Incorporating Mouse Genome
Mammalian Genome
From Genotype to Phenotype

Alternative splicing and imprinting control of the *Meg3/Gtl2-Dlk1* locus in mouse embryos

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Abstract

The distal part of the mouse Chr 12 contains a cluster of reciprocally imprinted genes. Recently we found a grandparental origin-dependent, transmission-ratio distortion (TRD) in this region. The TRD resulted from postimplantation loss of embryos that inherited the distal Chr 12 alleles from the maternal grandfather. These data suggested that imprinting of one or more genes in this region was not uniformly well established or maintained in all the embryos. To elucidate the mechanism underlying such a variation, we examined the expression of two genes from the distal Chr 12 imprinted region, the maternally expressed gene 3/gene-trap locus 2 (*Meg3/Gtl2*), and the delta-like homolog 1 (*Dlk1*) gene. We demonstrated that the *Meg3/Gtl2* gene had two major mRNA forms. One form, *Meg3*-proximal (*Meg3p*), contained exons 1–3. The second form, *Meg3*-distal (*Meg3d*) did not contain exons 1–3 and was present in oocytes and in 1- and 2-cell embryos. We observed cross-dependent and splice form-specific relaxation of imprinting of the *Dlk1* and *Meg3d*, but not *Meg3p*. Expression patterns of *Dlk1* and *Meg3/Gtl2* in embryos from crosses between different mouse strains suggest that 1) imprinting of the *Dlk1* and *Meg3/Gtl2* genes is not strictly coordinated; 2) parental origin-dependent expression of these genes is under control of a strain-specific, *cis*-acting modifier located in a 1.5-Mb region that includes the *Meg3/Gtl2-Dlk1* locus. Biallelic expression of *Dlk1* and *Meg3d* did not affect embryo viability and, therefore, cannot be responsible for the lethal phenotypes in UPD12 embryos or for the transmission-ratio distortion.

Genomic imprinting is a differential modification of DNA depending upon the parental origin of the allele. Such differential DNA modification usually results in monoallelic parent-of-origin-dependent expression of imprinted genes. One of the possible functions of genomic imprinting is fine regulation of genes in a tissue-specific and stage-specific fashion. Emerging evidence suggests that genomic imprinting may vary among individuals, thereby providing an additional source of phenotypic variation in the population. Individual variation of the imprinting status of several human genes (*IGF2*, *IGF2R*, *WT1*, and *HTR2A*) has been documented (Xu et al. 1993, 1997; Giannoukakis et al. 1996; Jinno et al. 1994; Bunzel et al. 1998). Different epigenetic variants may be neutral or may influence the susceptibility of an individual to disease or cancer. Our study of transmission ratios in imprinted regions of the mouse genome identified a 9-cM region in distal Chr 12 that showed a grandparental origin-dependent, transmission-ratio distortion (TRD) (Croteau et al. 2002). The TRD resulted from postimplantation loss of embryos that inherited the distal Chr 12 alleles from the maternal grandfather. Our data suggested that imprinting of one or more genes from the Chr 12 distal imprinted region was not uniformly well established, maintained, or recognized in all the embryos. To elucidate the mechanism underlying such a variation, we examined in detail the expression of two genes from the distal Chr 12 imprinted region, the maternally expressed gene 3/gene-trap locus 2 (*Meg3/Gtl2*) (Miyoshi et al. 2000), and the paternally expressed delta-like homolog 1 (*Dlk1*) gene (Schmidt

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et al. 2000). Expression analysis of these genes revealed a high incidence of biallelic expression in embryos and placentas, which is consistent with imprinting variation predicted by TRD in this chromosomal region.

Materials and methods

Strains and mouse crosses. C57BL/6 mice were purchased from Charles River Laboratories (Wilmington, Mass), NOD Lt/J mice were purchased from The Jackson Laboratory (Bar Harbor, Me.). (C57BL/6 × MOLF/Ei) F_1 mice were kindly provided by Danielle Malo. Crosses between either NOD Lt/J mice or MOLF/Ei mice and C57BL/6 were conducted to obtain postimplantation embryos. Consecutive backcrosses between C57BL/6 and MOLF/Ei mice were conducted to generate congenic mice for Chr 12 distal region. The sizes of litters of these crosses were used to assess the effect of biallelic expression of *Meg3d* and *Dlk1* on embryo viability.

Sample collection. Embryos were collected at day 7.5 or 11.5 post coitum (d.p.c.). Mature oocytes were collected according to the standard protocol (Hogan et al. 1994).

RT-PCR. RNA and DNA extractions and RT-PCR were performed as described previously (Croteau et al. 2001). The primers were purchased from Sigma-Aldrich Canada (Oakville, ON, Canada). The sequences for the primers are given in Table 1. For strand-specific RT-PCR, primers Meg3 4F and Meg3 10R were used as RT primers to detect antisense and sense transcripts, respectively. Trace amounts of α ^{32}P-dCTP (Perkin Elmer, Boston, Mass., USA) were added to label the PCR product. The *Meg3*/*Gtl2* PCR products were digested by restriction endonucleases *Bsr*DI and *Bst*UI to assay an SNP in exon 10, and *Eco*NI or *Alw*261 to assay SNPs in exon 3. The *Dlk1* PCR products were digested by *Dra*III (exon 2 SNP) or *Dra*I (exon 5 SNP) depending on the set of primers used. The digested PCR products were separated in a denaturing 5% PAG or in a 2% agarose gel. Quantitation of the RT-PCR was done by using a phosphorimager Storm 860 (Molecular Dynamics, Amersham Pharmacia Biotech, Arlington Heights, Ill.).

Cloning and sequencing. RT-PCR products were cloned by using a TA cloning kit (Invitrogen, Groningen, The Nederlands). Individual clones were analyzed by RFLP analysis. Selected clones were sequenced at the Montreal Genome Center to validate the RFLP results.

