RESEARCH COMMUNICATION

Bidirectional action of the *Igf2r* imprint control element on upstream and downstream imprinted genes

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Imprinting of the maternally-expressed *Igf2r* gene is controlled by an intronic imprint control element (ICE) known as Region2 that contains the promoter of the noncoding *Air* RNA, whose transcript overlaps the silenced paternal *Igf2r* promoter in an antisense orientation. Two novel imprinted genes, *Slc22a2* and *Slc22a3* are described here that lie 110 and 155 kb 3' to *Igf2r* and that are not overlapped by the *Air* transcript but are regulated by the *Igf2r*-ICE, as previously shown for *Igf2r*. These results identify a new cluster of imprinted genes whose repression by the bidirectional action of the Region2-ICE is independent of transcript overlap by the *Air* RNA.

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Cis-acting epigenetic mechanisms that regulate gene expression are of critical importance in understanding how gene regulation is controlled in development and disease, but are currently understood only at a basic level. Of the many mammalian systems currently used to investigate epigenetic mechanisms, parental-specific gene expression induced by genomic imprinting has the best potential to identify relevant DNA sequences and modifications, since both the active and silent parental alleles are retained in the same nuclear environment and can be directly compared (for reviews, see Reik and Walter 2001; Sleutels and Barlow 2001). At present, 50 imprinted genes have been identified with approximately equal numbers of maternally and paternally expressed examples (Beechey et al. 2000). This may represent a near complete collection, since the total number of imprinted genes, predicted to be in the range of 200, should now be reduced by 60%, following the reappraisal of the total number of genes in the mammalian genome (Claverie 2001).

[Key Words: *Slc22a3*-*Orct3*-*EMT*; *Slc22a2*-*Orct2*; *Igf2r*; genomic imprinting]

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A large body of evidence indicates that imprinted genes are regulated by *cis*-linked DNA imprint control elements (ICEs) that are epigenetically modified by an imprint in either the maternal germ line (for maternally imprinted genes) or paternal germ line (for paternally imprinted genes). Experimental data also indicate that DNA methylation is the imprint or at least is involved in maintaining the imprint for most but not all genes (Caspary et al. 1998; Jackson-Grusby et al. 2001). In contrast to early expectations, the data clearly show that imprints can act either as epigenetic activators to activate one allele of an imprinted gene, or as epigenetic repressors to silence one allele (Obata et al. 1998; Kato et al. 1999; Jackson-Grusby et al. 2001). The observation that imprinted genes are clustered within a chromosomal region has led to the suggestion that the ICE may also serve as an epigenetic domain regulator that controls the imprinted expression of more than one imprinted gene. To date, seven clusters have been identified in the mouse genome that are also conserved in the human genome (Beechey et al. 2000). The clusters show generally similar behavior whereby a majority of the clustered genes are expressed from one parental chromosome and only a minority from the reciprocal parental chromosome. Two well-known examples are the *Prader-Willi syndrome* (PWS) region (human 15q11–15 syntenic to mouse 7D) that contains 11 paternally expressed transcripts and one maternally expressed transcript, and the *Beckwith-Wiedemann syndrome* (BWS) region (human 11p15 syntenic to mouse 7C) that contains 13 maternally expressed transcripts and four paternally expressed transcripts (Beechey et al. 2000).

