A 178-kb BAC transgene imprints the mouse \textit{Gtl2} gene and localizes tissue-specific regulatory elements

Aleksey Yevtodiyenko,\textsuperscript{a} Ekaterina Y. Steshina,\textsuperscript{a} Scott C. Farner,\textsuperscript{a} John M. Levorse,\textsuperscript{b} and Jennifer V. Schmidt\textsuperscript{a},* \\
\textsuperscript{a}Department of Biological Sciences, University of Illinois at Chicago, 900 S. Ashland Avenue, MBBR 4202, Chicago, IL 60607, USA \\
\textsuperscript{b}Department of Molecular Biology, Princeton University, Washington Road, Princeton, NJ 08544, USA \\
Received 26 January 2004; accepted 8 April 2004 \\
Available online 25 May 2004 \\

Abstract

The regulation of genomic imprinting, the allele-specific expression of an autosomal gene, is complex and poorly understood. Imprinted genes are organized in clusters, where \textit{cis}-acting regulatory elements are believed to interact to control multiple genes. We have used BAC transgenesis in the mouse to begin to delineate the region of DNA required for proper expression and imprinting of the mouse \textit{Delta-like1} (\textit{Dlk1}) and \textit{Gene-trap locus2} (\textit{Gtl2}) imprinted genes. We demonstrate that the \textit{Gtl2} gene is expressed from a BAC transgene in mouse embryo and placenta only upon maternal inheritance, as is the endogenous \textit{Gtl2} gene. \textit{Gtl2} is therefore properly imprinted on the BAC in an ectopic chromosomal location and must carry with it all necessary imprinting regulatory elements. Furthermore, we show that the BAC \textit{Gtl2} gene is expressed at levels approaching those of the endogenous gene only in the brain of adult animals, not in other sites of endogenous expression such as the pituitary, adrenal, and skeletal muscle. These data localize the enhancer(s) for brain \textit{Gtl2} expression, but not those for other tissues, to the DNA contained within the BAC clone. As the \textit{Dlk1} gene is not expressed from the BAC in any tissues, it must require additional elements that are different from those necessary for \textit{Gtl2} expression. Our data refine the interval for future investigation of \textit{Gtl2} imprinting and provide evidence for distinct regulation of the linked \textit{Dlk1} and \textit{Gtl2} genes. \\
© 2004 Elsevier Inc. All rights reserved. \\

Keywords: \textit{Dlk1}; \textit{Gtl2}; Mouse; BAC; Transgene; Genomic imprinting

A subset of mouse genes is subject to genomic imprinting, the expression of only one of the two parental alleles. Imprinted genes may be expressed from either the maternally or the paternally inherited allele, but for a given gene this pattern is typically conserved both within and between species [1]. Imprinted genes are found in clusters in the genome, an organization believed to reflect the use of shared regulatory elements. The \textit{Igf2}–\textit{H19} gene pair, for example, is controlled by a shared chromatin boundary element and multiple tissue-specific enhancers [2–5]. The mouse \textit{Delta-like1} (\textit{Dlk1}) and \textit{Gene-trap locus2} (\textit{Gtl2}) genes form a pair of linked reciprocally imprinted genes located on distal mouse chromosome 12 and human 14q32 [6–9]. \textit{Dlk1} is expressed only from the paternal allele, while \textit{Gtl2} is expressed only from the maternal allele. These genes were initially investigated because of their structural homology to \textit{Igf2}–\textit{H19} and the possibility that they might be similarly coregulated. Additional genes in the \textit{Dlk1}–\textit{Gtl2} region have been variously described in mouse, human, and sheep, including the maternally expressed \textit{Rian}, \textit{Meg8}, and \textit{Mirg} genes and the paternally expressed \textit{Peg11}, \textit{Dio3}, and \textit{Rtl1} genes [10–16]. This chromosomal region also contains clusters of maternally expressed microRNA- and C/D snoRNA-producing genes [12,13]. 