Table 1. Primers used for RT-PCR analysis of expression of *Meg3/Gtl2* and *Dlk1*

Name	*Sequence 5′-….-3′*	*Position*
Meg3 1F	gacgaagagctggaatag	1–20
Meg3 3F	attccaggaacccactacca	261–280
Meg3 3R	acggagttgccagcaagatg	624–643
Meg3 4F	agcccggactcactcatgagattg	988–1011
Meg3 4R	gttcaatctcatgagtgagtcc	992–1014
Meg3 5F	ccatcgaacggctctcgctca	1147–1167
Meg3 6F	gtcgaactcgaaatcctagcc	1215–1235
Meg3 8F	gactgaggaccccaggatg	1362–1381
Meg3 9F	gacacacggacacagacacc	1518–1537
Meg3 10R	aagcaccatgagccactagg	1856–1875
Dlk 1F	gtgcaaccctggctttcttcc	1022–1042
Dlk 1AF	gagaatcaggggtgtgctgt	2251–2270
Dlk 2F	tgtgacccccagtatggatt	2499–2518
Dlk 4F	aacaatggaacttgcgtgga	5600–5619
Dlk 4R	tgtgcaggagcattcgtact	5635–5654
Dlk 5F	cttcgagtgtctgtgcaagc	7392–7411
Dlk 5R	tcaccagcctccttgttT*aa	7822–7841

Positions are given for *Meg3*/*Gtl2* sequence GI 27392999 and for *Dlk1* sequence GI 13365690.
* This primer has a mismatch in order to create a *Dra*I site in the C57BL/6 allele.

Quantitative RT-PCR analysis and dot-blot hybridization (QADB). To document temporal expression patterns of the *Meg3*/*Gtl2* splice variants, we employed the QADB method of quantitative gene expression analysis (Rambhatla et al. 1995). With this method, the 3′ termini of the entire mRNA populations were amplified quantitatively, and the amplified material were applied to dot blots. Radiolabeled mRNA-specific probes are then hybridized and the hybridization signals quantified. This method has been used in many studies and its quantitative power extensively documented (Wang and Latham 2000).

Electronic resources. Sequences of the *Dlk1* gene, *Gtl2* gene, and the mouse genomic supercontig from the Genbank database (NCBI, http://www.ncbi.nih.gov) were used for retrieval of SNPs. NCBI BLAST program was used to align sequences of the RT-PCR clones and to search for ESTs. The Mouse Genome Database (http://www.informatics.jax.org) was used for selection of marker loci used in the mapping experiment.

Results

Expression of different forms of Meg3/Gtl2 and Dlk1 in oocytes and in preimplantation embryos. We analyzed the expression of *Meg3*/*Gtl2* in the oocytes of mouse females. Expression of *Dlk1* and *Meg3*/*Gtl2* exons 1–3 was not detected, whereas expression

of exons 9–10 was found in the oocytes. These data suggest that the murine *Meg3/Gtl2*, in addition to the known multiple splice forms, has an oocyte-specific form which lacks exon 1 or 3 and most likely is transcribed from a different promoter. Seventeen splice variants were reported for the human *MEG3* gene, and several of them are likely to have alternative promoters located downstream from exon 1 (NCBI, http://www.ncbi.nih.gov/LocusLink), which is consistent with our finding of the oocyte-specific *Meg3/Gtl2* mRNA that lacks the 5′ region.

Probes containing exons 1–3 and 9–10 were used in quantitative dot-blot hybridization experiments to examine expression of *Meg3/Gtl2* at different stages of preimplantation development. The mRNA containing exons 1–3 was not detected before the morula stage. Messages containing exon 10 were present in the oocytes (confirming the RT-PCR result) and in embryos up to the blastocyst stage. In 1- and 2-cell embryos, the detected exon 10-containing mRNA represented maternal supply of the mRNA, whereas the embryonic transcript appeared at the morula stage (Fig. 1A). We used different combinations of primers to amplify the oocyte-specific forms. In assays using primers for exons 1 and 4, 4 and 10, 3 and 10, and 3 and 4, the PCR product was not synthesized. The assays using exons 5 and 10, and 8 and 10, produced PCR bands in RT-PCRs from oocytes (data not shown). RT PCR of individual blastocysts showed strong expression of *Meg3/Gtl2* exons 9–10 and no detectable expression of exons 1–4 (Fig. 1B). All these data imply that there are different, most likely overlapping transcripts expressed from the *Meg3/Gtl2* gene in the embryo. We will refer to the transcript that contains exons 1–3 as *Meg3* proximal (*Meg3p*) and to the transcript that does not contain the exons 1–3 as *Meg3* distal (*Meg3d*).

RT-PCR assay did not detect *Dlk1* expression in oocytes or individual blastocysts. A probe containing *Dlk1* exons 4 and 5 did not produce a hybridization signal in oocytes or preimplantation embryos, confirming that *Dlk1* was not expressed at these stages.