Genomic deletions in mice and humans have confirmed that imprinting of more than one gene can be regulated by an ICE, but these data also show that not all genes present in one cluster are regulated by the same element. For example, in the mouse 7C cluster homologous to the human BWS region, deletion of the *H19* ICE that lies close to the *H19* promoter causes reexpression of the silent allele of the downstream *H19* gene and two upstream genes, *Igf2* and *Ins2*, but does not affect imprinted expression of the more distal upstream *Mash2*, *Cdkn1*, and *Kcnq1* genes (Caspary et al. 1998; Thorvaldsen et al. 1998). Deletion of the mouse PWS ICE that maps to the *Snrpn* gene promoter shows a similar effect on multiple genes but, in contrast, leads to silencing of the active allele of flanking upstream and downstream imprinted genes (Yang et al. 1998). Similar regulation has been observed following spontaneous ICE deletions in the human PWS region that resulted in a complete loss of expression of at least five imprinted paternally expressed transcripts located across a 1.5 Mb domain (Ohta et al. 1999). In addition, an *in vitro* model system has shown that targeted deletion of the *LIT1* (*KCNQ1OT1*) promoter from a paternal copy of human chromosome 11 and subsequent analysis in a hamster monochromosomal hybrid cell results in reexpression of the silent allele of the upstream *CDKN1* and *SMS4* genes and of the downstream *KCNQ1* promoter, but did not affect the more distal *IGF2/H19* genes (Horike et al. 2000).

In contrast to the finding of clusters of imprinted genes, the mouse *Igf2r* gene that encodes an intracellular transport receptor has been proposed to be a solo maternally expressed imprinted gene (Schweifer et al. 1997;

Verhaagh et al. 1999). Paternal repression of the *Igf2r* promoter is regulated by a 3.7 kb ICE called Region2 (Wutz et al. 1997, 2001). Region2 is located within intron 2 of the *Igf2r* gene and contains a 1.5 kb maternally methylated CpG-island that is the promoter for an antisense RNA named *Air* that overlaps the *Igf2r* promoter. The *Air* RNA is specifically expressed from the paternal unmethylated ICE, but not from the maternal methylated ICE. The finding that the mature *Air* RNA is a 108 kb-long, nonspliced, repeat-rich, noncoding RNA has led to the proposal that this may not be a legitimate gene and instead may represent genomic RNA (Lyle et al. 2000). An additional transcript in this region corresponding to the *Mas* locus and previously described as imprinted has now been shown to be part of the *Air* transcript where it overlaps the 3' *Mas* exons (Lyle et al. 2000). No other imprinted genes have been identified on mouse chromosome 17, and analysis of mice with maternal or paternal disomy for proximal parts of this chromosome does not predict their existence (Beechey et al. 2000).

We recently identified a new gene, *Slc22a3* (also known as *Orct3/EMT*), whose promoter is located 155 kb from the 3' end of the *Igf2r* gene, which lies in close association with two related genes, *Slc22a2/Orct2* and *Slc22a1/Orct1* (Fig. 1; Verhaagh et al. 1999). The *Slc22a1*, *Slc22a2*, and *Slc22a3* genes all encode membrane-spanning transporter proteins that show a highly tissue-specific expression pattern in the embryo and adult, in contrast to the ubiquitous expression of the *Igf2r* gene (Verhaagh et al. 1999; Wutz et al. 2001). We show here by expression analysis that the genes *Slc22a2* and *Slc22a3* are imprinted and maternally expressed in the placenta. Imprinted expression of *Slc22a2* and *Slc22a3* shows tissue and temporal specificity, in contrast to the widespread imprinted expression of the *Igf2r* gene. Despite their contrasting gene expression and imprinted expression profiles, and despite the fact that they are not overlapped by the antisense *Air* transcript, targeted deletion of the Region2 ICE results in derepression of the paternal alleles of *Slc22a2* and *Slc22a3*, as shown previously for the *Igf2r* gene (Wutz et al. 2001). These results identify the presence of a new cluster of imprinted genes spanning more than 400 kb on proximal mouse chromosome 17, the majority of which show maternal-specific expression. Furthermore, the data show that the *Igf2r*-ICE generates a long-range effect that acts in a bidirectional manner to repress upstream and downstream genes.

Figure 1. Map of all known genes within 600 kb of the mouse *Igf2r* locus relative to centromere (cen) and telomere (tel). Region2 is shown as a black oval; genes are shown as white boxes which represent the locus size. Arrows and arrowheads indicate the direction of transcription when known.

