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Imprinted silencing of *Slc22a2* and *Slc22a3* does not need transcriptional overlap between *Igf2r* and *Air*

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Silencing of the paternal allele of three imprinted genes (*Igf2r*, *Slc22a2* and *Slc22a3*) requires *cis* expression of the *Air* RNA that overlaps the promoter of one of them (*Igf2r*). *Air* is a non-coding RNA whose mode of action is unknown. We tested the role of the *Igf2r* promoter and the role of transcriptional overlap between *Igf2r* and *Air* in silencing in this cluster. We analyzed imprinted expression in mice in which the *Igf2r* promoter is replaced by a thymidine kinase promoter that preserves a transcription overlap with *Air*, and in mice with a deleted *Igf2r* promoter that lack any transcriptional overlap with *Air*. Imprinted silencing of *Air*, *Slc22a2* and *Slc22a3* is maintained by the replacement promoter and also in the absence of transcriptional overlap with *Air*. These results exclude a role for the *Igf2r* promoter and for transcriptional overlap between *Igf2r* and *Air* in silencing *Air*, *Slc22a2* and *Slc22a3*. Although these results do not completely exclude a role for a double-stranded RNA silencing mechanism, they do allow the possibility that the *Air* RNA has intrinsic *cis* silencing properties.

Keywords: genomic imprinting/*Igf2r*/non-coding RNA/*Slc22a2*/*Slc22a3*

Introduction

Mammalian genomic imprinting is an epigenetic gene regulatory mechanism that results in parental-specific gene expression of a small number of genes in diploid somatic cells (Beechey *et al*., 2001; Reik and Walter, 2001; Li, 2002; Sleutels and Barlow, 2002). Several features of the imprinting mechanism have been identified; however, it is not yet clear whether imprinting is regulated by a unique process or whether it is part of the general epigenetic apparatus used to regulate mammalian gene expression. Clustering and coordinate regulation is one feature imprinted genes share with non-imprinted genes (Engemann *et al*., 2000; Onyango *et al*., 2000), and it is now clear that many imprinted genes are functionally grouped such that imprinted expression of several genes is regulated by one long-range imprint control element (Thorvaldsen *et al*., 1998; Horike *et al*., 2000; Zwart *et al*., 2001; Fitzpatrick *et al*., 2002). The frequent occurrence of an imprinted non-coding RNA within an imprinted gene cluster has also been observed (Sleutels and Barlow, 2002); however, non-coding RNAs have also been identified in several 'normal' bi-allelic expressed genes such as *N-myc*, *BFGF*, *Hox11*, *RPS14* and *Kelch-like1* (Krystal *et al*., 1990; Tasheva and Roufa, 1995; Li *et al*., 1996; Potter and Branford, 1998; Benzow and Koob, 2002), and, in the case of the human β-globin cluster, a non-coding RNA has been linked to chromatin remodeling (Gribnau *et al*., 2000). Approximately one-quarter of imprinted transcripts are non-coding, and the majority is expressed from the parental chromosome that carries the silent allele of an imprinted protein-coding gene (Beechey *et al*., 2001; Sleutels and Barlow, 2002). This reciprocal parental expression pattern has been proposed as either the cause or the consequence of imprinted expression, and evidence exists to support both these proposals.

An example of where reciprocal expression of a non-coding RNA is proposed to occur as a consequence of imprinting is shown by the imprinted cluster on mouse chromosome 7 that contains two paternally expressed protein-coding genes (*Ins2* and *Igf2*) located 80 kb upstream of a maternally expressed non-coding RNA (*H19*). A methylation-sensitive insulator element known as the 'H19 DMR' is located between *Ins2/Igf2* and *H19* and controls their access to a common enhancer, located downstream of all three genes, that activates either *H19* or *Ins2/Igf2* (Hark *et al*., 2000; Szabo *et al*., 2000; Bell *et al*., 2001). On the paternal chromosome, the H19 DMR inherits a methylation imprint that inactivates its insulator function and allows the access of *Ins2/Igf2* to the enhancer, thus permitting their expression on this allele. On the maternal chromosome, the H19 DMR insulator is unmethylated and active, which blocks the access of *Ins2/Igf2* to the downstream enhancer. H19 gains access by default and thus shows maternal-specific expression (Schmidt *et al*., 1999; Thorvaldsen and Bartolomei, 2000). Thus, the maternal-specific expression of the non-coding *H19* RNA is a consequence of the imprinted expression of *Ins2/Igf2*.

In contrast, a direct silencing function for a non-coding RNA has been demonstrated in an imprinted gene cluster on mouse chromosome 17 that contains three maternally expressed protein-coding genes (*Igf2r*, *Slc22a2* and *Slc22a3*) and one paternally expressed non-coding RNA (*Air*). The *Air* promoter lies within intron 2 of *Igf2r* and expresses, on the paternal chromosome only, a non-coding RNA that overlaps the *Igf2r* promoter in an antisense orientation and extends 79 kb upstream (Lyle *et al*., 2000). The *Air* promoter is methylated and silent on the maternal chromosome. Thus, maternal expression of *Igf2r*, *Slc22a2* and *Slc22a3* correlates with methylation and silencing of the *Air* promoter *in cis*. *Igf2r* expression has previously

