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The two-domain hypothesis in Beckwith–Wiedemann syndrome: autonomous imprinting of the telomeric domain of the distal chromosome 7 cluster

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A large cluster of imprinted genes is located on the mouse distal chromosome 7. This cluster is well conserved in humans and its dysregulation results in the overgrowth- and tumour-associated Beckwith–Wiedemann syndrome. Two imprinting centres (IC1 and IC2) controlling different sets of genes have been identified in the cluster, raising the hypothesis that the cluster is divided into two functionally independent domains. However, the mechanisms by which imprinting of genes in the IC2 domain (e.g. *Cdkn1c* and *Kcnq1*) is regulated have not been well defined, and recent evidence indicates that distantly located *cis*-acting elements are required for IC2 imprinting. We show that the maternal germ-line methylation at IC2 and the imprinted expression of five genes of the IC2 domain are correctly reproduced on an 800 kb YAC transgene when transferred outside of their normal chromosomal context. These results, together with previous transgenic studies, locate key imprinting control elements within a 400 kb region centromeric of IC2 and demonstrate that each of the two domains of the cluster contains the *cis*-acting elements required for the imprinting control of its own genes. Finally, maternal, but not paternal, transmission of the transgene results in fetal growth restriction, suggesting that during evolution the acquisition of imprinting may have been facilitated by the opposite effects of the two domains on embryo growth.

INTRODUCTION

Genomic imprinting consists of gamete-of-origin-dependent epigenetic modifications of genes, which result in differential expression of their maternal and paternal alleles (1–5). The majority of about 70 imprinted genes identified so far are organised in clusters (www.otago.ac.nz/IGC). Imprinting centres (ICs) have been described to control the expression of imprinted genes over long distances. The ICs consist of DNA sequences that are methylated on one of the two parental alleles (differentially methylated regions, DMRs), with differential methylation arising in the parental germ cells, and loss of methylation resulting in loss of imprinted expression (6–9).

A large cluster of imprinted genes (>1 Mb of DNA) is located on the mouse distal chromosome 7 and is largely conserved in human chromosome 11p15.5. The cluster contains

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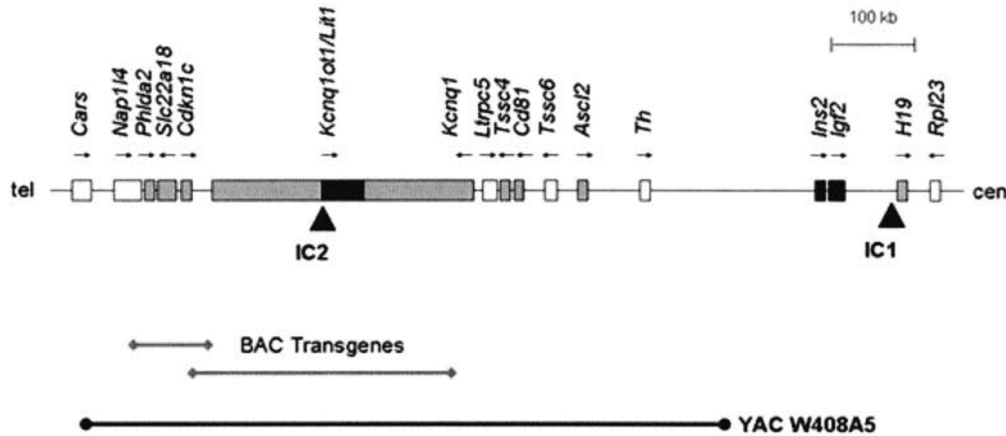

Figure 1. Map of the mouse distal chromosome 7 imprinting cluster and extension of the IC2 domain transgenes. Grey rectangles indicate maternally expressed genes, black rectangles paternally expressed genes and white rectangles biallelically expressed genes. Arrows above each gene indicate the direction of transcription and black triangles indicate the position of the IC (IC1 and IC2). The location of the previously described BAC transgenes (grey bars) and of the 800 kb YAC W408A5 transgene (black bar) is depicted below the map of the locus. Tel, telomere; cen, centromere. The YAC transgene extends 400 kb centromerically more than the BAC transgenes.