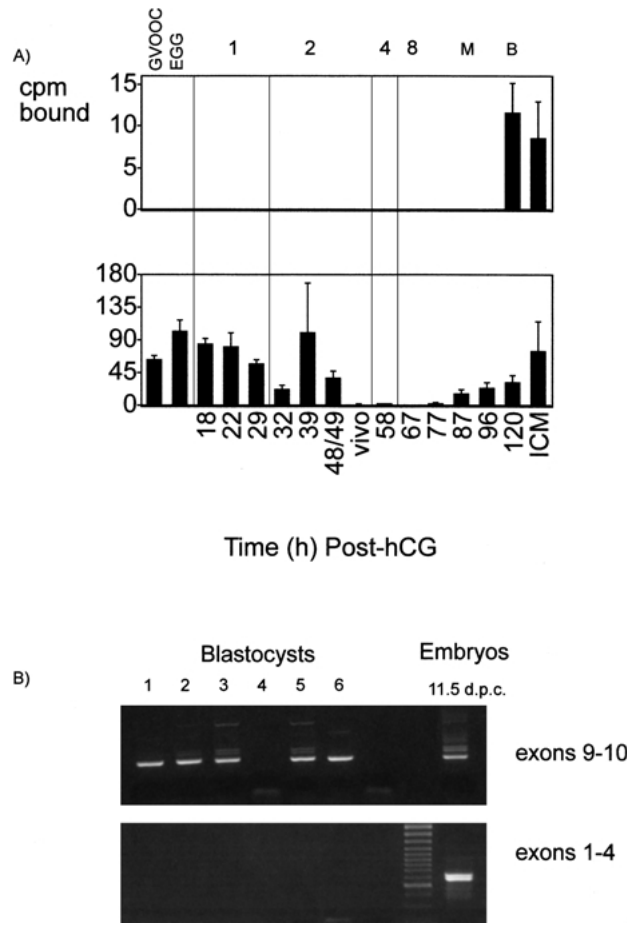

Fig. 1. Expression of *Meg3/Gtl2* gene in oocytes and preimplantation embryos. **A**. Quantitative RT-PCR. Upper panel shows data obtained using the probe containing *Meg3/Gtl2* exons 1–3. Lower panel shows data obtained using the probe containing *Meg3/Gtl2* exons 9–10. Data are expressed as normalized cpm bound. Morphological stages are shown at top. Times indicated below are the hours post-hCG injection for superovulation. All data are obtained with embryos from (C57BL/6 × DBA2) F_2 mice. **B**. Expression of *Meg3/Gtl2* in individual blastocysts. RT-PCR was performed by using total lysates from individual 3.5-d.p.c. C57BL/6 embryos.

Expression patterns of the Meg3 and Dlk1 genes in postimplantation embryos. Expression of *Meg3/Gt12* and *Dlk1* was assessed in embryos derived from reciprocal backcrosses between MOLF/Ei (a *Mus. musculus molossinus* strain) and C57BL/6 strains. Animals that inherited the *molossinus Meg3/Gtl2-Dlk1* allele from the mother and the C57BL/6 allele from the father will be referred to as MB12, whereas the animals that inherited the C57BL/6 *Meg3/Gtl2-Dlk1* allele from the mother and the MOLF/Ei allele from the father will be referred to as BM12 (Fig. 2A). We identified six single nucleotide polymorphisms (SNPs) between the MOLF/Ei and C57BL/6 *Meg3/Gtl2* sequences and two SNPs for *the Dlk1* sequences. These SNPs allowed distinguishing the parental origin of the RT-PCR product by RFLP analysis or sequencing. We compared the expression patterns of *Meg3/Gtl2* in 7.5 d.p.c. embryos derived from two reciprocal backcrosses (Fig.2A, B). When C57BL/6 females were mated to (C57BL/6 × MOLF/Ei) F_1 males, all the heterozygous embryos expressed only the maternal *Meg3/Gtl2* allele. In the second backcross, (C57BL/6 × MOLF/Ei) F_1 females were mated to C57BL/6 males. In Chr 12 heterozygotes, the paternal C57BL/6 *Meg3/Gtl2* allele was expressed in the majority of