The \textit{Dlk1} gene (also known as FA1, Pref-1, Zog, pG2, and SCP-1) encodes a transmembrane signaling molecule related to Delta/Notch, while the \textit{Gtl2} gene produces a poorly spliced noncoding RNA [17–22]. Little is known about either the regulation of the \textit{Dlk1} and \textit{Gtl2} genes or the functional roles of their gene products. The \textit{Dlk1} protein appears to be a growth regulator involved in
maintaining the proliferative state of undifferentiated tissues. For example, its downregulation is required for the differentiation of preadipocytes to mature adipocytes [19,23]. Dlk1 is also implicated as a growth factor by the phenotype of mice lacking Dlk1, which are growth retarded and display increased adiposity and skeletal and eye abnormalities [24]. The functional role of the Gtl2 gene is unknown, as is the case for the majority of imprinted noncoding RNA genes. A mouse insertion mutation located upstream of Gtl2 results in a paternally inherited dwarfism phenotype, suggesting that perturbation of Gtl2 expression might cause altered growth [25].

Dlk1 is the more thoroughly characterized of the two genes and is known to be expressed in such tissues as preadipocytes, pituitary, skeletal muscle, adrenal cortex, pancreatic islets, placenta, and bone marrow and thymic stromal cells, as well as in tumors of neuroectodermal origin [26–32]. Limited characterization of Gtl2 expression has shown it to be coexpressed with Dlk1 in many tissues, including the pituitary, pancreas, placenta, preadipocytes, and skeletal muscle [6,22]. These patterns are not entirely overlapping, however, as Gtl2 is expressed at high levels in the brain, while our previous data have shown that Dlk1 is not expressed in this tissue [6].

Imprinting regulation is poorly understood, but epigenetic processes such as DNA methylation and histone acetylation are known to be involved [33]. Also required is the concerted interaction of cis-acting regulatory elements, including promoters, enhancers, repressors, and imprinting control regions, that may be distributed over many kilobases of DNA. The understanding of imprinting regulation for a given gene, therefore, requires knowledge of the positions and functional roles of all these elements. Several elements essential for appropriate expression and imprinting of the Dlk1 and Gtl2 genes have been identified. Expression of Dlk1 in mouse preadipocytes, for example, requires an Egr/GC element located 45 bp upstream of the Dlk1 transcriptional initiation site, while the downregulation of Dlk1 preceding preadipocyte differentiation is dependent upon the SAD (suppressor of adipocyte differentiation) element located at −170 bp [34,35]. An imprinting control region required for the imprinting of Dlk1 and Gtl2, the intergenic differentially methylated region (IG-DMR), has been identified in the region between Dlk1 and Gtl2 [36]. This IG-DMR is involved in establishing the imprinted expression of Dlk1–Gtl2 only on the maternal chromosome; those elements required for paternal imprinting are unknown. Lastly, two elements with sequence similarity to the human IGF2–H19 endodermal enhancers were reported to lie downstream of the human GTL2 gene [9]. The Gtl2 gene described in this paper was not full length, however, and these elements can now be placed within intronic GTL2/Gtl2 sequence (see Results). Despite these data, many essential regulatory elements for the Dlk1 and Gtl2 genes remain unidentified. These include the elements that regulate Dlk1–Gtl2 paternal imprinting, as well as the enhancers that can be predicted to drive their distinct expression pattern.

Fig. 1. Structure and copy number of the 28G5 BAC transgene. (A) Organization of the Dlk1 and Gtl2 genes on mouse chromosome 12. Dlk1–Gtl2 exons are indicated by numbers and introns by letters. The position of the 28G5 BAC clone is shown, as is the Srf1 site within the BAC insert; the positions of the putative enhancer sequences homologous to those at H19 are indicated by arrows. (B) Schematic of the 28G5 BAC clone. Gray arrows represent the Dlk1 and Gtl2 genes, and the black box represents the pBeloBAC vector with the T7 and SP6 ends shown. Two Srf1 restriction sites are present, one in the BAC vector and one 10 kb from the 3′ end of the insert. (C) Southern blot determination of 28G5 transgene copy number. DNAs were digested with BstXI and hybridized to probes from the T7 end of the pBeloBAC vector and from the single-copy mouse H19 gene. Signal intensity of the BAC probe was compared to that of the H19 gene using a Molecular Dynamics PhosphorImager. Calculated copy numbers are indicated below the blot.
One commonly employed mechanism for the localization of transcriptional regulatory elements is the use of transgenes in the mouse. Upon random integration into the mouse genome (and disregarding the potential for position effects), the expression pattern of a transgene reflects the regulatory elements present within that transgene. The size limit of plasmid-based transgenes is approximately 20 kb, however, too short to encompass the distant regulatory elements of many mammalian genes. For imprinted genes in particular, shared elements may lie 100 kb or more from the genes they regulate [37]. Ideal for the analysis of imprinting regulation, therefore, is the use of BAC transgenes, which can be 100–200 kb in length [38,39]. That BAC clones may carry multiple linked genes allows for the identification of shared regulatory elements as well.