at least 11 imprinted genes (Fig. 1), and in the human, it is associated with the fetal overgrowth and tumour-associated Beckwith–Wiedemann syndrome (BWS, MIM 130650) (10,11). The majority of individuals affected by BWS have epigenetic defects at either one of two DMRs (12–14). In both human and mouse, deletion of these DMRs results in dysregulation of different subsets of genes in the cluster, suggesting that this region is divided into two domains controlled by functionally independent ICs (15–20). The domain controlled by IC1 (centromeric in the mouse) includes the paternally expressed insulin-like growth factor 2 (*Igf2*) and the maternally expressed *H19* genes. IC1 is a paternally methylated CpG-rich region (*H19* DMR) containing a methylation-sensitive chromatin insulator that controls the access of either *Igf2* or *H19* to down-stream enhancers (21). Additional *cis*-acting elements contributing to the control of *Igf2* and *H19* imprinting have been identified within the IC1 domain (22,23). The domain controlled by IC2 is much larger and contains several maternally expressed genes including the cyclin-dependent kinase inhibitor *Cdkn1c*. IC2 is a maternally methylated CpG-island (KvDMR1) and includes the promoter of a paternally expressed non-coding RNA gene (*Kcnq1ot1*), which is transcribed anti-sense to the maternally expressed protein-coding *Kcnq1* gene. IC2 and/or the *Kcnq1ot1* transcript regulate negatively the maternally expressed genes of the IC2 domain on the paternal chromosome (18,24,25). IC2 is demethylated in about half of the individuals affected by BWS, and this is associated with down-regulation of *CDKN1C* (11,26).

Although the *cis*-acting elements required for the establishment and maintenance of imprinting at the IC1 domain have not been completely defined, it is clear that they lie relatively close to IC1 because a 15.7 kb transgene, including the *H19* gene, and a 130 kb transgene, including both *Igf2* and *H19*, display correct imprinting at ectopic loci (23,27). A similar transgenic approach produced different results with the IC2 domain (28). In this case, a 260 kb transgene including *Cdkn1c* and IC2 (Fig. 1) did not reproduce the imprinted expression and the imprinted methylation of the locus, indicating that distantly located *cis*-regulatory elements, in addition to IC2, are needed for imprinting establishment and/or maintenance, and thus challenging the two independent domain-hypothesis of the BWS region.

We further investigated the imprinting requirements of the IC2 domain by generating transgenes covering larger genomic regions. YAC transgenesis has proven to be a useful tool; in several instances, it was invaluable in order to analyse regulatory mechanisms acting over long genomic distances (29). In this paper, we report the generation and analysis of the 800 kb YAC transgene derived from the IC2 domain. This transgene spans from the *Cars* to the *Th* gene and includes all the imprinted genes that are currently known to be regulated by IC2 but none of the IC1 genes (Fig. 1). We demonstrate that the imprinted methylation of IC2 and the imprinted expression of *Kcnq1ot1* and four maternally expressed genes are correctly maintained outside of their normal chromosomal context when such a large genomic region is transferred. These results have important implications both for studies of imprinting mechanisms and for molecular analysis of BWS.

RESULTS

Generation of transgenic mice carrying the IC2 domain of the mouse distal chromosome 7 cluster

The 800 kb YAC (W408A5, Fig. 1) containing genomic sequence from the mouse distal chromosome 7 and extending from the *Cars* to the *Th* gene was described by Kato *et al.* (30). To study the expression and imprinting of the genes included in the telomeric domain of the BWS imprinting cluster, we generated mice transgenic for YAC W408A5. ES cell lines carrying YAC W408A5 were obtained by yeast spheroplast–ES cell fusion, because this is considered as the most

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efficient method for generating transgenic mice with DNA molecules >500 kb (29). Using sequence polymorphisms for distinguishing the transgene (C57BL/6J genotype) from the endogenous locus (129Sv genotype), an ES cell line carrying a full-length and single-copy YAC was identified and used to establish transgenic mice (data not shown). In order to analyse the expression and methylation of all the imprinted genes present on the 800 kb transgene, the number of polymorphisms between the transgene and the endogenous locus was increased by crossing the transgenic line with SD7 mice (a *Mus musculus domesticus* strain containing the distal portion of chromosome 7 of *Mus spretus* origin).