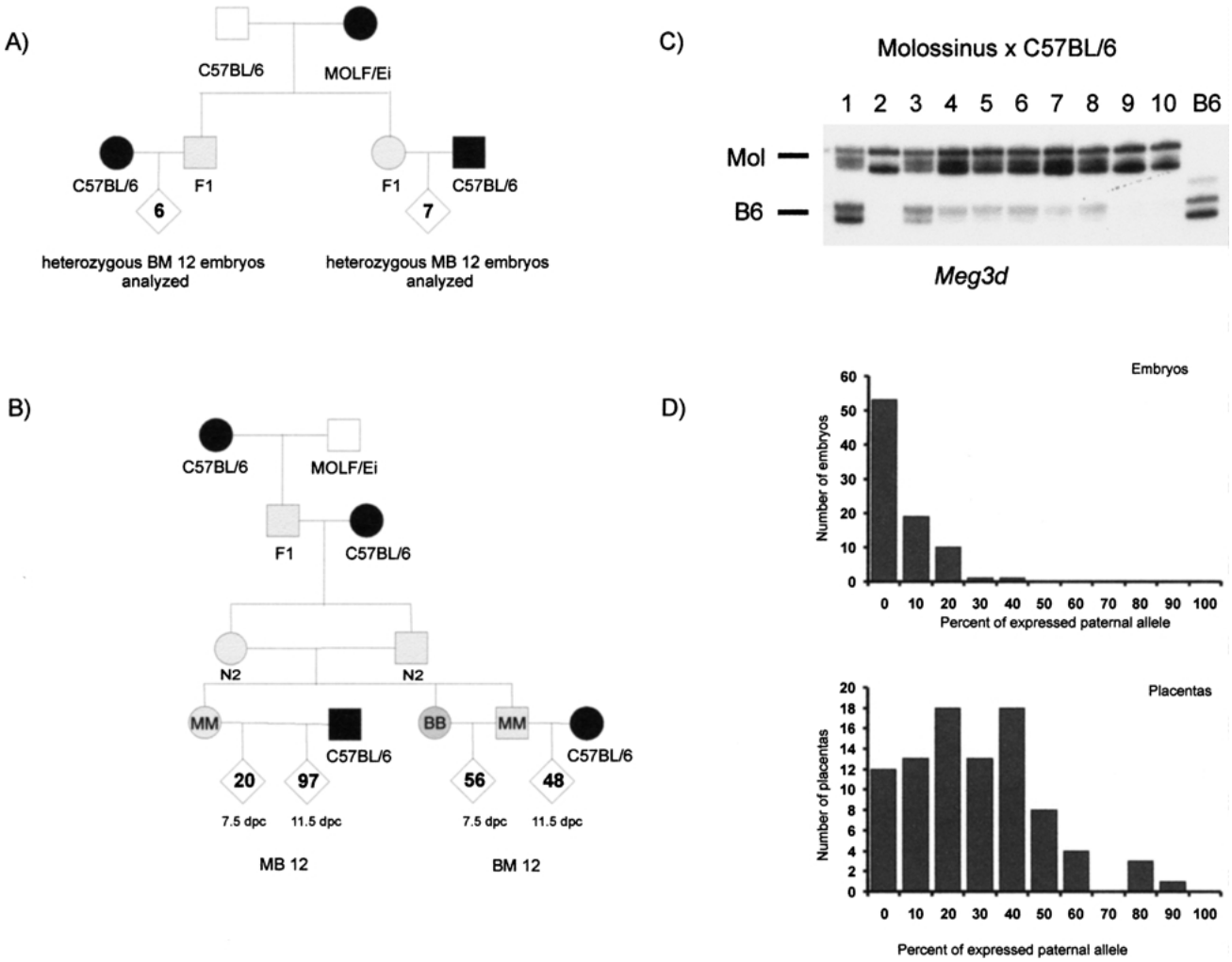

Fig. 2. Expression analysis of the *Meg3/Gtl2* gene in 7.5- and 11.5-d.p.c. embryos and placentas. **A** and **B**. Crosses used to obtain 7.5- and 11.5-d.p.c. embryos for analysis. The diamonds represent embryos. The number in the diamond corresponds to the number of embryos, for which expression analysis of *Meg3/Gtl2* was done. **C.** RFLP analysis of the RT-PCR products synthesized using primers for exons 9 and 10 of *Meg3/Gtl2* gene. The PCR products were cut with *Bsr*DI that recognizes an SNP in exon 10 of the C57BL/6 allele. **D**. Distribution of 11.5-d.p.c. MB12 embryos (upper panel) and placentas (lower panel) with different proportions of the paternal *Meg3d* allele expressed.

the embryos (Fig. 2C). To determine whether relaxation of imprinting of *Meg3/Gtl2* observed in 7.5 dpc MB embryos was associated with the embryo proper or the extraembryonic tissues, we generated 11.5 dpc embryos. Embryos and placentas were analyzed separately. Most of the placentas had biallelic expression of *Meg3/Gtl2*. The paternal allele was also expressed in about 30% of the embryos (Fig. 2D).

As with *Meg3/Gtl2*, imprinting of *Dlk1 was* maintained in the BM12 cross, but not in the MB12 cross. All embryos and placentas in the MB12 cross had partial relaxation of imprinting of the maternal *Dlk1* allele. Between 12% and 50% of the total mRNA detected by PCR with primers for exons 1 and 4 (Fig. 3A, B) or 4 and 5 (data not shown) of *Dlk1* was transcribed from the maternal *molossinus* allele. In contrast, in the reciprocal BM cross, 100% of the *Dlk1* message was expressed from the paternal allele. Since the parental origin of the expressed alleles was determined by RFLP analysis, possible experimental errors could arise from incomplete restriction endonuclease digestion of the RT-PCR product. To rule out such an error, the RT-PCR products were cloned. Individual clones were analyzed by restriction analysis and sequencing. The results of the analysis confirmed that a proportion of *Dlk1* transcripts originated from the maternal *molossinus* alleles in the MB12 cross (Table 2).

Some of the embryos investigated were heterozygous for the imprinted *HI9* gene (Chr 7 distal imprinted region). To determine whether the relaxation of imprinting affected the whole genome or was

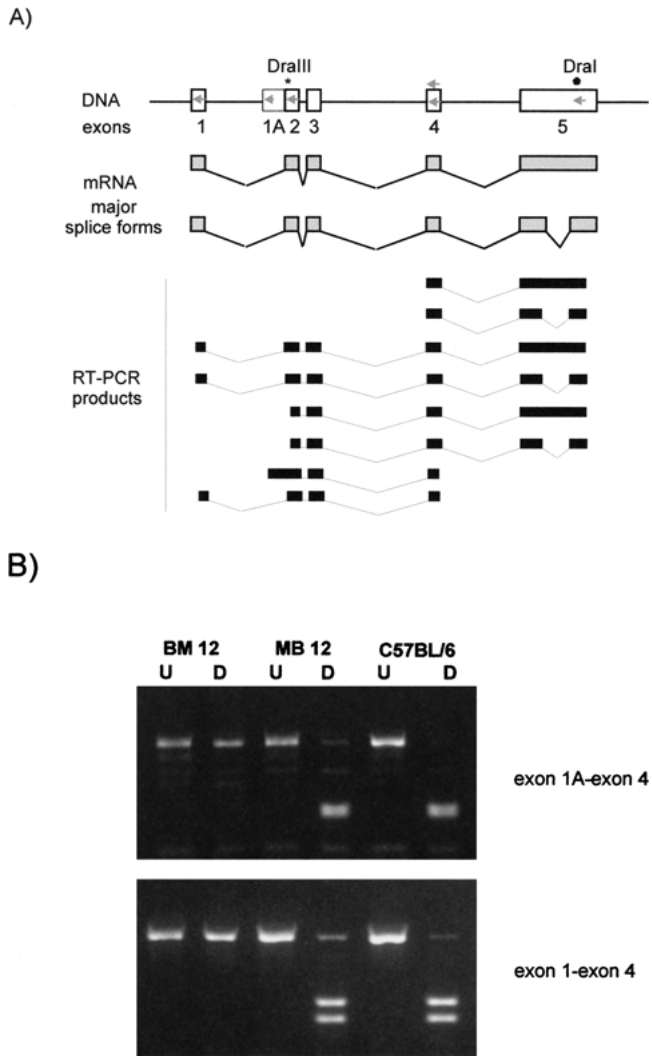

Fig. 3. *Dlk1* expression analysis. **A**. Exon-intron structure of the *Dlk1* gene and splice variants detected using RT-PCR and different combinations of primers. Locations of primers are denoted as arrows. **B**. RT-PCR and RFLP analysis of *Dlk1* expression. The upper panel represents the assay for the RT-PCR product detected by RT-PCR with exon 1A-exon 4 primers. The lower panel represents the assay for the RT-PCR product detected by RT-PCR with exon 1-exon 4 primers. In both cases, the PCR products were digested with the *Dra*III enzyme that recognizes an SNP in the 2nd exon of *Dlk1.*

confined to the Chr 12 imprinted region, we assayed the expression of the *H19* gene in the MB12 embryos. None of the 15 embryos and placentas analyzed had biallelic expression of *HI9* (data not shown). This implies that there is no global defect of genomic imprinting in this cross.