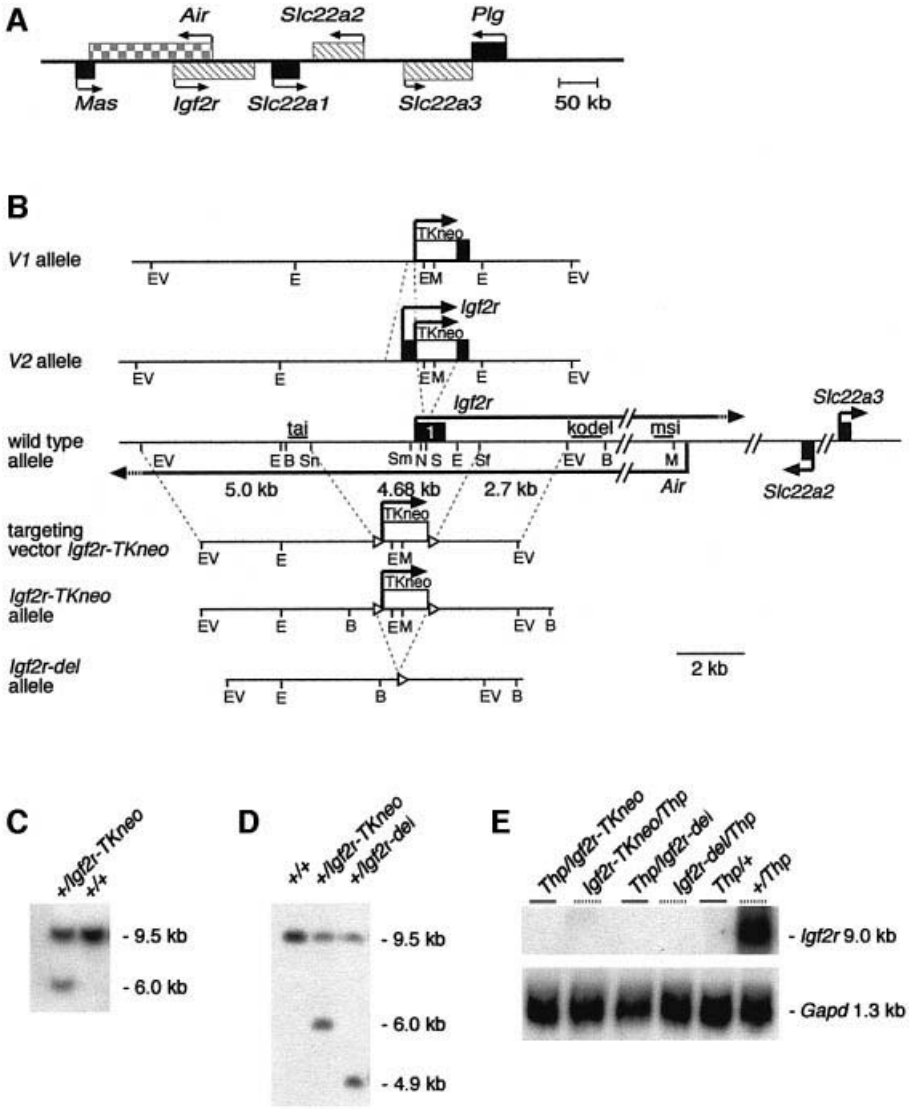


Fig. 1. Four *Igf2r* promoter replacement/deletion alleles. (**A**) A map of the *Igf2r*, *Air*, *Slc22a2* and *Slc22a3* imprinted cluster with arrows marking transcriptional orientation. Black box, genes with bi-allelic expression; gray cross-hatched box, imprinted protein-coding genes with maternal-specific expression; gray checked box, imprinted non-coding *Air* RNA with paternal-specific expression. (**B**) The *V1* and *V2* alleles in which, respectively, 444 and 29 bp of the *Igf2r* allele were replaced by a thymidine kinase promoter–neomycin (TKneo) cassette (Ludwig *et al*., 1996) are drawn above the wild-type allele. The targeting construct for the *Igf2r-TKneo* allele is shown below and contains a 12.4 kb *Eco*RV fragment (bp 89 965–102 345; AJ249895) from the *Igf2r* locus, from which a 4682 bp *Sna*BI–*Sfu*I fragment including the entire *Igf2r* CpG-island promoter and exon 1 was replaced by a 1200 bp loxP (open triangles) flanked cassette containing a TKneo resistance gene and polyadenylation signal (box labeled TKneo). Homologous recombination in embryonic stem (ES) cells yielded the *Igf2r-TKneo* allele, and *in vivo* Cre-mediated deletion of the TKneo cassette generated the *Igf2r-del* allele. Fragments: *Eco*RV (EV); *Eco*RI (E); *Bgl*II (B); *Sna*BI (Sn); *Sma*I (Sm); *Not*I (N); *Sal*I (S); *Sfu*I (Sf); and *Mlu*I (M). The probes (tai, kodel and msi) used for the methylation analyses are shown as black bars above the wild-type allele. (**C**) Correctly targeted *Igf2r-TKneo* ES clones were identified by DNA blot of *Bgl*II-digested DNA and probe kodel, yielding a 6 kb fragment instead of the 9.5 kb wild-type fragment. (**D**) The *Igf2r-del* allele was generated by crossing *Igf2r-TKneo* mice with mice carrying a CMV-Cre transgene (Schwenk *et al*., 1995), identified by DNA blot of *Bgl*II-digested DNA and probe kodel. Cre-mediated deletion of the TKneo cassette changes the 6.0 kb fragment from the *Igf2r-TKneo* allele to 4.9 kb, generating the *Igf2r-del* allele. (**E**) Absence of *Igf2r* mRNA from the *Igf2r-TKneo* and *Igf2r-del* alleles. RNA blot of 11.5 d.p.c. embryo RNA hybridized with cDNA probes detecting *Igf2r* exons 3–6 or *Gapd* used as loading control.

been shown to require DNA methylation (Li *et al*., 1993). Deletion of the *Air* promoter from the paternal chromosome demonstrated its action as a *cis*-acting bidirectional silencer on the upstream *Igf2r* promoter and on the downstream *Slc22a2* and *Slc22a3* (Zwart *et al*., 2001). Recent experiments that truncated *Air* to within 3 kb of its promoter have identified the *Air* RNA itself or its active transcription as the cause of *Igf2r*, *Slc22a2* and *Slc22a3* silencing (Rougeulle and Heard, 2002; Sleutels *et al*., 2002).