Imprinted expression of IC2 genes on the 800 kb transgene

We examined the expression of one paternally expressed (*Kcnq1ot1*) and six maternally expressed (*Phlda2*, *Slc22a18*, *Cdkn1c*, *Kcnq1*, *Tssc4* and *Ascl2*) genes of the IC2 domain in E13.5 mice with maternal or paternal inheritance of the 800 kb transgene. It has been previously shown that the endogenous alleles of *Kcnq1ot1*, as well as *Phlda2*, *Slc22a18*, *Cdkn1c* and *Kcnq1*, are imprinted both in the fetus and placenta, whereas *Tssc4* and *Ascl2* are imprinted only in the placenta (31–33). Expression from the 800 kb transgene was analysed in the tissues where the endogenous alleles are imprinted and was distinguished from that of the endogenous locus by typing for transcribed sequence polymorphisms. After maternal transmission, we observed expression from the transgenic *Phlda2*, *Slc22a18*, *Cdkn1c*, *Kcnq1*, *Tssc4* and *Ascl2* alleles at levels similar to those of the endogenous alleles, but no expression from the transgenic *Kcnq1ot1* allele both in the fetus and placenta (Fig. 2 and data not shown). After paternal transmission, expression comparable to the endogenous locus was detected on the transgene from *Kcnq1ot1*, but no or little expression was evident from *Phlda2*, *Slc22a18*, *Cdkn1c* and *Kcnq1*, indicating that the imprinting of these genes was maintained (Fig. 2). This was not the case for *Tssc4* and *Ascl2*, because these genes were expressed in the placenta at similar levels after maternal or paternal transmission of the transgene (Fig. 2).

We have then determined by real-time RT–PCR the absolute mRNA levels of the genes present on the YAC in the embryo and placenta of the mice with maternal and paternal transmission of the 800 kb transgene and compared them with those present in the wild-type littermates (Fig. 2). The results showed that in the placenta, the expression of *Phlda2*, *Slc22a18*, *Cdkn1c* and *Kcnq1* was increased by 30–60% in the mice with maternal transmission of the 800 kb transgene when compared with wild-type mice, whereas it was equivalent or slightly decreased in the mice with paternal transmission. In addition, after paternal transmission, the expression of *Kcnq1ot1* was twice that observed in the wild-type mice or after maternal transmission. In contrast, *Ascl2* expression was almost doubled after either maternal or paternal transmission, and the level of *Tssc4* mRNA was increased about 2-fold after maternal transmission and by 33% after paternal transmission of the transgene. The levels of *Cdkn1c* and *Kcnq1ot1* RNAs were also determined in the embryo, with results comparable to those obtained in the placenta (data not shown). Although some dosage compensation effects may have occurred in the transgenic mice, overall these data indicate that the 800 kb transgene contains most of the *cis*-acting elements required for the expression of the genes of the IC2 domain in the placenta (and in the embryo), whereas the previously described 260 kb *Cdkn1c* transgene lacked appropriate placenta-specific expression (28). Thus, the 800 kb transgene reproduces ectopically the tissue- and gamete-of-origin-specific expression of all the IC2 genes that are normally imprinted both in the fetus and placenta and the tissue-specific expression of the genes imprinted exclusively in the placenta.