The observed asymmetry of imprinting in the Chr 12 distal region between the BM12 and MB12 crosses could be explained (i) by loss of ability to correctly interpret imprinting marks in intersubspecific crosses, (ii) by monoallelic expression of particular *Meg3*/*Gtl2* and/or *Dlk1* splice forms from the C57BL/6 or MOLF/Ei chromosome respectively, (iii) by a parent-of-origin-dependent relaxing effect on imprinting produced by *cis*- or *trans*-acting factor(s) encoded by the MOLF/Ei or C57BL/6 genome.

Relaxation of imprinting affects specific splice forms. To discriminate among these three possibilities, we first analyzed the expression of different splice forms of *Meg3*/*Gtl2* and *Dlk1* genes by RT-PCR, using different combination of primers.

Meg3/Glt2. *Meg3*/*Gtl2* has multiple splice variants (Miyoshi et al. 2000; Schuster-Gossler et al. 1998). All of them are believed to be transcribed from the maternal chromosome (Miyoshi et al. 2000; Schmidt et al. 2000; Wylie et al. 2000). To determine whether imprinting relaxation affected all splice variants of *Meg3*/*Gtl2*, we performed RT-PCR with different combinations of PCR primers and cloned the PCR products. Then the clones were analyzed by restriction enzyme digestion and sequencing (Fig. 4 and Table. 3). We found that relaxation of imprinting of *Meg3*/*Gtl2* was splice-variant specific. All the RT-PCR products that were amplified using primers for exons 1 and 3, 1 and 4, 3 and 4, and 3 and 10 were derived from the maternal alleles (Fig. 4 a–d; Table 3). Primers for exons 5 and 10, and 4 and 10 amplified messages from both parental alleles. Splice variants that did not contain exon 9 were expressed mostly from the paternal C57BL/6 allele (forms j–m), whereas the splice forms e, f, and g originated from the maternal allele only (Table 3).

To test the possibility that our RT-PCR assays were detecting putative antisense transcript, we did reverse transcription in a strand-specific manner. PCR with primers for exons 4 and 10 (Meg3 4F and Meg3 10R) was performed. If antisense transcripts containing exons 4 and 10 were present in the placenta, the as-RT-PCR assays would produce bands. However, PCR products were detected only with the sense RT reaction. Therefore, if there is a gene in this locus that is transcribed in an antisense direction with regard to the *Meg3*/*Gtl2* and *Dlk1* genes, we could not detect it with this set of primers.

Expression of *Meg3*/*Gtl2* was examined in 97 11.5-dpc embryos. Three of them showed signs of delayed development, and one of these had biallelic expression of the *Meg3p* transcript, which suggested that it was the only case of loss of imprinting of all *Meg3*/*Gtl2* forms.

Dlk1 has several splice forms (Smas et al. 1994). The two major forms that we detected in embryos contain exons 1, 2, 3, 4 and alternatively spliced exon 5. The search for mouse ESTs corresponding to

Table 2. Parental origin of the major splice forms of *Dlk1* in 11.5-d.p.c. embryos

		Cross/parental origin			
		MB12		*BM12*	
RT-PCR	*Splice form*	*Paternal*	*Maternal*	*Paternal*	*Maternal*
ex1-ex5	Large	12	1	7	0
	Small	4	0	7	0
ex2-ex5	Large	8	4	nt	nt
	Small	4	0	nt	nt
ex4-ex5	Large	0	7	3	0
	Small	6	2	8	0

The numbers in the cells represent the number of RT-PCR derived clones that contained inserts corresponding to particular splice form of one or the other parental origin. Large form refers to the *Dlk1* mRNA with unspliced exon 5; small form refers to the *Dlk1* mRNA in which a 222-bp sequence from exon 5 is spliced out. Nt, not tested.

