uniparental disomy for chromosome 12 (UPD12), we found that Gtl2 is expressed from the maternal allele and methylated at the 5’ end of the silent paternal allele. A reciprocally imprinted gene, Delta-like (Dlk), with homology to genes involved in the Notch signalling pathway was identified 80 kb upstream of Gtl2. Dlk was expressed exclusively from the paternal allele in both the embryo and placenta, but the CpG-island promoter of Dlk was completely unmethylated on both parental alleles. Rather, a paternally methylated region was identified in the last exon of the active Dlk allele. The proximity, reciprocal imprinting and methylation in this domain are reminiscent of the co-ordinatorly regulated Igf2–H19 imprinted domain on mouse chromosome 7. Like H19 and Igf2, Gtl2 and Dlk were found to be co-expressed in the same tissues throughout development, though not after birth. These results have implications for the regulation, function and evolution of imprinted domains.

A bacterial artificial chromosome (BAC) clone containing Gtl2 was isolated from a 129/Sv mouse genomic library (Invitrogen), and a 100 kb stretch encompassing Gtl2 was mapped (Figure 1). Northern blots using RNA isolated from E15.5 mUPD12, pUPD12 and normal conceptuses were probed with Gtl2 exon 3 (Figure 2b). Gtl2 transcripts were expressed in mUPD12 embryos at levels at least twice those seen in normal embryos, but were absent in pUPD12 embryos, indicating that the gene is imprinted. Southern blot analysis of mUPD12 and pUPD12 DNA, cut with methylation-sensitive restriction enzymes, showed that the promoter region of Gtl2 was differentially methylated (Figure 3c); the inactive paternal promoter was hypermethylated and the promoter of the active maternal allele completely unmethylated. The paternal promoter was not methylated in sperm (Figure 3d, probe G1), suggesting that the promoter methylation on this allele is not the germ-line imprinting signal. The methylation status is summarised in Figure 1b.

We also identified, from genome databases, candidate genes and expressed sequence tags (ESTs) mapping to distal chromosome 12 or the region of syntenic homology on human chromosome 14q. One of these, Delta-like (Dlk) was found to map 80 kb upstream of Gtl2 on the same mouse BAC clone (Figure 1a). Dlk encodes an EGF-repeat-containing protein and has been shown to function in several cell types, including pre-adipocytes [10], thymocytes [11], adrenal glomerulosa cells [12], pancreatic
β-cells [13], hematopoietic stromal cells and B lymphocytes [14]. A secreted form of the protein has been isolated from amniotic fluid [15]. In mammalian systems, \(Dlk\) plays a role in differentiation (reviewed in [16]). Although murine \(Gtl2\) has previously been mapped to chromosome 12, the map position of the \(Dlk\) gene is ambiguous [17,18]. Using a sequence from intron 2 of the \(Dlk\) gene, we verified the location of the cloned genomic DNA fragment to mouse chromosome 12 on a mouse × hamster radiation hybrid mapping panel (Research Genetics). The highest anchor LOD (10.6) was obtained for linkage to the marker \(D12Mit280\), and the best-fit position for this locus was 9.5 cR proximal to \(D12Mit141\), and 1.4 cR distal to \(D12Mit280\) (data not shown).

Allele-specific expression of \(Dlk\) was analysed using RNA isolated from mUPD12 and pUPD12 embryos (and placentae, data not shown). Four different transcripts were evident in both embryos and placentae. \(Dlk\) was not expressed in mUPD12 material, and was expressed at approximately twice the normal level in pUPD12 conceptuses (Figure 2a), making it the first paternally expressed imprinted gene identified on mouse chromosome 12. Furthermore, \(Dlk\) and \(Gtl2\) are imprinted reciprocally. To determine whether the imprinting of \(Dlk\) is associated with allele-specific methylation, DNA from UPD12 conceptuses was digested with methylation-sensitive restriction enzymes. The promoter of \(Dlk\) is a CpG island, at which no parental-origin specific methylation differences were observed (Figure 3a, probe D1). Systematic analysis of all the \(HpaII\) sites in the gene identified a single differentially methylated site in a smaller CpG island within the last exon. This \(HpaII\) site was completely unmethylated.

![Figure 1](image1)

**Figure 1**

Genomic organisation and differential methylation of the \(Dlk\) and \(Gtl2\) genes. (a) \(Dlk\) (blue exons) and \(Gtl2\) (red exons) gene structures. Yellow boxes, CpG islands; open yellow box, CpG-rich domain at the \(Gtl2\) promoter; green triangle, lacZ insertion site in the \(Gtl2lacZ\) mouse [7,8]; black bars, probes; arrows, positions of primers used in the reverse transcription (RT)–PCR analysis. A restriction map of the region is shown above, and other relevant sites associated with probes used are shown below. \(Hpa\) sites are indicated as unlettered vertical lines. N, Nof; S, Sph; M, Mlu; P, Pst; Nh, Nhel. (b) Summary of differential methylation and opposite imprinting of the \(Dlk\) and \(Gtl2\) genes. M and P, the maternally and paternally inherited chromosomes, respectively; circles, methylation status; white, black and half-filled circles, unmethylated, fully methylated and partially methylated \(Hpa\) sites, respectively; arrows, expression of allele and direction of transcription.