Imprinted methylation of IC2 DMRs on the 800 kb transgene

A germ-line derived (primary) and maternally methylated DMR (KvDMR1 or IC2) and two somatically acquired (secondary) and paternally methylated DMRs (*Cdkn1c* and *Tssc4* up-stream regions) are present in the IC2 domain (34) (Fig. 3A). KvDMR1 and *Cdkn1c* DMRs display their gamete-of-origin-specific methylation both in embryonic and extraembryonic tissues, whereas the *Tssc4* DMR is only methylated in the placenta (34,35). We investigated whether the imprinted methylation of these DMRs was reproduced on the 800 kb transgene. KvDMR1 methylation was analysed by digestion with the methylation-sensitive *Sma*I enzyme and Southern blotting. An *Apa*I RFLP was used to distinguish the transgenic from the endogenous alleles (Fig. 3C). Because the IC2 CpGs are homogeneously methylated on the maternal chromosome and homogenously non-methylated on the paternal chromosome (34), the methylation of the centrally located *Sma*I site was taken as an indication for the methylation status of the entire DMR. The results demonstrated that this region of the transgene was methylated both in the fetus and placenta only when maternally inherited, as at the endogenous locus (Fig. 3C and data not shown). Methylation of the *Cdkn1c* DMR was analysed by bisulphite sequencing in neonatal kidney and placenta and that of the *Tssc4* DMR in the placenta. The results showed that the *Cdkn1c* DMR was methylated only after paternal transmission of the transgene both in embryonic and extraembryonic tissues (Fig. 3B and data not shown). Differently from the endogenous locus, the transgenic *Tssc4* DMR was unmethylated on both maternal and paternal transmission (Fig. 3D and data not shown). Thus, the 800 kb transgene ectopically reproduced the imprinted methylation of the DMRs that, at the endogenous loci, are differentially methylated both in the fetus and in the placenta but not of the DMRs showing differential methylation only in the placenta.

Life-cycle of KvDMR1 methylation on the 800 kb transgene

Methylation at the endogenous KvDMR1 locus is erased in the primordial germ cells and re-established in the mature oocyte, but not in the sperm (34,36,37). We investigated whether the maternal methylation of the transgenic KvDMR1 allele was also acquired in the oocyte and whether it was correctly reprogrammed when passed through the male germ-line. KvDMR1 methylation was first analysed by bisulphite