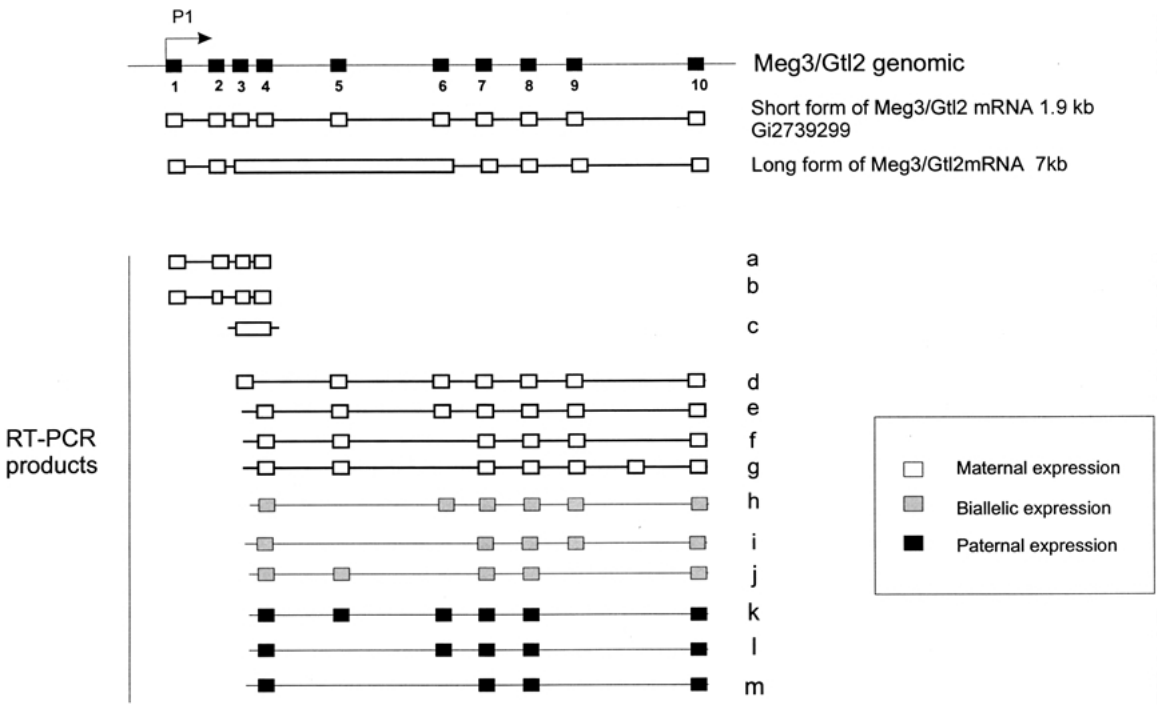

Fig. 4. Exon-intron structure of the *Meg3/Gtl2* gene and splice variants detected with a RT-PCR and different combinations of primers.

the *Dlk1* gene identified another form that does not contain exon 1, but contains the 3′ part of intron 1 (we refer to it as exon 1A) and is expressed in embryos (GenBank, Gi 1744491; Fig. 3). This mRNA variant was expressed exclusively from the paternal *Dlk1* allele (Fig. 3B). Parental origin of *Dlk1* transcripts in 11.5-d.p.c. embryos was assayed with primers for exons 1 and 5, and 2 and 5 (Fig. 3, Table 2). The PCR products were cloned, and the allelic origin of each clone was determined by RFLP analysis with *Dra*I and *Dra*III restriction endonucleases. Among the 28 clones analyzed, 5 were derived from the maternal *Dlk1* allele. All of these maternal clones contained the unspliced exon 5 (Table 2). The analysis of the PCR products synthesized by using exon 4 and 5 primers showed predominance of the maternal *molossinus* alleles (Table 2). Thus, relaxation of imprinting of *Dlk1* shows a splice-form-specific bias similar to the relaxation of imprinting of *Meg3/Gtl2*. However, at this time the data are insufficient to determine whether imprinting relaxation of *Dlk1* is associated with alternative promoter usage.

To determine whether relaxation of imprinting of the *Meg3/Gtl2-Dlk1* region was unique to our crosses, we assayed the expression of these genes in embryos from an intraspecific cross between two *M. domesticus* strains: C57BL/6 and NOD/LtJ. Ex-

Table 3. Alternative splicing and parental origin of the *Meg3/Gtl2* transcripts determined by RT-PCR

				MB12 cross		*BM12 cross*	
Form	*Primers*	*Exons*	*Source*	*Proportion of clones*	*Parental origin*	*Proportion of clones*	*Parental origin*
a	Meg3 1F-4R	1-2-2a-3-3b-4	fetus	2/4	maternal		nt
			placenta	5/9	maternal	2/3	maternal
b	Meg3 1F-4R	1-2-3-3b-4	fetus	2/4	maternal		nt
			placenta	4/9	maternal	1/3	maternal
e	Meg3 4F-10R	4-5-6-7-8-9-10	fetus	1/13	maternal	6/17	maternal
			placenta	5/23	maternal	11/25	maternal
			adult muscle	1/5	maternal		nt
f	Meg3 4F-10R	4-5—7-8-9-10	fetus	11/13	maternal	10/17	maternal
			placenta	1/23	maternal	3/25	maternal
			adult muscle	2/5	maternal		nt
g	Meg3 4F-10R	4-5—7-8-9-9a-10	adult muscle	2/5	maternal		nt
h	Meg3 4F-10R	4–6-7-8-9-10	fetus	1/13	maternal	1/17	maternal
			placenta	9/23	biallelic	7/25	maternal
i	Meg3 4F-10R	4—7-8-9-10	placenta	4/23	biallelic	1/25	maternal
j	Meg3 4F-10R	4-5–7-8—10	fetus	1/13	paternal		
			placenta	1/23	paternal	1/25	maternal
l	Meg3 4F-10R	4–6-7-8—10	placenta	1/23	paternal		
k	Meg3 4F-10R	4-5-6-7-8—10	placenta	1/23	paternal		
m	Meg3 4F-10R	4—7-8—10	placenta	1/23	paternal		

Different number of clones were analyzed from fetuses and placentas from the MB12 and BM12 crosses. For example, 13 clones from MB 12 fetuses and 23 clones from MB12 placentas derived from PCR using primers 4 and 10 were analyzed. ''Proportion of clones'' column shows the number of clones corresponding to a particular splice variant in the numerator and the total number of clones analyzed from a particular source in the denominator. As control, adult muscle from MB12 animal was used.

pression of both imprinted genes, the *Meg3d* and *Dlk1* gene in F_1 (NOD/LtJ × C57BL/6) embryos and placentas, was fully biallelic, suggesting that imprinting of these genes was not properly maintained or recognized in this cross. Furthermore, the *Meg3p* mRNA containing exons 1–4 of the *Meg3/Gtl2* gene was expressed from the maternal allele as in the MB12 cross. Again, as in the BM12 cross, imprinting of both genes was maintained in the (C57BL/6 × NOD/LtJ) F_1 embryos and placentas. Overall, the expression patterns in these crosses were very similar to those observed in the crosses with the MOLF/Ei strain. Thus, we identified two mouse strains, MOLF/Ei and NOD/LtJ, that when crossed to C57BL/6 mice produce offspring with imprinting relaxation of *Meg3d* and *Dlk1*.