Results and Discussion

The position of genes analyzed in this study is shown in Figure 1. The *Igf2r*, *Air*, *Slc22a2*, and *Slc22a3* gene loci span ~400 kb. The *Igf2r* promoter lies 30 kb downstream of Region2 and is overlapped by the *Air* RNA. The *Slc22a2* and *Slc22a3* gene promoters lie 170 kb and 210 kb upstream of Region2 and are not overlapped by *Air*. The T^{hp} (hairpin) mouse mutant used for the analyses below carries a 3 cM deletion that includes the entire region shown in Figure 1. Paternal inheritance of the T^{hp} allele (+/ T^{hp}) is viable, whereas maternal inheritance (the maternal allele is written on the left-hand side; $T^{hp}/+$) is lethal during 16.5–18.5 days post-conception (dpc) due to the absence of *Igf2r* expression. Parental allele-specific expression can be analyzed before the lethal period (Wutz et al. 2001).

Slc22a3 is a new imprinted gene linked to the Igf2r locus

Imprinted expression of *Slc22a3* was analyzed in placentas of embryos heterozygous for a paternally or maternally inherited T^{hp} allele. *Slc22a3* expression is restricted to placenta in development but shows widespread expression in adult organs (Verhaagh et al. 1999). Figure 2A shows that at 11.5 dpc, *Slc22a3* was absent in $T^{hp}/+$ placentas, whereas +/ T^{hp} placentas showed expression equal to wild-type placentas (+/+). Thus *Slc22a3* shows maternal-specific expression at 11.5 dpc. In contrast, at 15.5 dpc, expression is detected in both paternal and maternal T^{hp} heterozygotes, and thus *Slc22a3* is expressed from both parental alleles at this stage, as reported previously (Verhaagh et al. 1999). These results show that *Slc22a3* imprinted expression is restricted to an early time window in development, in contrast to the imprinted expression of *Igf2r* (Fig. 2A).

$T^{hp}/+$ placentas show increased growth from 13.5 dpc (Ludwig et al. 1996). To exclude the possibility that absence of *Igf2r* leads directly to loss of *Slc22a3* expression, we also analyzed imprinted expression in placentas of embryos heterozygous for a targeted deletion of the *Slc22a3* gene. Mice with a null mutation of *Slc22a3* ($\Delta 3$) show impaired transport of the neurotoxin MPP⁺ in placenta and adult heart, but lack any obvious biological or cytological phenotype and are viable and fertile (Zwart et al. 2001). Figure 2B shows that *Slc22a3* expression is imprinted at 11.5 dpc (cf. $\Delta 3/+$ with +/ $\Delta 3$) in placentas with a wild-type *Igf2r* allele. The $\Delta 3$ mutant mice were further used to test imprinted expression in adult organs. In Figure 2C, adult brain, heart, and skeletal muscle from maternal ($\Delta 3/+$) and paternal (+/ $\Delta 3$) heterozygous knockout mice show equal levels of expression, equivalent to 50% of wild-type expression (quantitated by PhosphorImager; data not shown), showing an absence of imprinting in adult tissues. These results show that *Slc22a3* is imprinted in a tissue-specific fashion in placenta, and in a manner temporally restricted to early postimplantation stages. Several examples of placental- or extraembryonic-specific imprinted expression have been described: for example, the human *WT1* gene (Jinno et al. 1994) and the mouse genes *Ins2*, *Tssc4*, *Obph1*, and *Kcnq1* (Deltour et al. 1995; Caspari et al. 1998; Engemann et al. 2000; Paulsen et al. 2000). Temporal changes of imprinted expression in placenta have also been reported for *Mash2* and *Ins2* (Deltour et al. 1995; Caspari et al. 1998).