The *Air* RNA originates from an antisense-orientated promoter within intron 2 of *Igf2r* and overlaps by 29 kb the 5′ part of *Igf2r* including the promoter (Figure 1A). The *Slc22a2* and *Slc22a3* promoters lie, respectively, 170 and 215 kb upstream of the *Air* promoter and thus are not overlapped by the *Air* RNA (Zwart *et al*., 2001). Since the *Air* RNA silences *Slc22a2* and *Slc22a3* without overlapping them, this may indicate that *Air* or its active transcription has a direct action on its susceptible target genes. However, it is also possible that silencing of this

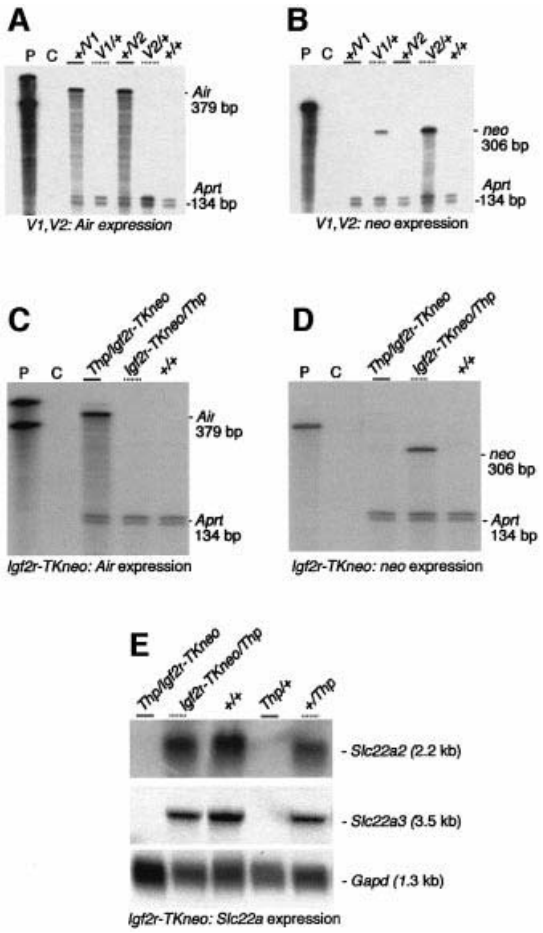


Fig. 2. Parental-specific expression in three *Igf2r* promoter replacement alleles. (**A** and **B**) The *V1* and *V2* alleles have imprinted paternal expression of *Air* (gray solid bar) and imprinted maternal expression of neomycin (*neo*, gray dotted bar). RNase protection analysis (RPA) on 16.5 d.p.c. embryonic RNA with probe Airneo that detects the *Air* RNA at the position of the thymidine kinase promoter–neomycin (TKneo) cassette (A) or with probe tkneo that detects the neomycin RNA produced from the TK promoter (B). The probes Airneo and tkneo do not recognize endogenous *Air* RNA. *Aprt* exon 3: RNA loading control. Lane P, input probes; lane C, tRNA hybridization control. (**C** and **D**) The *Igf2r-neo* allele has imprinted paternal expression of *Air* (gray solid bar) and imprinted maternal expression of neomycin (*neo*, gray dotted bar). RPA on 11.5 d.p.c. embryo RNA from T^{hp}*/Igf2r-neo* reciprocal heterozygous crosses with probe Airneo that detects the *Air* RNA at the position of the TKneo cassette (C) or with probe tkneo that detects the neomycin RNA produced from the TK promoter (D). Controls as above. (**E**) The *Igf2r-neo* allele shows imprinted maternal expression of *Slc22a2* and *Slc22a3*. RNA blot of 11.5 d.p.c. placenta RNA from T^{hp}*/Igf2r-neo* reciprocal heterozygous crosses hybridized with a cDNA probes detecting *Slc22a2* (top), *Slc22a3* (middle) or *Gapd* (bottom) used as loading control.

gene cluster acts in a two-step manner that is initially dependent on the transcript overlap between *Air* and *Igf2r* (Rougeulle and Heard, 2002). In a two-step model, silencing of the non-overlapped *Slc22a2* and *Slc22a3* would depend on the initial silencing of the *Igf2r* promoter by the overlapping *Air* RNA.

In this study, we tested the role of the *Igf2r* CpG-island promoter itself and the role of transcriptional overlap between the *Air* and *Igf2r* transcripts in regulating imprinted silencing of *Air* on the maternal chromosome and of *Slc22a2* and *Slc22a3* on the paternal chromosome. Three different alleles in which part or all of the *Igf2r* promoter were replaced by a thymidine kinase (TK) promoter–neomycin cassette were analyzed, and each showed normal imprinted expression of *Air*, *Slc22a2* and *Slc22a3* when the *Igf2r* promoter was replaced by a foreign promoter. In addition, a fourth allele, deleted for the entire *Igf2r* promoter, also preserved normal imprinted expression of *Air*, *Slc22a2* and *Slc22a3*. These results show that paternal-specific silencing of two coding genes plus maternal-specific silencing of one non-coding RNA do not require the *Igf2r* promoter, a silent promoter at the same position or transcriptional overlap between *Igf2r* and *Air*. Although these results do not exclude a role for a double-stranded RNA silencing mechanism, the data presented here, in combination with the recent demonstration of the direct involvement of the *Air* RNA in gene silencing (Sleutels *et al*., 2002), allow the possibility that *Air* has intrinsic *cis* regulatory properties and can act directly to silence autosomal genes.

Results

Replacement of the Igf2r promoter: three alleles

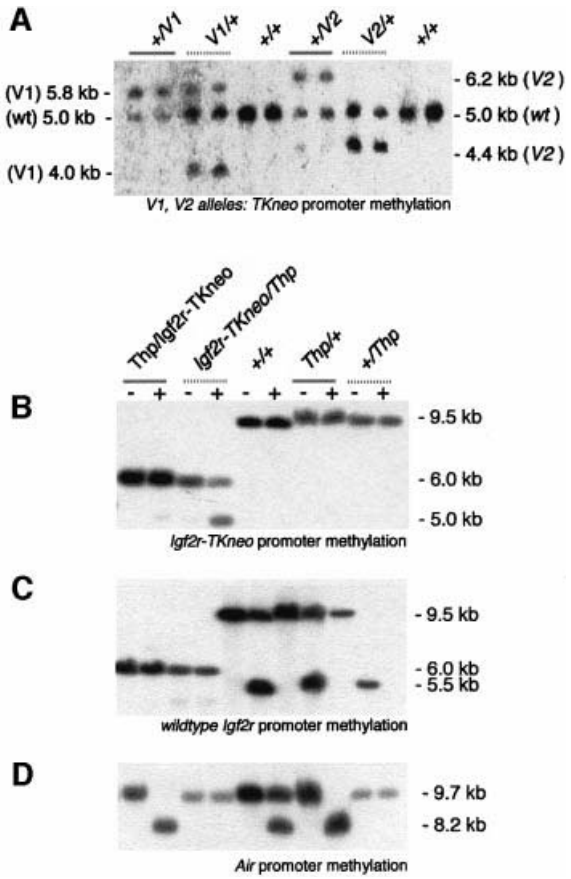
The replacement *V1* and *V2* alleles have, respectively, a 444 and 29 bp fragment from within the *Igf2r* CpG-island promoter region replaced by a 1100 bp fragment containing a herpes simplex TK promoter linked to neomycin/poly(A) (TKneo). The *V1* 444 bp deletion removes 330 bp of upstream promoter sequences, including up to codon 38 of *Igf2r* exon 1, and the smaller *V2* allele deletion is contained within exon 1 and removes codons 28–38. These alleles do not express *Igf2r* from the targeted allele due to the poly(A) signal included in the neomycin gene and show maternal-specific embryonic lethality (Ludwig *et al*., 1996). The *Igf2r-TKneo* allele generated for this work contains a 4682 bp deletion spanning the complete *Igf2r* CpG-island promoter and exon 1 replaced by a 1200 bp TKneo cassette flanked by loxP sites. Figure 1B–E shows the relationship of the *V1* and *V2* alleles to the wild-type locus and the derivation of the *Igf2r-TKneo* allele in embryonic stem (ES) cells and mice.