Our previous studies of the Dlk1–Gtl2 region utilized a BAC clone (28G5) that spans a region from 3.5 kb upstream of the Dlk1 gene to 69 kb downstream of Gtl2 [6]. This region contains both known and proposed regulatory elements for Dlk1–Gtl2, including the Egr/GC and SAD elements, the IG-DMR, the putative intronic enhancers, and the differentially methylated CpG islands present within each gene. To begin to delimit the genomic region required for imprinting regulation of these genes, we used the 28G5 BAC to generate a transgene in the mouse. Analysis of 28G5 transgenic mice allows us to draw three conclusions. First, the 28G5 BAC transgene is able to direct expression and imprinting of the Gtl2 gene in the embryo, placenta, and adult brain of transgenic animals. These data indicate that the necessary elements for Gtl2 imprinting regulation in these tissues lie on the BAC clone. Second, the Gtl2 gene is expressed from the BAC at only low levels in its other known sites of expression, such as the adult pituitary, adrenal, and skeletal muscle. The elements directing high-level expression in these tissues must, therefore, lie outside the BAC region. Third, expression of the Dlk1 gene is dependent upon additional sequences from those that direct Gtl2 expression, even in those tissues in which the two genes are coexpressed. This conclusion is drawn from the fact that the Dlk1 gene was not expressed from the BAC in any of the tissues examined, even those such as embryo and placenta, in which transgenic Gtl2 expression was seen.

**Results**

**Generation of 28G5 BAC transgenic mice**

The 28G5 mouse BAC clone has been described previously [6]. This clone was end-sequenced and compared to the mouse genome sequence (www.ensembl.org) and was found to span at least 188 kb, from 3.5 kb upstream of Dlk1 to 69 kb downstream of Gtl2 (Fig. 1A). Small gaps within the mouse genome sequence make it impossible to deter...
mine the exact size of the BAC clone, but 188 kb is consistent with data from pulsed-field gel electrophoresis (data not shown). To generate a linear transgene from this clone, we used the restriction enzyme SrfI, which cuts once within the BAC vector and once at a site 10 kb from the 3′ end of the BAC insert (Figs. 1A and 1B). This digest releases two fragments, one containing the 5′-most 178 kb of the BAC insert and the majority of the pBeloBAC vector (T7 end) and the other containing the 3′-most 10 kb of the BAC insert and 256 bp of the SP6 end of the BAC vector.

To generate 28G5 transgenic mice, the 178- and 10-kb fragments were co-injected into fertilized FVB/N mouse eggs and the eggs implanted in foster females. A total of 22 pups were born and were genotyped from tail DNA for the presence of the BAC clone by PCR using primers specific for the T7 end of the BAC vector (data not shown). Three founder animals (designated 28G5126, 28G5130, and 28G5137) were identified and these mice were analyzed for the presence of the 10-kb fragment using a PCR assay for the SP6 BAC end (data not shown). This fragment was found to be present in the 28G5126 and 28G5137 founder animals, but was not detected in the 28G5130 founder. Founder animals were bred to wild-type FVB/N mice and independent lines were established; all founders transmitted the large transgenic fragment to their progeny at expected ratios. The 10-kb fragment segregated with the large transgene in the 28G5126 line, indicating that these DNAs are likely co-integrated at a single location. In the 28G5137 line, however, the small fragment must have integrated at a site unlinked to the larger transgene, as it segregated independently in the N1 generation. All transgenic lines were maintained as heterozygotes, and all 28G5137 animals analyzed in this work lacked the small transgene fragment. To determine the copy number of the BAC transgene in the three lines, Southern blotting followed by PhosphorImager analysis was used to normalize band intensity of the BAC vector fragment to that of the single-copy mouse H19 gene (Fig. 1C). Copy numbers were estimated to be, line 28G5126, 2 copies; line 28G5130, 6 copies; and line 28G5137, 6 copies.