Figure 2. The *Slc22a3* gene is imprinted in placenta. (A) Blot of placental RNA from $T^{hp}/+$, $+/T^{hp}$, and $+/+$ offspring isolated at 11.5 and 15.5 dpc. The two ($T^{hp}/+$) lanes are independent samples. The same blot was hybridized with *Igf2r* and *Pai1*. (B) RNA blot of 11.5-dpc placentas isolated from wild-type mice ($+/+$) and mice with a maternally ($\Delta a3/+$) or paternally ($+/ \Delta a3$) inherited *Slc22a3* targeted allele. (C) RNA blot of adult heart, skeletal muscle, and brain RNA isolated from wild-type mice ($+/+$) and mice with a maternally ($\Delta a3/+$) or paternally ($+/ \Delta a3$) inherited *Slc22a3* targeted allele. (D) Methylation analysis of the *Slc22a3* CpG-island. A 4.3-kb genomic *Bam*HI fragment that contains *Slc22a3* exon 1 (gray box) embedded in a CpG-island is shown (top) with the positions of two methyl-sensitive *Bss*HIII restriction enzyme sites. The complete 4.3 kb *Bam*HI fragment was used as a probe on a DNA blot of genomic DNA of 11.5-dpc and 15.5-dpc wild-type embryos and placentas digested with *Bam*HI (–) or *Bam*HI and *Bss*HIII (+). Only a 3.3-kb genomic fragment was detected upon digestion with *Bss*HIII, showing that the *Slc22a3* CpG-island is unmethylated. (E) RNA blot of 13.5-dpc placental RNAs isolated from wild-type mice ($+/+$) with a maternally ($\Delta a3/+$) or paternally ($+/ \Delta a3$) inherited targeted allele, and homozygous targeted mice ($\Delta a3/\Delta a3$). The 2.8-kb *Slc22a3* RNA is specific for the targeted allele.

The *Slc22a3* promoter is embedded in a CpG-island (Verhaagh et al. 1999; GenBank accession no. AF078748). To investigate whether the *Slc22a3* CpG-island is methylated in a manner that correlates with imprinted expression in placenta, we performed a methylation-sensitive DNA blot analysis. Complete digestion of two *Bss*HIII sites (Fig. 2D) and *Sac*II and *Hpa*II sites (present at several sites in the CpG-island promoter; data not

shown) was observed in both 11.5 dpc and 15.5 dpc embryonic, placental, and adult tail genomic DNA. Thus, the *Slc22a3* CpG-island is nonmethylated on both alleles in expressing and nonexpressing tissues, irrespective of the imprinted expression of *Slc22a3* in early placenta.

Targeted inactivation of *Slc22a3* removed a 4.3-kb fragment containing the complete CpG-island and exon 1. This deletion abolished *Slc22a3* gene expression in $\Delta a3/\Delta a3$ adult tissues and in 11.5-dpc placentas (Fig. 2B; Zwart et al. 2001). However, in placentas from 12.5 dpc onward, a 2.8-kb aberrant, nontranslated *Slc22a3* transcript that uses exons 3–11 was identified in mice carrying the targeted allele (Zwart et al. 2001). Because the original *Slc22a3* gene promoter (P1) was deleted, an alternative promoter sequence (P2) must be employed to drive expression of the 2.8-kb transcript. Figure 2E shows that expression of the 2.8-kb transcript is specific to the targeted allele and is expressed only from the maternal allele in 13.5-dpc placenta (cf. lanes $\Delta a3/+$ and $+/ \Delta a3$). Note that the $\Delta a3/+$ lane shows maternal expression of the 2.8-kb transcript and paternal expression of the 3.5-kb transcript, indicating the partial depression of the P1 promoter at this stage. The aberrant 2.8-kb transcript is expressed one day later than the 3.5-kb wild-type mRNA, and imprinted expression persists until 15.5 dpc in most but not all offspring, indicating a temporal difference between the P1 and P2 *Slc22a3* promoters (Fig. 2; data not shown).