Modifiers of Meg3 and Dlk1 imprinting in embryos. To test the hypothesis that relaxation of imprinting in the MB12 cross resulted from the action of unlinked modifiers, we generated a new set of BM12 embryos. Fifty-six 7.5-d.p.c. embryos were derived from matings between nine ($N_2 \times N_2$) females homozygous for the C57BL/6 allele at the *Meg3/Gtl2-Dlk1* locus and ($N_2 \times N_2$) males homozygous for the MOLF/Ei alleles at the same locus (Fig. 2B). If relaxation of imprinting (biallelic expression) of *Meg3d* and *Dlk1* were due to the effect of unlinked factors encoded by the MOLF/Ei genome, for example, it would be observed in a proportion of these embryos. However, none of these 56 embryos showed imprinting relaxation of *Meg3/Gtl2* or *Dlk1*, suggesting that *cis*-acting rather than *trans*-acting factors are responsible for this phenomenon.

In N_{10} embryos derived from a cross between a (C57BL/6 × MOLF/Ei) N_9 female and C57BL/6 male, both genes, *Meg3d* and *Dlk1*, showed biallelic expression patterns similar to those observed in the MB12 embryos. Since N_{10} embryos are expected to have very little contribution from the MOLF/Ei genome, it is unlikely that a MOLF/Ei trans-acting modifier causes biallelic expression.

Discussion

Alternative splicing of the Meg3 and Dlk1 genes. Expression analysis in oocytes and preimplantation embryos showed that the 5′ region of the *Meg3/Gtl2* gene was not transcribed, but the 3′ region was. Furthermore, a study of expression of *Meg3/Gtl2* and *Dlk1* in postimplantation embryos revealed a complex, cross-dependent pattern of expression. In crosses where the mother carried *Meg3/Gtl2* and *Dlk1* alleles from C57BL/6 and the father carried molossinus alleles (BM12 cross), both genes were expressed in a parent-of-origin-dependent fashion. In the reciprocal crosses (MB12), the expression of *Meg3d* and *Dlk1* was partially or com-

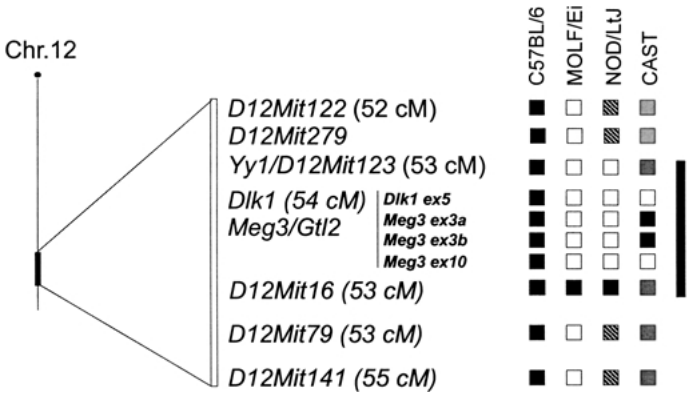

Fig. 5. Mapping the *cis*-acting modifier of Chr 12 imprinting. The order of marker loci is adopted from the genomic superconting Mm12_WIFeb01_237. SNPs from the *Dlk1* and *Meg3/Gtl2* exons were also used for genotyping purposes. The squares on the right represent the genotype for a particular marker locus in distal Chr 12 region.

pletely biallelic. The proximal form *Meg3p* was biallelically expressed in 1 of 97 11.5-d.p.c. embryos. The distal form *Meg3d* was biallelically expressed in about 30% of embryos and in 86% of the placentas. *Dlk1* was biallelically expressed in all the embryos and placentas analyzed, with about 50–80% of the transcripts derived from the paternal chromosome. These data led us to the following conclusions. First, the *Meg3*/*Gtl2* gene has at least one additional promoter. Second, imprinting of *Meg3*/*Gtl2* and *Dlk1* in embryos may not be strictly coordinated.

In the MB12 embryos and placentas, several splice variants containing the proximal and/or the distal part of *Meg3* were expressed only from the maternal allele, suggesting that imprinting was not completely lost in this cross, but rather bypassed or not recognized in a proportion of embryos. The paternal-specific splice forms of *Meg3d* that lacked exon 9 were rarely found in the placentas from the BM12 cross in which imprinting was maintained. Therefore, it is possible that the exon 9-minus splice forms are expressed only from the C57BL/6 alleles in the placenta, i.e., monoallelically in a strain-of-origin-specific fashion. Our data show that apparent relaxation of imprinting of *Meg3*/G*tl2* in the MB12 embryos and placentas results from change in alternative splicing and perhaps from promoter switching that leads to biallelic expression of the *Meg3d* form.

Expression of three major forms of *Dlk1* mRNA was assayed in our study. The large form that contains exons 1–4 and the unspliced exon 5 showed biallelic expression more often. However, it is not clear whether *Dlk1* transcripts that are expressed from the maternal allele use an alternative promoter or alternative 3′ end. Our findings are consistent with other reports of splice-form-specific imprinting in human and mouse genes. The insulin-like growth factor 2 (*IGF2*/*Igf2*) gene, for example, has four promoters, three of which direct expression of *IGF2*/*Igf2* from the paternal allele. The most proximal promoter, P1, which is active in placenta and liver, directs biallelic expression of *IGF2*/*Igf2*. It has been shown that apparent relaxation of imprinting (biallelic expression) of human *IGF2* associated with carcinogenesis or aging results from promoter switching (Issa et al. 1996; Kim et al. 2002).

Alternative splice forms of the human gene for growth factor receptor-bound protein 10 (*GRB10*) may be expressed from the maternal, paternal, or both parental alleles in a tissue-specific manner (Blagitko et al. 2000). A complex pattern of parent-of-origin and promoter-dependent expression of the guanine nucleotide-binding protein G-s, alpha subunit, (GNAS/*Gnas*) locus in humans and mice strongly supports the importance of alternative promoter usage and alternative splicing for imprinted genes (Wroe et al. 2000). Two other human imprinted genes, *H19* and *PEG1*/*MEST*, show partial relaxation of imprinting that is splice-form specific (Lin et al. 1999; Nakabayashi et al. 2002). Furthermore, gain of imprinting (parental-origin-dependent expression) resulting from a splice-site mutation in the retinoblastoma tumor-suppressor gene (RB1) in two human families (Klutz et al. 2002) raises the question whether alternative splicing is regulated by genomic imprinting and deserves special attention in imprinting studies.