Expression of the BAC Dlk1 and Gtl2 genes

To determine if the Dlk1 and Gtl2 genes were expressed from the BAC transgene, we used Northern blotting to analyze total message levels of these genes in transgenic and nontransgenic animals (Fig. 2A and data not shown). Heterozygous transgenic males and females were crossed to FVB/N animals, to give both maternal transmission and paternal transmission of the transgene (28G5+/− × FVB/N and FVB/N × 28G5+/−).1 Embryos and placenta were collected from wild-type and transgenic littermates at midgestation (E12–E14) for RNA extraction. This time point corresponds to high-level expression of Dlk1 and Gtl2 as analyzed previously [6]. Northern blots were sequentially hybridized with Dlk1, Gtl2, and β-actin probes, band intensity was quantitated by PhosphorImager analysis, and Dlk1–Gtl2 levels were normalized to the expression of β-actin (Fig. 2A and data not shown). Upon maternal inheritance, total levels of Gtl2 message were increased by 20–25% in both embryo and placenta of the 28G5126 and 28G5137 lines, but were not increased over wild type in the 28G5130 line (Fig. 2B). As demonstrated previously [6], the Gtl2 gene is highly alternatively spliced, giving rise to multiple RNA species particularly in the mouse embryo. For analysis of Gtl2 expression levels by Northern blotting, data were quantitated initially using the total signal of all RNA products (Fig. 2B). Subsequently these data were recalculated using the two major species alone; for a given sample these two values differed by less than 5% (data not shown). These data indicate that all Gtl2 splice forms produced from the endogenous Gtl2 gene are also produced from the transgene.

Upon paternal inheritance of the transgene, Gtl2 levels were not greater than those of wild type in any of the lines (Fig. 2B). Dlk1 message levels were not increased over wild type by Northern blotting in any of the three lines upon either maternal or paternal inheritance (Fig. 2B). These data were highly reproducible across multiple animals from several different litters and are confirmed by allele-specific expression analysis in Fig. 4. Gtl2, but not Dlk1, is thus expressed from the BAC transgene. Regulatory elements sufficient for the expression of Gtl2 in mouse embryo and placenta can therefore be localized to the region of DNA contained within the BAC. The Dlk1 gene, however, must require sequences outside the BAC region for expression. As the BAC extends only 3.5 kb upstream of the Dlk1 gene, it is possible these element(s) lie farther upstream.

Previously it was reported that a pair of sequences downstream of the human GTL2 gene bore similarity to the enhancers known to regulate endodermal expression of the imprinted Igf2 and H19 genes [4,9]. The GTL2 sequence reported in this paper was truncated, however, and these putative enhancers are properly placed within intron G of the human GTL2 gene, at positions +11,880 and +13,610 relative to the transcriptional initiation site (data not shown). To determine if similar sequences existed for the mouse Gtl2 gene, and are contained on the 28G5 BAC, we analyzed the sequence for mouse intron G, between Gtl2 exons 7 and 8. We found that one of the putative enhancers (CTGCAAAACA, which we will call element A) is conserved within mouse intron G at position +10,564 relative to the transcriptional initiation site (Fig. 1A). The other sequence, however (TGTCCTGCAG, which we will call element B), is not conserved in intron G between human and mouse. Searching a larger region of the mouse Gtl2 gene for this sequence identified the presence of element B in intron E, between Gtl2 exons 5 and 6 at position +6997 (Fig. 1A). This element is not present in intron E of the human GTL2 gene.

1 By convention, in mouse crosses the female is listed first.
Further investigation is required to understand the role of these sequences in regulating the expression of the Gtl2 gene.

**Imprinting of the BAC Gtl2 gene**

Expression of Gtl2 from the transgene only upon maternal inheritance suggests that this gene can be imprinted on the BAC in an ectopic chromosomal location. Verifying the imprinted expression of Gtl2 would indicate that all regulatory elements required for the imprinting process are contained within the transgene. Imprinting analysis in these animals is complicated by the fact that the 28G5 BAC transgene is derived from mouse DNA. We chose to use a mouse BAC for this project because human transgenes often do not imprint properly in the mouse [40, 41], but this approach precludes the use of sequence polymorphisms to identify transgene-derived mRNA. To analyze the allele-specific expression of the BAC Gtl2 gene, and to distinguish BAC-derived Gtl2 mRNA from endogenous Gtl2, we exploited a congenic mouse line (Cg12) available in our laboratory [14]. Cg12 mice carry a distal chromosome 12 derived from *Mus musculus castaneus* (Cast/Ei strain, abbreviated C) on a *Mus musculus domesticus* background (C57BL/6 strain, abbreviated D) (Fig. 3A). Established assays exist to distinguish the C allele of Gtl2 found in Cast/Ei and Cg12 animals from the D allele found in C57BL/6, FVB/N, and 129/Sv mice [6]. We bred transgenic females from all three 28G5 lines to male mice from the Cg12 line, using microsatellite markers to follow the integrity of the congenic interval during breeding as described previously [14] (Fig. 3A).