The flanking *Slc22a2* gene is imprinted in placenta

The finding that both the *Slc22a3* wild-type P1 and the aberrant P2 promoter were imprinted in early placenta prompted us to reexamine imprinted expression of other genes flanking *Igf2r*. We had shown previously in 14.5-dpc embryos that the *Plg*, *Tcp-1*, and *Sod2* genes (Fig. 1) were not imprinted; however, placental tissues were not tested (Barlow et al. 1991). We retested these genes and found no imprinted expression in 11.5-dpc embryos, nor in 11.5-dpc or 15.5-dpc placentas (data not shown). Other genes in this region include the *Mas1* gene, initially described as imprinted in embryonic tissue, but later shown to be expressed solely in adult brain and testis and to lack any imprinted expression (Schweifer et al. 1997; Lyle et al. 2000), and the *Slc22a1* and *Slc22a2* genes that lie between *Slc22a3* and *Igf2r*. *Slc22a1* is not expressed during embryonic development, and expression in adults has been shown to be biallelic (Schweifer et al. 1997). *Slc22a2* expression had been reported previously only in adult kidney and brain (Mooslehner and Allen 1999). However, hybridization of wild-type placental RNA with an *Slc22a2* cDNA probe detects abundant expression (Fig. 3A). Analysis of embryos heterozygous for the T^{hp} allele shows that in both 11.5-dpc and 15.5-dpc placentas, *Slc22a2* was repressed on the paternal allele ($T^{hp}/+$), but showed normal expression from the maternal allele ($+/T^{hp}$). Imprinted maternal-specific *Slc22a2* expression was also confirmed at these two developmental ages in placenta of mice carrying a targeted *Slc22a2* null mutation that are wild-type for the *Igf2r* allele (H. Jonkers and A. Schinkel, unpubl.). Methylation analysis of the *Slc22a2* promoter region in 13.5-dpc placental DNA from $T^{hp}/+$ reciprocal crosses demonstrated an absence of parental-specific methylation at *Hpa*II sites (data not shown). Paternal expression of *Slc22a2* in later placental stages could not be examined in $T^{hp}/+$ offspring due to

Figure 3. *Slc22a2* is imprinted in placenta but not in adult tissue. (A) RNA blot of 11.5- and 15.5-dpc wild-type (+/+) and maternal T-hairpin ($T^{hp}/+$) and paternal T-hairpin (+/ T^{hp}) placentas. The blot was hybridized successively with *Slc22a2* and *Pai1*. (B) RNA blot of adult liver and kidney from maternal (T^{hp}/PWD) and paternal (PWD/T^{hp}) placentas. The blot was hybridized successively with *Igf2r*, *Slc22a2*, and *Actin*.

lethality at 16.5 dpc; however, adult mice can be obtained in crosses with the PWD *Mus m. musculus* subspecies (Forejt and Gregorova 1992). Figure 3B shows that while imprinting of *Igf2r* is maintained in adult kidney, the *Slc22a2* gene is expressed from the paternal allele. However, comparison with *Actin* indicates that expression of the paternal *Slc22a2* allele is reduced compared to the maternal allele.

In development, imprinting of *Slc22a2* and *Slc22a3* is restricted to placenta. Neither gene is expressed in embryonic tissues assessed by nonamplification techniques, although low levels of *Slc22a2* can be detected by RT-PCR in early embryos (R. Zwart, unpubl.; Mooslechner and Allen 1999; Verhaagh et al. 1999). *Slc22a3* shows a loss of imprinted expression in late placenta, contrasting with *Slc22a2*, which maintains imprinted expression at this stage. The *Igf2r* gene differs from *Slc22a2* and *Slc22a3* in that it shows imprinted expression in embryos that is maintained in adult tissues (Fig. 3B; Forejt and Gregorova 1992). Adult brain has been reported as showing a loss of *Igf2r* imprinting (Hu et al. 1999). However, whereas the paternal *Igf2r* allele is re-expressed to some extent in brain, it remains repressed compared to the maternal allele when analyzed using nonamplification techniques (Schweifer et al. 1997). Therefore, these three genes show differences in both the regulation of their imprinted expression and in their general expression patterns. The biological significance of *Slc22a2* and *Slc22a3* imprinting is not clear at this time, as neither gene has an essential developmental function in embryos or adults (Zwart et al. 2001; H. Jonker and A. Schinkel, pers. comm.). Taken together, these results identify a new cluster of imprinted genes in the proximal part of mouse chromosome 17.