Strain-specific modifier of imprinting. The fact that none of the 56 embryos from the $(N_2 \times N_2)$ females that were homozygous for the C57BL/6 alleles of *Meg3* had biallelic expression of *Meg3d* or *Dlk1* suggests that the observed relaxation of imprinting resulted from an effect of a *cis*-acting modifier. This conclusion is supported by the biallelic expression of *Meg3d* and *Dlk1* in N_{10} embryos derived from a cross between a (C57BL/6 × MOLF/Ei) N_9 female and C57BL/6 male and in (NOD/LtJ × C57BL/6) F_1 embryos.

Comparison of the Chr 12 distal regions from C57BL/6, NOD/LtJ, and MOLF/Ei showed that the NOD/LtJ and MOLF/Ei strains shared about a 1- to

2-cM region that was highly similar between them, but different from other strains (Fig. 5). The physical distance, based on the data from the mouse genomic supercontig Mm12_WIFeb01_237 encompassing this region, is estimated to be a maximum of 1.5 Mb. Therefore, it is likely that this 1.5-Mb region contains the *cis*-acting element(s) that is responsible for imprinting relaxation. It is conceivable that this 1.5-Mb region harbors a gene for a maternally expressed factor that is required for correct imprinting or correct recognition of imprinting marks of the *Dlk1* and *Meg3d* genes in embryos and placentas. Embryos from crosses between another strain of mice, *Mus musculus castaneus* (CAST), and C57BL/6 have been reported to have normal imprinting of the *Dlk1* gene (Schmidt et al. 2000). The data of Schmidt et al. argue against the possibility that relaxation of imprinting was caused by the C57BL/6 genome. Moreover, although the CAST mice have two SNPs similar to the MOLF/Ei and NOD strains in the 1.5-Mb candidate region, they have an overall different haplotype (Fig. 5). These observations also support the existence of a *cis*-acting modifier of imprinting in the vicinity of the *Meg3d*/*Gtl2-Dlk1* locus.

Relaxation of imprinting and embryo viability. The *Dlk1* gene (also known as Pref-1, FA1, pG2, and ZOG) encodes a protein that is implicated in the Notch-signaling, prenatal and postnatal growth and development (Moon et al. 2002). Less is known about the *Meg3*/*Gtl2* gene. It is believed that *Meg3*/*Gtl2* is a non-coding mRNA that may have regulatory functions. Uniparental disomies for the distal part of mouse Chr 12 cause late fetal and postnatal lethality (Georgiades et al. 2000), suggesting that distal Chr 12 harbors genes whose correct imprinting is necessary for embryo viability. If biallelic expression *of Meg3d* and *Dlk1* in the placenta or in embryos had a damaging effect on development, one would expect that the MB12 fetuses would be less viable than BM12 fetuses, and, therefore, the mean litter size of the $F_1 \times$ C57BL/6 backcross would be smaller than the mean litter size of the C57BL/6 $\times$ F_1 backcross. We recorded the sizes of litters from backcrosses between C57BL/6 and $N_{(2-9)}$ mice heterozygous for the Chr 12 distal region. The mean litter sizes in both backcrosses were similar: 8.1 and 8.0 in C57BL/6 $\times$ $N_{(2-9)}$ and $N_{(2-9)} \times$ C57BL/6, respectively. Therefore, relaxation of *Meg3d* and *Dlk1* imprinting in embryos and placentas does not have a major effect on fetal survival. In contrast, the only embryo that had biallelic expression of the *Meg3p* mRNA was not viable. Our data imply that lethal effects observed in fetuses with uniparental disomy 12 (UPD12) and grandparental origin-dependent, transmission-ratio distortion in Chr 12 are not likely to be caused by biallelic expression of *Dlk1* or the *Meg3d* genes. An important caveat here is that severity of phenotypes may be modulated by genetic background, and, hence, the consequences of biallelic expression in other crosses may be different.

A mouse model for polymorphic imprinting. Data collected in the human population reflect the level of variation of imprinting and its relevance to pathology (Xu et al. 1997; Wilkin et al. 2000), but are insufficient to elucidate the mechanisms underlying such variation. However, emerging evidence suggests that polymorphic imprinting of *IGF2R* and *WT1* depends upon *cis*-acting elements. The imprinting status of the human *IGF2R* gene in kidney and Wilms tumors depends upon the genotype at the *IGF2R* locus and most likely is controlled by a *cis*-acting modifier that is closely linked to *IGF2R* (Xu et al. 1997). This may also be true for the human gene *WT1*.

In mice, the genetic background influence on imprinting has been documented for the *Kvlqt1* gene (Jiang et al. 1998) and for mutations involving imprinted loci (Forejt and Gregorova 1992; Schuster-Gossler et al. 1996; Rogers et al. 1997). This is, perhaps, the phenomenon closest to polymorphic imprinting in humans. Cross-dependent imprinting relaxation has been reported for reciprocal crosses between two species of the deer mouse *Peromyscus* (Vrana et al. 1998). Most of the examined, paternally expressed genes were biallelically expressed in one cross, but imprinted in the other. Vrana et al. explained this asymmetry of imprinting by inability to correctly recognize the imprinting signals derived from different species. Despite the obvious parallels between our crosses and the *Peromyscus* hybrids, there are also significant differences. The *Peromyscus* hybrids had major developmental anomalies that were not observed in our crosses. Moreover, curiously enough, the *Dlk1*, which is biallelically expressed in our crosses, maintains its imprinting in the *Peromyscus* crosses (Schmidt et al. 2000). More importantly, in contrast to global imprinting relaxation in the *Peromyscus* interspecific crosses, imprinting variation in our crosses appears to be confined to Chr 12 imprinted region. We believe that our mouse model gives an opportunity to study the genetic mechanism of epigenetic variation in the absence of selective pressure that may be imposed by developmental anomalies.

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