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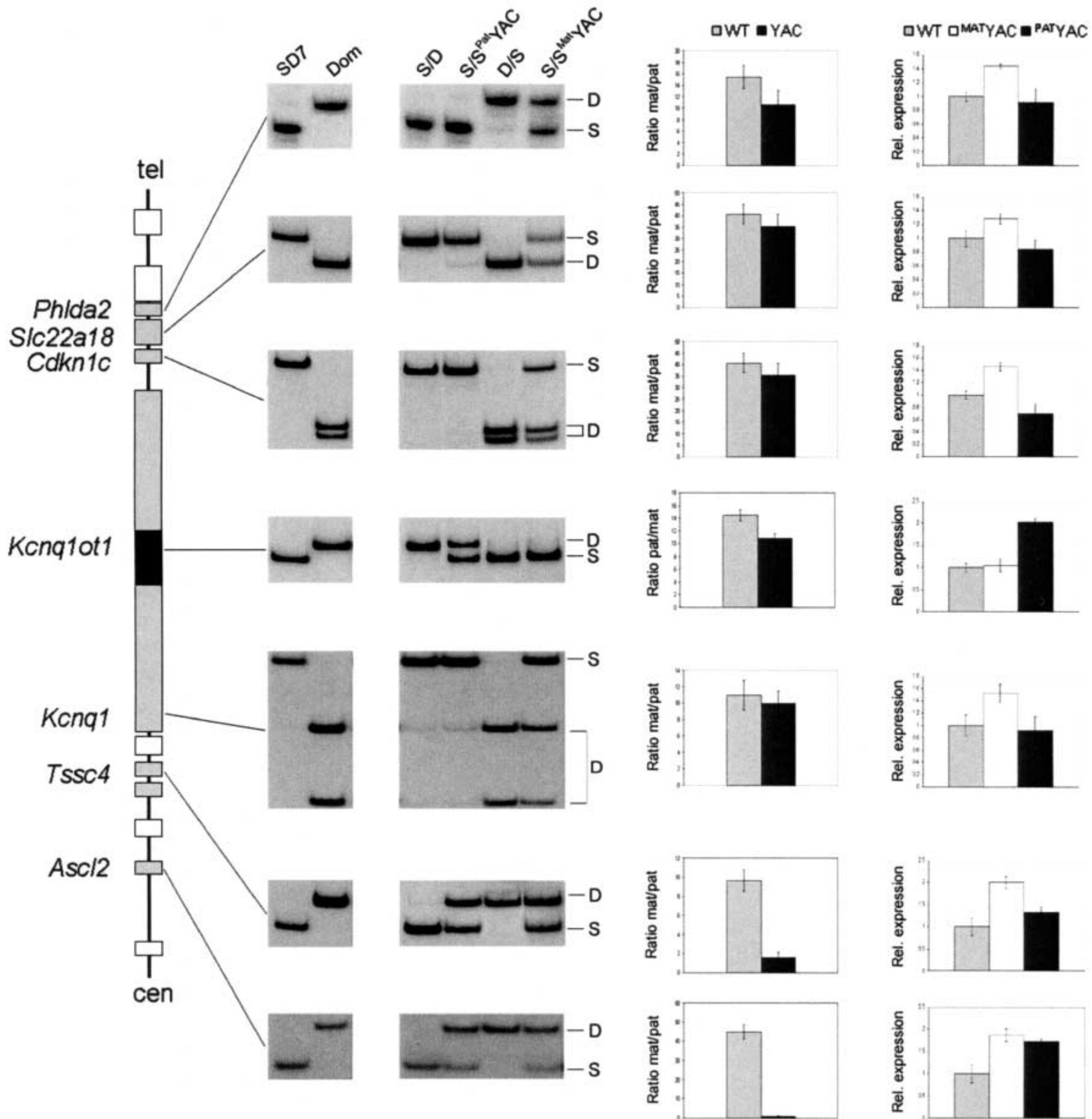

Figure 2. Imprinting and overall expression of IC2-dependent genes on the 800 kb transgene. RNA extracted from placentas of E13.5 mice following paternal and maternal transmission of the 800 kb transgene (SD7/SD7 $^{Pat/Mat}$YAC) and their corresponding wild-type littermates (Dom/SD7, SD7/Dom) was reverse-transcribed, the imprinting analysed by allele-specific RT–PCR and overall gene expression determined by quantitative real-time RT–PCR with primers specific for the *Phlda2*, *Slc22a18*, *Cdkn1c*, *Kcnq1ot1*, *Kcnq1*, *Tssc4*, *Ascl2* and *Gapd* genes. The polymorphisms used to distinguish between expression from the YAC transgene and that of the endogenous alleles are reported in Table 1. The figure shows one representative experiment chosen among three with similar results. The first series of histograms on the right of each panel indicates the mean ratios between the expression levels of the maternal and paternal alleles of wild-type and transgenic mice. The second series of histograms indicates the relative expression of the IC2 genes in mice with maternal or paternal transmission of the 800 kb transgene and their wild-type littermates. S, SD7; D, *Domesticus*. The imprinting of *Phlda2*, *Slc22a18*, *Cdkn1c*, *Kcnq1ot1* and *Kcnq1* is correctly reproduced on the 800 kb transgene.

sequencing in unfertilised oocytes derived from adult females that had paternally inherited the 800 kb transgene (Fig. 4A). The results showed that the transgenic, as well as the endogenous KvDMR1, alleles were methylated in the female gametes (Fig. 4A and B, II2–II5). We then asked whether, the methylation of the transgenic KvDMR1 was erased when passed through the male germ-line, as it happens at the endogenous locus. Methylation was therefore determined in the somatic cells of fourth generation-mice that had paternally inherited the 800 kb transgene (Fig. 4A and C). The analysis by bisulphite sequencing showed that the transgenic KvDMR1 (*Domesticus* allele) was unmethylated in such a mouse (IV2), whereas it was normally methylated in his father (III1) demonstrating that the life-cycle of the primary DMR of the IC2 domain was correctly reproduced on the 800 kb transgene (Fig. 4C).