Control of imprinted expression by Region2

The imprinted expression of the two *Slc22a3* P1 and P2 promoters and that of the *Slc22a2* gene resemble that of the *Igf2r* gene in that all transcripts show maternal-specific expression. The imprinted expression of *Igf2r* has

been shown, using targeted deletion in mice, to be controlled by the Region2 ICE that lies in intron 2 (Wutz et al. 2001). To investigate whether this ICE also regulates imprinted expression of the *Slc22a3* and *Slc22a2* genes, we performed gene expression analysis in placentas from double heterozygote crosses of T-hairpin (T^{hp}) and Region2 deletion ($\Delta R2$) mice (Fig. 4). $T^{hp}/+$ placentas showed no expression of the *Slc22a3*, *Slc22a2*, or *Igf2r* genes at 11.5 dpc, confirming the absence of expression from the paternal copy of these genes at this timepoint in this genetic background. When a Region2 deletion was introduced on the paternal allele ($T^{hp}/\Delta R2$), paternal expression of the three genes *Slc22a3* (P1 promoter), *Slc22a2*, and *Igf2r* was apparent to a level which approximates the maternal expression observed in the wild-type placentas (+/+). The *Slc22a3* P2 promoter was not tested, because the tight linkage (210 kb) would have required an excessive breeding program to place the *Slc22a3* P2 promoter *in cis* to the Region2 deletion. At 15.5 dpc, *Slc22a3* is expressed from the paternal allele in ($T^{hp}/+$) placenta, indicating that it has also lost its imprinted expression in placenta in this genetic background. Imprinted expression of the *Slc22a2* and *Igf2r* genes is maintained at this later timepoint in this genetic background, and is still dependent on the presence of Region2 (15.5 dpc, $T^{hp}/\Delta R2$).

Region2 is a bidirectional silencer

The data presented here show that the Region2 ICE is a bidirectional silencer on the paternal allele, able to simultaneously repress a cluster of genes in a 400-kb domain. The silencing activity of Region2 on the paternal chromosome correlates with absence of methylation and the presence of *Air* RNA. In contrast, expression of the *Igf2r/Slc22a2/Slc22a3* cluster on the maternal chromosome correlates with Region2 methylation and *Air* repression (Wutz et al. 1997). The presence of a methylation imprint on the active maternal allele indicates that interestingly, this allele is epigenetically activated by

Figure 4. The Region2-ICE regulates imprinting of *Slc22a2* and *Slc22a3*. RNA blot of 11.5 and 15.5 dpc wild-type (+/+), maternal T-hairpin ($T^{hp}/+$), paternal Region2 deletion (+/ $\Delta R2$), and maternal T-hairpin/paternal Region2 deletion double heterozygote ($T^{hp}/\Delta R2$) placentas. The blot was hybridized successively with *Igf2r*, *Slc22a2*, *Slc22a3*, and *Pai1*.

DNA methylation. This interpretation contrasts with results obtained in vitro based on demethylating agents (Hu et al. 1999), but is consistent with results obtained in vivo based on mouse mutants lacking the maintenance methyltransferase gene (Jackson-Grusby et al. 2001). A large number of imprinted genes have now been shown to be epigenetically activated by imprints (for review, see Sleutels and Barlow 2001).