In order to examine *Igf2r* expression from the targeted allele, heterozygous +/*Igf2r-TKneo* mice were mated to mice carrying the T^{hp} chromosome. The T^{hp} chromosome contains a 3 cM deletion that includes the *Igf2r*, *Air*, *Slc22a2* and *Slc22a3* region, and these crosses allow examination of parental-specific expression in the absence of the second parental allele. Paternal transmission of the T^{hp} allele in wild-type laboratory mouse strains (+/T^{hp}; note that the maternal allele is written on the left side) has no effect on viability, but maternal transmission (T^{hp}/+) is lethal between 15.5 and 17.5 days post-coitum (d.p.c.) because the absence of *Igf2r* leads to a lethal excess of *Igf2* (Wutz *et al*., 2001). RNA blots of 11.5 d.p.c. embryos show that a maternally transmitted *Igf2r-TKneo* allele lacks *Igf2r* expression, in contrast to a wild-type maternal allele (Figure 1E; compare lanes *Igf2r-TKneo/Thp* and +/*Thp*), and that *Igf2r* is not expressed from a paternal *Igf2r-TKneo* allele (T^{hp}*/Igf2r-TKneo*) or a paternal wild-type allele (T^{hp}/+). These results demonstrate that, although the targeting event abolished maternal *Igf2r* expression downstream of the inserted TKneo cassette, it did not affect

silencing on the paternal allele. The absence of maternal *Igf2r* expression explains the absence of viable offspring with a maternally inherited *Igf2r-TKneo* allele (data not shown), as has been reported for other *Igf2r* loss-of-function alleles (Lau *et al*., 1994; Wang *et al*., 1994; Ludwig *et al*., 1996).

Parental-specific expression in the Igf2r promoter replacement alleles

Parental-specific expression of the replacement TK promoter and the downstream imprinted promoters (*Air*, *Slc22a2* and *Slc22a3*) was tested in the *Igf2r* promoter replacement/deletion alleles in embryonic and placental tissue. Both the *V1* and *V2* alleles showed complete imprinted expression in 16.5 d.p.c. embryos of both *Air* and *TKneo*, such that *Air* is expressed only from the paternal allele and *TKneo* is expressed only from the maternal allele (Figure 2A and B; note that the Airneo probe does not detect endogenous *Air* RNA). Maternal expression of *TKneo* from the *V1* allele is reduced, compared with the *V2* allele (Figure 2B). The *Igf2r-TKneo* replacement allele similarly showed paternal-specific *Air* expression and maternal-specific *TKneo* expression in 11.5 d.p.c. embryos (Figure 2C and D). In addition, imprinted expression of the downstream *Slc22a2* and *Slc22a3* was also fully maintained and unchanged from the wild-type state in 11.5 d.p.c. placenta on the *Igf2r-TKneo* allele (Figure 2E; note that imprinted expression of *Slc22a2* and *Slc22a3* is restricted to the embryonic placenta; Zwart *et al*., 2001). In summary, analysis of parental-specific expression in these three alleles, with a TK promoter inserted into or replacing the *Igf2r* promoter, shows that the *Igf2r* promoter itself is not required for imprinting the other genes in this cluster and that an exogenous promoter that fully replaces the *Igf2r* promoter can acquire full imprinted expression.



Parental-specific methylation of the Igf2r promoter replacement alleles

Parental-specific methylation of the TK promoter in the replacement alleles was tested in embryos. Figure 3A shows that maternally inherited *V1* and *V2* alleles have either reduced or an absent methylation, compared with paternally inherited alleles, in 16.5 d.p.c. embryos. Maternal methylation (as quantified by PhosphorImager; data not shown) was present to ~40% of the paternal level on the *V1* but completely absent on the *V2*. Paternal methylation was complete for *V1* but incomplete for *V2* in 16.5 d.p.c. embryos (Figure 3A; lanes *V1/+* or *V2/+* show the presence of smaller fragments not found in lanes *+/V1* and seen as faint bands in *+/V2*; note that methylation of