Among the offspring of a cross between a 28G5+/− female and a Cg12 male, 50% of the progeny will be heterozygous for the congenic chromosome 12 and carry the BAC transgene (28G5+/− Cg12+/−) (Fig. 3A, row II). Females of this genotype were again crossed to Cg12 males to give mice that were homozygous for the congenic distal chromosome 12 region and heterozygous for the 28G5 transgene (Fig. 3A, row III). These animals are called 28G5+/− Cg12+/+, and both males and females of this genotype were again bred to Cg12 mice. Pregnant females were sacrificed at midgestation and their embryos and placenta were collected (Fig. 3A, row IV). Samples were analyzed using an RT-PCR-based Gtl2 imprinting assay that exploits an SfcI restriction site present in the D allele, but absent in the C allele, of the Gtl2 message [6].
As the transgenic animals carry only C endogenous alleles, any D message detected must be derived from the transgene. In embryo and placenta of the 28G5\(^{126}\) and 28G5\(^{137}\) lines, transgene-derived Gtl2 message was detected upon maternal inheritance of the transgene, but not upon paternal inheritance (Fig. 3B). The Gtl2 gene carried on the BAC transgene is therefore properly imprinted in both lines. No BAC-derived Gtl2 message was detected in the 28G5\(^{130}\) line, after inheritance in either direction, confirming the lack of transgene expression in this line by Northern blotting.

**Tissue-specific expression from the 28G5 BAC**

Northern blotting indicated that both the 28G5\(^{126}\) and the 28G5\(^{137}\) transgenic lines expressed the BAC Gtl2 gene at only 25% of the wild-type level despite the presence of multicopy arrays. This could indicate that the BAC Gtl2 gene was unable to be fully expressed due to repressive chromatin at the sites of integration. As the two lines presumably have different integration sites, however, it seemed more likely that BAC Gtl2 was active in only a subset of the endogenous expressing tissues. To determine the tissue-specific expression pattern of the BAC Gtl2 gene, we isolated individual tissues known to express Gtl2 (brain, pituitary gland, adrenal gland, and skeletal muscle) from 28G5\(^{126}\)Cg12\(^{+/+}\) and 28G5\(^{137}\)Cg12\(^{+/+}\) mice and their nontransgenic littermates (Cg12\(^{+/+}\)) at 4–6 weeks of age after maternal inheritance of the transgene (Fig. 3A, row IV animals). We used our Gtl2 imprinting assay to investigate the allelic expression of the BAC Gtl2 gene in these tissues (Fig. 4). We found that while the endogenous Gtl2 gene (C allele) is expressed in all four tissues analyzed, the BAC Gtl2 gene (D allele) is expressed at levels similar to endogenous only in the brain (Fig. 4 and data not shown). BAC Gtl2 is expressed at only low levels in pituitary, skeletal muscle, and adrenal gland, and these results are highly reproducible for both lines across multiple litters (data not shown). These data suggest an explanation for the low levels of transgene-derived Gtl2 message seen in whole embryo analysis, as it may be only the embryonic brain that is expressing Gtl2.

**Tissue-specific imprinting of the endogenous Dlk1 and Gtl2 genes**

The expression of Gtl2, but not Dlk1, from the 28G5 transgene suggested that these two genes may be under the control of independent regulatory elements, even in those tissues such as embryo and placenta where they are normally coexpressed. This apparent dissociation of Dlk1–Gtl2 regulation was surprising, as it was expected that these two linked genes would share enhancer elements as shown for the Igf2–H19 genes [4,5]. For Igf2–H19, in tissues in which their expression is not coregulated (the choroid plexus and leptomeninges of the brain), imprinting of the genes is also not maintained [42]. As tissue-specific imprinting had not been previously explored for Dlk1–Gtl2, these data led us to investigate the imprinted expression of Dlk1–Gtl2 in individual tissues of wild-type mice. To be able to utilize D/C polymorphisms for imprinting analysis, we crossed wild-type C57BL/6 mice (D allele) to our Cg12 animals (C allele). Offspring from D female × C male (and C female × D male) matings were allowed to reach the age of 4–6 weeks, and Dlk1–Gtl2-expressing tissues were isolated for analysis of Dlk1–Gtl2 imprinting (Figs. 5A and 5B). The Gtl2 imprinting assay has already been described; the Dlk1 imprinting assay was performed using RT-PCR followed by direct sequencing of the PCR products [6]. The Dlk1 polymorphic base is an “A” in D mice and a “G” in C mice. When C × D offspring were analyzed, both Dlk1 and Gtl2 were properly imprinted, with only the maternal allele of Dlk1 and the paternal Gtl2 allele expressed in all tissues examined (Figs. 5A, top, and 5B, 1–4). The demonstration of Dlk1 expression in the brain by RT-PCR analysis was surprising, as we had shown previously that Dlk1 is not expressed in this tissue by Northern blotting [6]. It may be that Dlk1 levels in brain are too low to be detectable by Northern, or levels of Dlk1 expression may vary among animals of different ages. Our current analysis used young adult mice, while the previous data were generated with somewhat older animals.