The earlier demonstration that deletion of the Region2 ICE containing the *Air* promoter leads to derepression of the *Igf2r* promoter suggested several models to explain its action (Wutz et al. 1997). The expression-competition model proposed that competition for limiting transcription factors, or for the use of shared *cis*-acting elements, could prevent coexpression of the *Igf2r* and *Air* promoter *in cis*. However, the identification of four additional examples of imprinted protein-coding genes overlapped by antisense noncoding RNAs on their silent allele [*UBE3A-as* overlapping *UBE3A* (Rougeulle et al. 1998), *KCNQ1OT1* overlapping *KCNQ1* (Smilnich et al. 1999), *Nesp-as* overlapping *Nesp* (Wroe et al. 2000), and *Cop2-as* overlapping *Cop2* (Lee et al. 2000)] indicated a function for sense/antisense transcription, for example, by promoter occlusion or by the formation of double-stranded interfering RNA on one chromosome. The demonstration here of the bidirectional action of Region2 is a surprise, and although it does not directly exclude a function for the *Air* RNA, it does indicate that neither the antisense orientation nor the physical overlapping of coding/noncoding transcripts is necessary for repression, at least of *Slc22a2* and *Slc22a3* (see Fig. 1). While all three genes are regulated by the Region2 ICE, it remains possible that a dual mechanism operates for transcriptional silencing, and a role for the *Air* RNA in silencing *Igf2r* is not yet excluded. In vitro deletion of the antisense *KCNQ1OT1* promoter in a hamster/human hybrid cell line leads to derepression of upstream and downstream genes, indicating that it may also be a bidirectional silencer (Horike et al. 2000).

The recent demonstration that imprinting of the *Igf2/H19* genes is caused by the insulator CTCF protein binding to the unmethylated maternal allele at the *H19* ICE, thus preventing interaction between the *Igf2* promoter and enhancer, showed that repressed genes lie on one side of the ICE and indicates that repression is unidirectional (for review, see Thorvaldsen and Bartolomei 2000). It has also recently been proposed that that 'epigenetic regulation of access to shared enhancers' may be a common feature of imprinting mechanisms (Bell et al. 2001). Although enhancers for none of the genes studied here have been identified, the location of the *Slc22a2* and *Slc22a3* genes on the opposite side of the Region2 ICE from the *Igf2r* promoter shows the bidirectional action of Region2 and argues against the generality of the shared enhancer model. The data presented here are compatible with a model that the Region2 ICE has a long-range effect on the paternal allele leading to silencing of upstream and downstream susceptible gene promoters. The details of the silencing action are not yet known but may involve chromatin changes such as indicated by the observation that a 1 Mb domain around the *Igf2r* locus shows a parental-specific replication asynchrony whereby the paternal allele replicates before the maternal (Simon et al. 1999). Since the early replicating allele is predicted to indicate a less compact chromatin structure, this pattern reflects the paternal-specific expression

of *Air* and may provide a possible explanation as to why Region2 ICE fails to silence the paternal *Air* promoter.

The data presented here show the existence of a cluster of three imprinted and maternally expressed protein-coding genes on proximal mouse chromosome 17 that are silenced on the paternal chromosome by the unmethylated Region2 ICE. Although these results cannot directly exclude a role for *Air* expression, because the 3.7 kb deleted in the $\Delta R2$ allele included the *Air* promoter, they do show clearly that silencing mechanisms in genomic imprinting can act bidirectionally on several genes in a subchromosomal domain.

Materials and methods

Mice

All mouse strains were maintained on an *FVB/N* background. Genotyping as described: T^{hp} , $\Delta R2$ (Wutz et al. 1997), $\Delta a3$ (Zwart et al. 2001). Timed matings (vaginal plug at 0.5 dpc) were used to collect samples at different developmental stages.

Expression analysis

RNA was isolated using LiCl/urea and analyzed using standard formaldehyde gels and hybridization techniques. Probes: *Igf2r*, exon 3 to exon 6 RT-PCR fragment; *Slc22a2*, 626 bp RT-PCR fragment (bp 989–1605; AJ006036); *Slc22a3*, 2.7 kb *EcoRI* cDNA fragment detects the wild-type and aberrant transcript (bp 1–2766; AF078750); *Pai1*, 500 bp cDNA fragment (Zwart et al. 2001); *Plg*, 526 bp RT-PCR fragment (bp 2129–2655; NM008877); *Sod2*, 1-kb cDNA fragment, and *Tcp-1*, 0.8-kb cDNA fragment (Barlow et al. 1991).

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