Fig. 3. Parental-specific methylation of three *Igf2r* promoter replacement alleles. (**A**) Thymidine kinase promoter–neomycin (TKneo) methylation analysis in *V1* and *V2* alleles. Heterozygous 16.5 d.p.c. embryonic DNA carrying either paternally derived (gray solid bars) or maternally derived (gray dotted bars) *V1* and *V2* replacement alleles was digested with *Eco*RI and hybridized with probe tai (Figure 1B). Note that *Eco*RI is methyl sensitive when G flanks the GAATTC recognition site and the CG dinucleotide is methylated (Ludwig *et al*., 1996). The *V1* allele generates 4.0 kb when the *Eco*RI is unmethylated and 5.8 kb for a methylated site. *V1* paternal transmission (gray solid bar) shows full methylation at this site, and *V1* maternal transmission (gray dotted bar) shows partial (~40%) methylation at this site. The *V2* allele yields 4.4 or 6.2 kb for an unmethylated and methylated sites, respectively. *V2* paternal transmission (gray solid bar) shows partial (~80%) methylation at this site, and *V2* maternal transmission (gray dotted bar) shows no methylation at this site. The *Igf2r* wild-type (wt) promoter fragment is 5.0 kb and does not contain a methyl-sensitive *Eco*RI site. (**B**) TKneo promoter methylation analysis in the *Igf2r-TKneo* allele. Heterozygous 13.5 d.p.c. embryonic DNA carrying either a paternally derived (gray solid bar) or maternally derived (gray dotted bar) *Igf2r-TKneo* replacement allele was digested with *Bgl*II (–) or *Bgl*II and *Mlu*I (+) and hybridized with probe kodel (Figure 1B). Note that, in crosses with the T^{hp} allele, only bands from the opposite allele are seen. The *Igf2r-TKneo* allele yields 5.0 or 6.0 kb for an unmethylated and methylated sites, respectively. *Igf2r-TKneo* paternal transmission (gray solid bar) shows almost complete methylation at this site (a faint 5.0 kb unmethylated can be seen on the original image), and *Igf2r-TKneo* maternal transmission (gray dotted bar) shows partial (~50%) methylation at this site. The wild-type *Igf2r* promoter fragment is 9.5 kb and does not contain an *Mlu*I site. (**C**) *Igf2r* promoter (*Not*I) methylation analysis in the wild-type *Igf2r* promoter. T^{hp}/+ heterozygous 13.5 d.p.c. embryonic DNA carrying either a paternally derived (gray solid bar) or maternally derived (gray dotted bar) wild-type *Igf2r* promoter was digested with *Bgl*II (–) or *Bgl*II and *Not*I (+) and hybridized with probe kodel (Figure 1B). The wild-type *Igf2r* promoter yields 5.5 or 9.5 kb for an unmethylated and methylated sites, respectively. *Igf2r* wild-type promoter paternal transmission (gray solid bar) shows partial (~50%) methylation at this site, and *Igf2r* wild-type promoter maternal transmission (gray dotted bar) shows no methylation at this site. The *Igf2r-TKneo* promoter fragment is 6.0 kb and lacks this *Not*I site (it was deleted in the targeting event). (**D**) *Air* promoter (*Mlu*I) methylation analysis in the *Igf2r-TKneo* and wild-type *Igf2r* alleles. Heterozygous 13.5 d.p.c. embryonic DNA carrying either a paternally derived (gray solid bar) or maternally derived (gray dotted bar) *Igf2r-TKneo* or a wild-type *Igf2r* allele was digested with *Bgl*II (–) or *Bgl*II and *Mlu*I (+) and hybridized with probe msi (Figure 1B). The *Air* promoter yields 8.2 or 9.7 kb for an unmethylated and methylated sites, respectively. Both the *Igf2r-TKneo* and wild-type *Igf2r* alleles show no methylation at this site on paternal transmission (gray solid bar) and full methylation on maternal transmission (gray dotted bar).

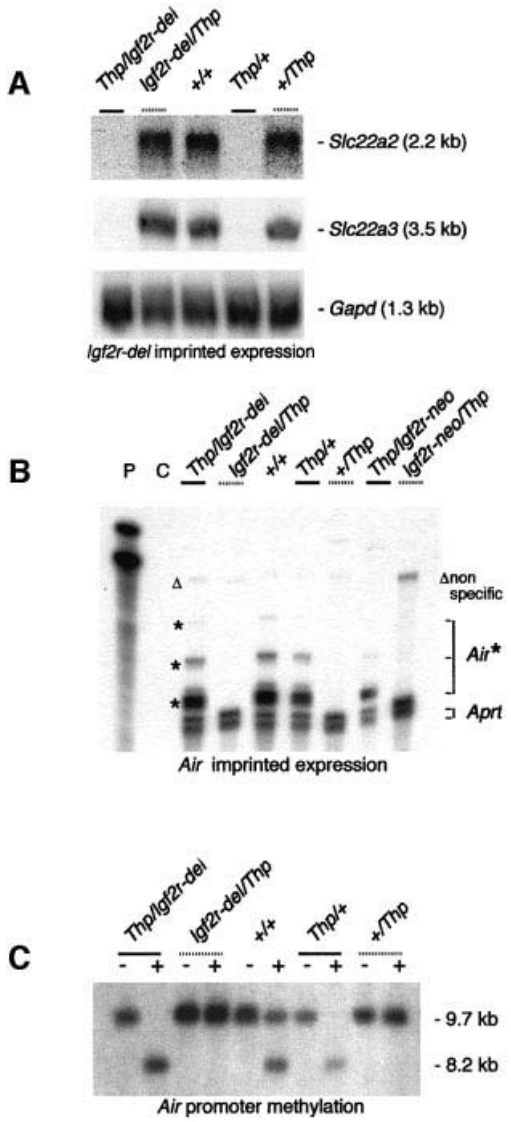


Fig. 4. Imprinted expression and methylation in the *Igf2r-del* allele. (**A**) *Slc22a2* and *Slc22a3* imprinted expression from the *Igf2r-del* allele is maternal specific (gray dotted bars), identical to the wild-type situation. RNA blot of 11.5 d.p.c. placenta RNA from T^{hp}/*Igf2r-del* and T^{hp}/wild-type reciprocal crosses hybridized with cDNA probes detecting *Slc22a2*, *Slc22a3* or *Gapd* used as loading control. Note that imprinted expression of *Slc22a2* and *Slc22a3* is restricted to the embryonic placenta (Zwart *et al*., 2001). (**B**) *Air* RNA imprinted expression from the *Igf2r-del* allele is paternal specific (gray solid bars), identical to the wild-type situation. RNase protection analysis using probe MlMs1 on 11.5 d.p.c. embryo RNA from T^{hp}/*Igf2r-del* and from T^{hp}/wild-type reciprocal crosses. This probe detects multiple fragments (asterisk) as it overlaps multiple *Air* transcription start sites. The open triangle indicates non-specific protected bands. Controls as in Figure 3. (**C**) *Air* promoter methylation on the paternal (gray solid bar) and maternal (gray dotted bar) *Igf2r-del* allele is identical to the wild-type *Air* promoter. Heterozygous 13.5 d.p.c. embryonic DNA from T^{hp}/*Igf2r-del* and from T^{hp}/wild-type reciprocal crosses was cut with *Bgl*II (–) or *Bgl*II and *Mlu*I (+) and hybridized with probe msi (Figure 1B).The unmethylated and methylated alleles are 8.2 and 9.7 kb, respectively.