In the offspring of the reverse D × C cross, the Gtl2 gene was found to be imprinted in all tissues examined, and the Dlk1 gene was imprinted in the brain, skeletal muscle, and pituitary (Figs. 5A, bottom, and 5B, 5–7). In adrenal gland, however, we consistently observed expression of the maternal allele of Dlk1, which should be silent (Fig. 5B, 8 and 9). As intercrosses that generate sequence polymorphisms are required for imprinting analysis, we cannot establish whether this phenomenon is
present in matings between like strains of mice, for example C57BL/6 × C57BL/6, or whether it may be a result of the F1 cross used for analysis (see Discussion).

Discussion

The 28G5 BAC localizes tissue-specific regulatory elements for the Gtl2 gene

We have shown that a BAC transgene carrying the Dlk1 and Gtl2 genes flanked by 3.5 kb of 5′ and 59 kb of 3′ flanking sequence allows expression of Gtl2 in mouse midgestation embryo and placenta. Gtl2 is expressed at levels lower than that seen for the endogenous gene, however, quantitated by Northern blotting at approximately 25% of wild type. To determine the tissue-specific pattern of BAC Gtl2 expression, individual tissues were analyzed for transgene expression and imprinting in young adult animals. We found that the BAC Gtl2 gene is expressed at high levels in the brain, but at only low levels in other known sites of endogenous expression, such as pituitary, skeletal muscle, or adrenal. These data suggest that the reduced levels of Gtl2 expression seen in whole embryos may be the result of expression only in embryonic brain. Interestingly, the BAC Gtl2 gene was also expressed at only 25% of wild-type levels in the placenta. The cell-type-specific expression of Gtl2 in the mouse placenta is unknown, and there is currently no way to determine if the reduced transgene expression in placenta is also a result of gene activity in only a subset of the expected cells.

The expression pattern of the transgenic Gtl2 gene begins to delineate the locations of the enhancers regulating the expression of this gene. Our data localize the enhancers driving expression in embryo, placenta, and adult brain to the sequences contained within the transgenic fragment. Since we cannot rule out that the embryonic expression we observe is derived from embryonic brain alone, the enhancers for embryonic and adult brain may be the same or different. Our data also show that elements required for high level expression in pituitary, adrenal, and skeletal muscle must lie outside the BAC region. We have found that the 28G5 line carries the 10-kb BAC fragment but the 28G5 line does not, yet the expression patterns for these two lines are identical. While these data suggest it is unlikely that essential regulatory elements lie within this region, we must consider that integration of the 10-kb fragment in a position different from that of the endogenous sequence (relative to the rest of the transgene) may have affected its function.

In contrast to Gtl2, the Dlk1 gene was not expressed from the BAC transgene in any of the three lines. One possible explanation is that the transgene lacks the enhancer elements that allow Dlk1 to be expressed in those tissues in which Gtl2 expression is seen—embryo, placenta, and brain. The chromosomal linkage and known coregulation of Dlk1 and Gtl2 suggested these two genes should share enhancers in those tissues in which they are coexpressed, as do the Igf2 and H19 genes [4,5]. Instead,
our data can be interpreted as evidence that the Dlk1 and Igf2 genes are under the control of unique regulatory elements, even in tissues in which both genes are active. It is also possible that the BAC Dlk1 gene is missing basal regulatory elements that function to allow high-level expression when under the control of tissue-specific enhancers. Without such putative basal elements, the BAC Dlk1 gene might be silent even in the presence of enhancers. Since the transgene carries only 3.5 kb of sequence 5' of the Dlk1 gene, there may be essential upstream promoter elements missing from the BAC. The integrated BAC transgene retains approximately 7 kb of vector sequence upstream of the Dlk1 gene, and an inhibitory effect of this sequence on Dlk1 expression must also be considered. We are currently generating transgenes with adjacent BAC clones, aimed at localizing those elements identified in the present study, as well as identifying elements required for Dlk1 expression and for the expression of both genes in additional tissues.