![Figure 2](image2)

**Figure 2**

Imprinted expression of \(Dlk\) and \(Gtl2\). (a) \(Dlk\) is exclusively expressed from the paternal allele. Total RNA (10 µg) prepared from normal (lane 1), mUPD12 (lanes 2–4) and pUPD12 mice (lanes 5,6) at E15.5 were analysed by northern blot hybridisation [23] using D2 as a probe. RNA loading was confirmed with a probe for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Expression of \(Dlk\) was 1.9 ± 0.3 times stronger in pUPD12 compared with normal embryos, as determined by densitometric analysis on a Storm 860 phosphorimager (Amersham). (b) \(Gtl2\) is expressed exclusively from the maternal allele. PolyA+ RNA (0.5 µg) prepared from normal (lanes 1,2), mUPD12 (lane 3) and pUPD12 mice (lane 4) was analysed as in (a). \(Gtl2\) exon 3 (G2) was used as a probe. Expression of \(Gtl2\) was 2.7 ± 0.3 times stronger in mUPD12 compared with normal embryos, as determined by densitometric analysis.
Identification of the differentially methylated regions of Dlk and Gtl2. (a) The Cpg island at the start of Dlk is completely unmethylated on both parental alleles in embryos and adults. Genomic DNA prepared from normal, mUPD12 and pUPD12 mice at E15.5 and kidney from an adult C57BL/6J mouse was digested with PstI (–), further digested with MspI (M), HpaII (H), or HpaI (Hh) and analysed by Southern blot hybridisation using D2 as a probe. (b) The Cpg island in exon 5 of Dlk is differentially methylated on the two parental alleles. Genomic DNA, prepared as in (a), was digested with Nhel (–), further digested with MspI or HpaII and analysed by Southern blot hybridisation using D1 as a probe. (c) The Cpg-rich 5′ region at the Gtl2 promoter is hypermethylated on the paternal allele and unmethylated on the maternal allele. Genomic DNA, prepared as in (a), was digested with Nhel (–), further digested with MspI or HpaII and analysed by Southern blot hybridisation using G1 as a probe. (d) Analysis of genomic DNA isolated from sperm indicated that the paternal-specific methylation in Dlk exon 5 is inherited from sperm (probe D2). In contrast, the paternal methylation at the Gtl2 promoter was predominantly unmethylated in sperm (probe G1). Thus, this modification was acquired after fertilisation.

Dlk and Gtl2 represent a new pair of reciprocally imprinted genes. The imprinting characteristics of Dlk and Gtl2 are highly reminiscent of those seen for the Igf2–H19 locus. Like Igf2–H19, Dlk and Gtl2 are 80–100 kb apart, oppositely imprinted, and the 3′ gene, Gtl2, like H19, encodes an untranslated RNA. The methylation profiles are also very similar; H19 and Gtl2 have paternally methylated CpG-rich promoters on the inactive alleles, and Igf2 and Dlk have paternal-specific partial methylation in the last exon of the active allele. H19 and Igf2 are expressed in the same tissues in the embryo and the genomic organisation and epigenetic characteristics are important for this co-ordinate regulation (reviewed in [22]). We therefore predicted that Dlk and Gtl2 might also show co-ordinate regulation. Developmental expression of Gtl2 has been documented by in situ hybridisation [8], but Dlk expression data are limited. Co-expression of the genes was therefore assessed by PCR amplification of a tissue- and stage-specific panel of cDNAs generated for comprehensive developmental expression assays (E.C., A.C.F.-S., M.H.J. and T.F., unpublished). Gtl2 and Dlk transcripts were detected at most stages in all embryonic and extraembryonic tissues (see Supplementary material). Northern analysis of total RNA from E18.5 foetal tissues confirmed this Dlk expression (data not shown). In 88–90% of the prenatal tissues examined, the genes were co-expressed. In adult tissues, no Dlk expression was detected but Gtl2 was expressed in some adult tissues. Thus, at prenatal stages, Dlk is expressed in the majority of tissues that also
express Gtl2. The relative levels of expression varied between tissues and, because each gene encodes multiple transcripts, may reflect differences in the specific transcripts amplified. The sites of strongest expression were, however, consistent with previous reports [8,10]. Thus, Dlk and Gtl2 share the combination of common methylation imprints, common sites of expression, the same linkage pattern, the same absence of open reading frame in the 3’ gene, and the same reciprocity in imprinting as Igf2–H19. Further studies will determine whether the two genes share common regulatory elements, as has been shown for the Igf2–H19 locus.

Gtl2 was isolated originally as a gene, located 3 kb downstream of a transgene insertion, Gtl2lacZ, which caused a foetal and postnatal growth retardation phenotype on paternal transmission [7]. Maternal transmission of the insertion results in normal animals. Gtl2 expression is reduced though not absent in mutant embryos homozygous for the insertion. Thus Gtl2 is repressed on the paternal allele, and does not appear to be activated in the transgene homozygotes [8]. The growth retardation phenotype observed on paternal transmission might therefore be explained by the silencing of a paternally expressed imprinted gene, which is in the vicinity of Gtl2, with regulatory elements affected by the insertion. Consistent with this is the finding that all mUPD12 embryos are severely growth retarded [6]. Based on the results presented here, absence of Dlk expression might be responsible for this growth retardation. Analysis of Dlk expression in the Gtl2lacZ mouse, and comparison of the Gtl2lacZ phenotype with that of a Dlk knockout mouse, will test this explanation.

The lethality and accompanying array of mutant phenotypes identified in mUPD12 and pUPD12 concepsites indicate that imprinted genes on chromosome 12 play roles in multiple lineages that are not affected in the Gtl2lacZ mutant. It is therefore likely that other imprinted genes exist on mouse chromosome 12, and Dlk and Gtl2 may be two genes within a larger imprinted domain.

Supplementary material

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