the paternal allele is incomplete on the wild-type chromosome in embryos; Figure 3C, lane *Thp*/+; Stoeger *et al*., 1993). Identical results were obtained for 16.5 d.p.c. embryonic membrane and for the *Mlu*I site in the TK promoter and the *Sma*I sites that flank the replacement (data not shown). The *V2* methylation pattern is the same as that on wild-type *Igf2r* promoter, which is methylated over a 1500 bp region in late development; complete methylation is seen only after birth, and the maternal wild-type *Igf2r* promoter is always unmethylated (Stoeger *et al*., 1993). The wild-type *Air* promoter that lies 29 kb downstream of the *Igf2r* promoter is normally only methylated on the maternal allele (Stoeger *et al*., 1993), and this methylation (as determined by analysis of an *Mlu*I site inside the *Air* promoter) remains maternal specific in both the *V1* and *V2* alleles (data not shown). *Slc22a2* and *Slc22a3* lack parental-specific methylation (Zwart *et al*., 2001).

Parental-specific methylation in the *Igf2r-TKneo* allele was tested in a similar manner by restriction enzyme digest and DNA blot hybridization in 13.5 d.p.c. embryos in mice heterozygous for the T^{hp} deletion (Figure 3B, C and D). Methylation of the *Mlu*I site present in the TKneo promoter is almost complete on paternal transmission (Figure 3B, lane *Thp/Igf2r-TKneo*); however; this methylation is only reduced by 50% on maternal transmission (lane *Igf2r-TKneo/Thp*) as quantified by PhosphorImager (data not shown). This result is similar to that shown by the *V1* allele (Figure 3A) and contrasts to the strictly paternal-specific methylation that is incomplete in 13.5 d.p.c. embryo tissue (Stoeger *et al*., 1993) seen in the wild-type *Igf2r* promoter at the *Sal*I site (data not shown) and the *Not*I site (Figure 3C; compare lanes *Thp/+* and *+/Thp*). Methylation of the downstream *Air* promoter at the *Sfu*I site (data not shown) and the *Mlu*I site was restricted to the maternal allele in both the *Igf2r-TKneo* and wild-type alleles in 13.5 d.p.c. embryos (Figure 3D, lanes *Igf2r-TKneo/Thp* and *+/Thp*).

In summary, the methylation studies of the three *TKneo* alleles show that paternal methylation is retained after targeted insertion/replacement of the *Igf2r* promoter by a TK promoter. However, the absence of maternal methylation that is a feature of the wild-type *Igf2r* promoter is only seen in the *V2* allele that is deleted for 29 bp but retains the *Igf2r* promoter. The *V1* and *Igf2r-TKneo* alleles (which lack, respectively, 444 and 4682 bp) gain methylation on maternal transmission, albeit at a reduced level compared with paternal transmission. The gain of maternal methylation on the TK promoter in the *V1* allele, compared with the *V2* allele, likely explains the reduced TKneo expression from the *V1* allele (Figure 2B). The methylation status of the linked *Air* promoter remained unchanged in all three replacement alleles.

Deletion without replacement of the Igf2r promoter

An *Igf2r-del* allele that replaced the 4682 bp *Igf2r* promoter fragment with a single loxP site was generated by mating *+/Igf2r-TKneo* males with female mice containing a Cre transgene driven by a CMV promoter (Schwenk *et al*., 1995). Heterozygous offspring in which the *TKneo* cassette was deleted to produce the *Igf2r-del* allele (Figure 1D) were obtained and crossed with T^{hp} mice to generate embryos and placentas with a germline-transmitted *Igf2r-del* allele that could be analyzed for allele-specific expression. RNA blot analysis of 11.5 d.p.c. embryos showed that removal of this 4682 bp fragment abolished all transcription through the *Igf2r* locus as

detected by a cDNA probe spanning exons 3–6 (Figure 1A and E). Imprinted expression of *Slc22a2* and *Slc22a3* on the *Igf2r-del* allele was maintained, as in wild-type mice, in 13.5 d.p.c. placentas (data not shown) and in 11.5 d.p.c. placentas (Figure 4A; compare lanes *Thp/Igf2r-del* and *Igf2r-del/Thp).* Expression from the *Air* promoter was analyzed in 13.5 d.p.c. embryos (data not shown) and in 11.5 d.p.c. embryos (Figure 4B). These results show that the *Air* promoter on the *Igf2r-del* allele is expressed only after paternal transmission and thus retains its normal wild-type parental-specific expression pattern (Figure 4B; compare lanes *Thp/Igf2r-del* and *Igf2r-del/Thp*; protected multiple fragments are paternal specific and represent the multiple transcription starts mapped in the *Air* promoter; Sleutels *et al*., 2002). Analysis of imprinted methylation was only possible at the *Air* promoter (since the *Igf2r* promoter is deleted in this allele and since *Slc22a2* and *Slc22a3* lack parental-specific methylation), and this analysis in 13.5 d.p.c. embryos showed that *Sfu*I (data not shown) and *Mlu*I retained their wild-type pattern and were methylated only on the maternal chromosome (Figure 4C; compare lanes *Igf2r-del/Thp* and *Thp/Igf2r-del*; the unmethylated 8.2 kb fragment is paternal specific).

In summary, analysis of parental-specific expression in an allele completely deleted for the *Igf2r* promoter confirms that the *Igf2r* promoter has no role in a silent or an active form in imprinting genes in this cluster. These results also show that transcriptional overlap between the *Air* and *Igf2r* RNAs is not needed for silencing *Air* on the maternal chromosome and *Slc22a2* and *Slc22a3* on the paternal chromosome.