Imprinting of Gtl2 is maintained from the BAC transgene

Expression of Gtl2 from the BAC transgene is seen only following maternal inheritance, as is that of the endogenous Gtl2 gene, indicating that the BAC Gtl2 gene is able to imprint properly in ectopic chromosomal locations. These data indicate that all necessary regulatory elements for establishing the imprinting of the Gtl2 gene must be contained within the genomic region of the BAC transgene and delineate an interval for future study of Gtl2 imprinting regulatory elements.

Our laboratory and others have described the structural similarity of the Dlk1–Gtl2 locus to that of the well-characterized Igf2–H19 genes [6–8]. Both regions contain a paternally expressed gene involved in growth regulation that is tightly linked to a maternally expressed noncoding RNA gene. In addition, both gene pairs show significant degrees of coexpression of their respective genes; for Igf2–H19 this has been demonstrated to result from the use of shared enhancer elements located downstream of H19 [4]. These two loci have different patterns of expression, however, with Igf2–H19 expressed in endodermal and mesodermal tissues, while Dlk1–Gtl2 is expressed primarily in tissues of neuroectodermal origin. The structural homology of these two loci suggested that the positions of Dlk1–Gtl2 regulatory elements might also correspond to those for Igf2–H19. Identification of the IG-DMR challenged this theory, however, as the position of this element is not analogous to any known at Igf2–H19 [36]. Our data further support the unique regulatory features of the Dlk1–Gtl2 locus. A 130-kb YAC transgene that begins just upstream of the Igf2 gene and extends 30 kb downstream of H19 is smaller but located similarly to our 28G5 BAC [38]. This transgene allows expression and imprinting of both the Igf2 and the H19 genes in endodermal and mesodermal tissues. The brain-specific Gtl2 expression and lack of Dlk1 expres-

sion that we observe in 28G5 transgenic mice are therefore unlike what would be expected if the Dlk1–Gtl2 locus were organized as is Igf2–H19.

Loss of Dlk1 imprinting in wild-type D × C adrenal gland

Three phenomena led us to investigate the tissue-specific imprinting of the Dlk1 and Gtl2 genes in wild-type animals: (1) the growing number of genes reported to be imprinted only in certain tissues or at certain times [43], (2) evidence that Dlk1–Gtl2 imprinting is perturbed in F1 offspring between C57BL/6 and MOLF/Ei mice [44], and (3) the seeming dissociation of Dlk1–Gtl2 regulation seen in our 28G5 transgenic animals. Even for tightly imprinted genes, individual tissues may show escape from imprinting. The Igf2–H19 genes, for example, are coexpressed and imprinted in all tissues with the exception of the choroid plexus and leptomeninges of the brain, in which Igf2 is biallelic and H19 is silent [42]. We have not previously observed any relaxation of Dlk1–Gtl2 imprinting in embryo or placenta in crosses between C57BL/6 and Cast/Ei or Cg12 mice; however, tissue-specific imprinting had not been examined for these genes.

We generated F1 offspring from crosses of C57BL/6 (D allele) and Cg12 (C allele) mice in both directions and analyzed individual tissues from these animals at 4–6 weeks of age. We observed correct imprinting of Gtl2 in all tissues from both D × C and C × D crosses, indicating that Gtl2 is not subject to tissue-specific imprinting and that its imprinting is not lost in F1 animals. In C × D mice we also saw correct imprinting of Dlk1 in all tissues. In D × C mice, however, Dlk1 was imprinted in brain, skeletal muscle, and pituitary, but was consistently biallelically expressed in the adrenal gland. As interstrain or interspecific crosses that generate polymorphisms are required for imprinting analysis, we cannot currently determine if this result indicates a lack of imprinting in wild-type adrenal gland or if it is a result of the interspecific cross itself.