Discussion

Paternal expression of the *Air* RNA is required for silencing *Igf2r*, *Slc22a2* and *Slc22a3* on the same chromosome but has no effect on maternal expression of these three genes; thus, imprinted silencing is strictly a *cis*-acting mechanism (Sleutels *et al*., 2002). On the paternal chromosome, *Air* expression from an antisense-orientated promoter lying within intron 2 of *Igf2r* creates a potential transcriptional overlap of 29 kb with the 5′ end of that gene. A transcriptional overlap allows the possibility that *Air* could silence the overlapped gene by a mechanism different from those that silence non-overlapped genes. For example, *Igf2r* could be silenced by promoter occlusion (Villemure *et al*., 2001) or by the formation of a double-stranded RNA intermediate that could induce an RNA interference (RNAi) post-transcriptional silencing response (Hannon, 2002). RNAi was initially excluded from involvement in imprinting, since it was described as *trans*-acting and imprinting requires a *cis*-acting mechanism. However, the identification of a putative *cis*-acting form of RNAi in the nucleus of *Schizosaccharomyces pombe* involved in centromeric heterochromatin (Volpe *et al*., 2002) allows the possibility of a similar mechanism in mammals. If *Igf2r* was repressed because of transcriptional overlap with *Air* on the same chromosome, then silencing of the upstream *Slc22a2* and *Slc22a3* may be a secondary event due to spreading of repressive chromatin from the silenced *Igf2r* allele. The experiments described here were thus designed to test the role of the *Igf2r* promoter, the role of transcriptional overlap *in cis* between *Igf2r* and *Air* and, finally, whether the silencing of *Slc22a2* and *Slc22a3* is secondary to the action of *Air* on *Igf2r*.

The relevance of the Igf2r promoter in the imprinting mechanism

All three replacement alleles contained the same 1100 bp TKneo cassette combined with different deletions of the endogenous *Igf2r* promoter, and together they allowed the analysis of the 'foreign' TK promoter in different 'host' environments. The *V1* and *V2* alleles share the same distal deletion endpoint (codon 38 of exon 1). The *V1* deletion extends 330 bp upstream to exon 1, whereas the *V2* deletion extends to codon 28 of exon 1. The deletion in the *Igf2r-TKneo* allele extends 2733 bp upstream and 1560 bp downstream to exon 1 and thus removes all of the *Igf2r* promoter. All three alleles showed imprinted maternal-specific expression of the neomycin gene expressed by the foreign TK promoter and also maintained wild-type imprinted expression of *Air*, *Slc22a2* and *Slc22a3*. Thus, imprinting of the foreign TK promoter resembled that of the endogenous *Igf2r* promoter in the presence or the absence of this promoter. In addition, a role for the endogenous *Igf2r* promoter in the imprinting mechanism acting on this gene cluster is excluded. The TK and the *Igf2r* promoters are very different. *Igf2r* has a CpG-island-type promoter with a 1 kb CG-rich core (Stoeger *et al*., 1993). The TK promoter is composed of a 141 bp CpG-poor polyomavirus late-region fragment and a 129 bp CpG-rich herpes simplex promoter fragment (see Materials and methods). The common imprinting of these different promoters indicates that *Air* may silence in a non-specific, promoter-independent manner that supports arguments that *Air* has intrinsic silencing properties. A comparable promoter-independent mode of silencing has been observed for the *Xist* non-coding RNA that is responsible for X-chromosome inactivation (Wutz and Jaenisch, 2000). Although the presence or the absence of the *Igf2r* promoter had no influence on the imprinted expression of the replacement TK promoter, the absence of methylation on the maternal allele was affected. The wild-type *Igf2r* promoter is normally free of methylation on maternal transmission but becomes partially methylated following paternal transmission in late embryogenesis (full paternal methylation is only found in postnatal stages; Stoeger *et al*., 1993). The TK promoter showed 'wild-type' methylation behavior only in the *V2* allele that also contains the full *Igf2r* promoter. The *V1* and *Igf2r-TKneo* alleles that are deleted for part or all of the *Igf2r* promoter were methylated on maternal transmission to a level of 40–50% of that seen following paternal transmission. This indicates that the *Igf2r* promoter contains sequences that act to prevent maternal methylation that are not present in the TK promoter.

Imprinted silencing of Slc22a2 and Slc22a3 is not a two-step mechanism

The *Igf2r-del* allele substituted a loxP site for a 4682 bp fragment containing the complete *Igf2r* promoter and exon 1 and lacked any transcription downstream from the deleted promoter (Figures 1E and 4B). This allele was used to test the role of transcriptional overlap *in cis* between *Igf2r* and *Air* in silencing this imprinted gene cluster. The results show that imprinted expression of the

remaining genes (*Air*, *Slc22a2* and *Slc22a3*) was unaffected by the removal of the *Igf2r* promoter. All three genes maintained their wild-type pattern of imprinted expression and, in addition, the methylation imprint on the maternal *Air* promoter was unchanged. Thus, transcriptional overlap between *Air* and *Igf2r* is not needed for paternal silencing of *Slc22a2* and *Slc22a3* or maternal silencing of *Air*. Since the absence of the *Igf2r* promoter precluded the existence of a 'silenced' promoter, these results also exclude models in which silencing of a promoter *in cis* is necessary for subsequent silencing of the neighboring *Slc22a2* and *Slc22a3* (Zwart *et al*., 2001). Models based on maternal *Igf2r* transcription playing a role in maintaining maternal methylation on the *Air* promoter are also now excluded. In addition, this results question the general applicability of a multiple CpG-island requirement for an imprinting mechanism (Onyango *et al*., 2000).

Slc22a2 and *Slc22a3* are paternally silenced by *Air* but have no transcription overlap with it (Zwart *et al*., 2001). The demonstration here that this silencing is independent of *Igf2r* indicates that *Air* has a direct action on these genes, supporting arguments that *Air* has intrinsic silencing properties. Despite their common regulation by *Air*, imprinted silencing of *Slc22a2* and *Slc22a3* is different in quality from that of *Igf2r*. The molecular basis of this difference is unknown. One correlation can be made: the degree of imprinted silencing on the paternal allele correlates with distance from the *Air* promoter. The *Igf2r* promoter is closest (29 kb) and is silenced in embryo and adult, *Slc22a2* is 170 kb distant and is silenced in embryonic placenta and partly silenced in adult kidney (expression is limited to these tissues) and *Slc22a3* is 215 kb distant and is silenced in 11.5 but not 15.5 d.p.c. placentas and has widespread bi-allelic expression in adult tissues (Zwart *et al*., 2001). Thus, *Air* may have reduced ability to affect promoters with distance. Currently, we have no explanation of why two non-CpG-island promoters in this region (*Mas* and *Slc22a1*) escape imprinted silencing despite close proximity to the *Air* promoter.

Homology and imprinted gene silencing

The physical arrangement of the *Air* non-coding RNA in this imprinted gene cluster (Figure 1A), as well as the demonstration that other non-coding RNAs are expressed from antisense-orientated promoters within introns of imprinted protein-coding genes (Rougeulle *et al*., 1998; Smilinich *et al*., 1999; Lee *et al*., 2000; Wroe *et al*., 2000), presents a strong argument that homology and double-stranded RNA formation between non-coding RNAs and target gene transcripts may be involved in imprinted gene silencing. The results presented here, however, show that transcription overlap of 29 kb between *Igf2r* and *Air* is not needed for paternal silencing of *Air* or maternal silencing of the upstream *Slc22a2* and *Slc22a3*. Although these data argue against a role for homology in silencing these specific genes, other possibilities exist whereby homology could play a role in the silencing mechanism at this locus. One possibility is that homology exists between the interspersed repeats present in the mature *Air* RNA and in the unspliced precursor *Slc22a2* and *Slc22a3* RNAs. Another possibility is that transcripts in the opposite orientation to *Air* could exist in the region upstream of the *Igf2r* promoter and contribute to gene silencing in the *Igf2r-del* allele described here. At this time, however, an analysis of all expressed sequence tags (ESTs) mapped to this interval (mouse chromosome 17; 12.168–12.288 Mbp; http://www.ensembl.org/Mus_musculus/contigview) shows an abundance of antisense ESTs corresponding to *Air* but no significant sense transcription in this region. Since silencing of *Igf2r* cannot be tested in the *Igf2r-del* allele, it also remains a possibility that *Air* has two independent silencing modes that act in parallel to silence genes in this cluster: one based on transcriptional overlap, acting on *Igf2r* (and on foreign promoters replacing *Igf2r*); and one independent of transcriptional overlap, acting on *Slc22a2* and *Slc22a3*.

The Air RNA may have intrinsic cis silencing properties

In summary, the results presented here show that neither prior silencing of *Igf2r* nor the transcriptional overlap between *Air* and *Igf2r* are necessary for *Slc22a2* and *Slc22a3* silencing. Thus, models based on this 29 kb transcriptional overlap and those based on a two-step mechanism for silencing *Slc22a2* and *Slc22a3* are excluded from operating at this imprinted gene cluster. The absence of a two-step mechanism for silencing *Slc22a2* and *Slc22a3*, combined with the lack of specificity in promoters susceptible to *Air*, allows the possibility that the *Air* RNA has intrinsic promoter-independent *cis* silencing properties. However, the existence of nearby genes that are not silenced by *Air* also indicates that not all promoters are equally susceptible to silencing. Based on these findings, the silencing model we propose for the *Air* RNA is analogous but different to the *Xist* model for X-chromosome inactivation in mammals (Avner and Heard, 2001). This model proposes that the *Air* RNA would generate a *cis* silencing effect that can repress susceptible gene promoters within a specific region whose boundaries are not yet known. Although the limitation of silencing to a small subchromosomal region marks a major difference between *Air* and *Xist*, the suggestion that X-chromosome inactivation evolved from a localized form of imprinting that initially affected only a small region of the X chromosome supports this model (Graves, 1996; Lyon, 1999; Lee, 2003).

Materials and methods

Generation of the Igf2r-TKneo allele

The targeting vector contained a 12.4 kb *Eco*RV fragment (bp 89 965–102 345; AJ249895) isolated from BAC 18p11 (Research Genetics). A 4682 kb *Sna*BI–*Sfu*I fragment (bp 95 005–99 687; AJ249895) that includes the entire *Igf2r* CpG-island promoter and exon 1 was replaced by a loxP-flanked cassette of 1.2 kb containing a herpes simplex TK promoter, neomycin resistance gene and polyadenylation signal obtained from pMC1neo-poly(A) (bp 455–1597; U43612; Stratagene). This construct left 5.0 and 2.7 kb for recombination at the 5′ and 3′ ends, respectively. E14 ES cells (15×10^6) were electroporated with 0.02 mg of *Not*I linearized targeting construct and selected with 0.2 mg/ml G418; the targeting efficiency was 2%. Correctly targeted ES cells were identified by DNA blot, and chimeric mice were subsequently generated by injecting heterozygous *Igf2r-neo* ES cells into C57/Bl6 blastocysts (Hogan *et al*., 1994). All mice, except the V1 and V2 mice, were maintained on an FVB/N background and identified by DNA blot analyses. The V1 and V2 mice were maintained on a C57/Bl6 background. Embryos and placentas (including membranes) were collected after timed mating where the vaginal plug counts as 0.5 d.p.c.

DNA and methylation analyses

Genomic DNA preparation and DNA blots were performed according to standard procedures. Digestion of methyl-sensitive enzymes was monitored by hybridization with mitochondrial DNA (Walsh *et al*., 1998). The following methylation analyses probes were used (Figure 1B): kodel, a 325 bp fragment (bp 102 813–103 137; AJ249895); msi, an *Sfu*I–*Mlu*I fragment (bp 124 992–126 086; AJ249895); and tai, an *Eco*RI–*Hin*dIII fragment (bp 94 104–94 986; AJ249895).

RNA analyses

Total RNA was isolated with Tri Reagent (Molecular Research Center). RNase protection analysis (RPA) was performed with the RPAIII kit (Ambion). The probes for RPA were tkneo and Airneo: a 685 bp *Pst*I fragment (bp 923–1554; U43611) from the neomycin resistance gene taken from plasmid pMC1neo-poly(A) (Stratagene) and cloned into pBluescriptII (Stratagene) cut with *Nco*I to generate the Airneo and the tkneo template. The Airneo probe (T3) is 446 bp and protects 379 bp of *Air* RNA. The tkneo probe (T7) is 365 bp and protects 305 bp from the neomycin gene. The *Aprt* probe is a 252 bp *Xho*I–*Xba*I fragment (bp 2165–2417; M11310) that protects 134 bp of *Aprt* exon 3. The MlMs1 probe is an *Mlu*I–*Mse*I fragment (bp 126 086–126 293; AJ249895) that overlaps multiple *Air* transcription starts and detects 207, 171 and 148 bp for *Air* RNA (Sleutels *et al*., 2002). For RNA blot analyses, the following probes were used: *Igf2r*, exons 3–6 cDNA fragment; *Slc22a2* (bp 989–1605; AJ006036); *Slc22a3* (bp 1–2766; AF078750); and *Gapd* (bp 1–1066; NM_008084).

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