Numerous examples of tissue-specific imprinting have been documented, but there is also precedent for relaxation of imprinting among interspecific hybrids in both Peromyscus and Mus [44, 45]. The F1 offspring of reciprocal crosses between Peromyscus maniculatus and Peromyscus polionotus display opposite growth phenotypes and widespread loss of imprinting in both directions [45]. Interestingly, different sets of genes are affected depending on the direction of the cross. Loss of Dlk1–Gtl2 imprinting in M. m. domesticus (C57BL/6) × M. m. molossinus (MOLF/Ei) crosses was observed only when females carrying a maternally inherited C57BL/6 chromosome 12 and a paternally inherited MOLF/Ei chromosome 12 were backcrossed to the MOLF/Ei strain [44]. Despite these examples, this is the first time we have observed such an effect between D and C crosses for any of the many imprinted genes examined [6, 46].
Materials and methods

Generation of 28G5 BAC transgenic mice

The 28G5 BAC clone was isolated from the CITB 129/Sv mouse genomic library and has been described previously [6]. The 5’ end of the clone lies 3.5 kb upstream of the Dlk1 transcriptional initiation site and the 3’ end lies 69 kb downstream of the Gtl2 gene. The BAC clone was digested with the restriction enzyme SrfI, which yields two fragments—one that carries the bulk of the transgene and the majority of the pBeloBAC vector (T7 end) and another that carries the 3’most 10 kb of the BAC insert and the remaining 256 bp of the vector (SP6 end). The two fragments were extracted together with phenol:chloroform, ethanol precipitated, and resuspended in 100 mM Tris, pH 7.5/0.1 mM EDTA at a concentration of 2 ng/ml. DNA was microinjected into fertilized embryos from FVB/N mice and the embryos were implanted into foster mothers. Founder animals were bred to wild-type FVB/N mice to establish independent transgenic lines.

Transgene detection and copy number determination

Animals were genotyped for the presence of the 28G5 transgene using genomic DNA from either tail snips (adult animals) or yolk sacs (embryos). Genomic DNAs were purified by proteinase K digestion and phenol:chloroform extraction, followed by ethanol precipitation. For detection level of 2 ng/ml. DNA was microinjected into fertilized embryos from FVB/N mice and the embryos were implanted into foster mothers. Founder animals were bred to wild-type FVB/N mice to establish independent transgenic lines.

Expression and imprinting analysis

Embryos and placentae were recovered at midgestation (E12–E14), and adult tissues were obtained from 4- to 6-week-old animals. For adult animals, RNA was extracted from brain and skeletal muscle of individual mice, but due to the small size of the pituitary and adrenal these tissues were pooled from three or four littermate animals of like genotype for analysis. Total RNA was purified from embryos, placentae, and adult tissues using LiCl–urea precipitation [47]. For Northern analysis, 10–15 μg of total RNA was separated on 1% formaldehyde agarose gels and transferred to Hybond N+ membranes. Membranes were hybridized with the following probes: Dlk1, a 735-bp fragment generated by PCR using primers Dlk1F3, 5’-ATGGGGTGCTGACCCAGACAC-3’, and Dlk1R3, 5’-CACACACATAGAGGAAACTCCAGG-3’, and Dlk2, a 366-bp fragment generated by PCR using primers Gtl2F3, 5’-GCTTCTAGAGCATTGCGGTG-3’ and Gtl2R3, 5’-GCTTCTAGAGCATTGCGGTG-3’ and a 1.2-kb fragment of the mouse β-actin gene. Northern blot hybridization was carried out using ExpressHyb (Clontech) at 65°C 1 h. Blots were washed three times with 2× SSC, 0.05% SDS for 5 min followed by two washes with 0.1× SSC, 0.1% SDS for 20 min. Signal intensity was quantitated by phosphorimaging and normalized to the expression level of β-actin. For RT-PCR analysis, 2 μg of total RNA was reverse transcribed using Superscript III (Invitrogen), RT reactions were diluted 1:10, and 2 μl was used for all subsequent PCR analyses. The Dlk1 imprinting assay was performed using RT-PCR followed by direct sequencing, and the Gtl2 imprinting assay was performed using RT-PCR followed by SfeI digestion; both have been described previously [6]. The RT-PCR imprinting assays have been shown previously by mixing experiments to amplify both the D and the C alleles equally. For all RT-PCR assays, control reactions lacking reverse transcriptase were performed and showed no amplification.

Acknowledgments

We thank Teresa Orenic, Pete Okkema, and Angela Tyner for critically reading the manuscript. This work was supported by a Kimmel Scholar Award from the Sidney Kimmel Foundation for Cancer Research and by grants from the Human Growth Foundation and the National Institutes of Health (HD042013).

References