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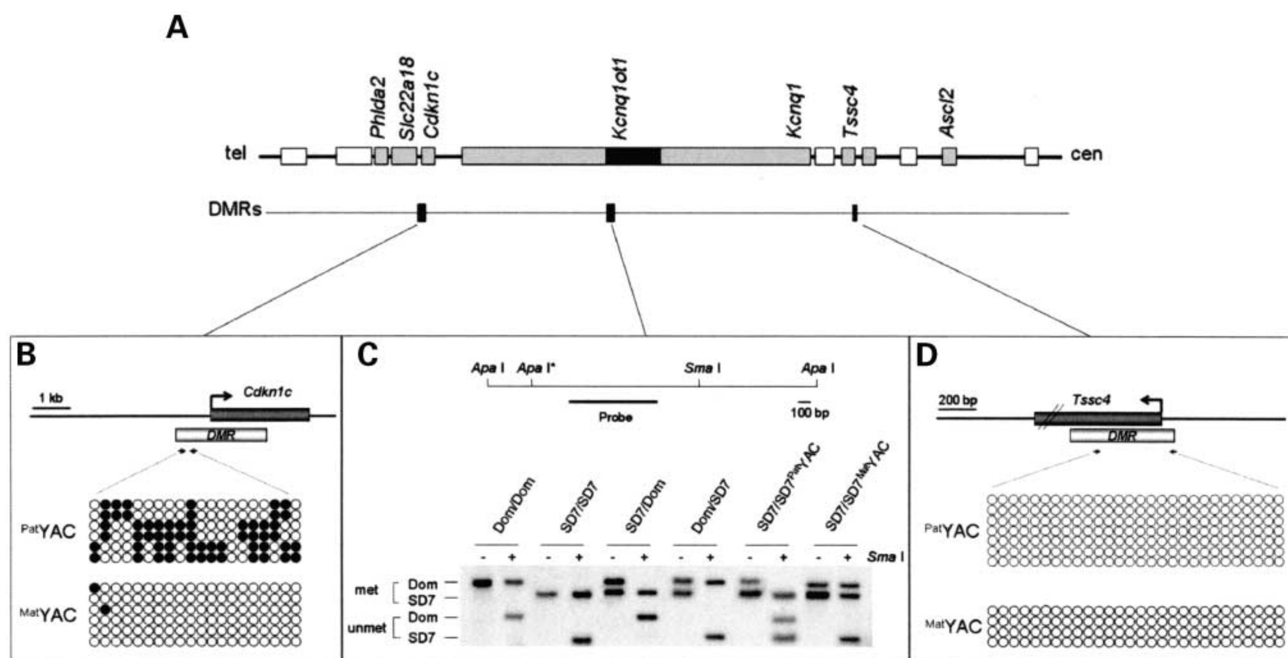

Figure 3. Imprinted methylation of IC2 DMRs on the 800 kb transgene. (**A**) Diagram showing the relative positions of the DMRs in the IC2 domain. (**B–D**) DNA methylation analysis of the *Cdkn1c* DMR (B), KvDMR1 (C) and *Tssc4* DMR (D) on the 800 kb transgene. Genomic DNA was extracted from the placenta of E13.5 conceptuses with paternal (^{Pat}YAC) or maternal (^{Mat}YAC) transmission of the 800 kb transgene. For the analysis of the *Cdkn1c* (B) and *Tssc4* (D) DMRs, DNA samples were treated with sodium bisulphite, amplified by PCR, cloned and sequenced. Each line corresponds to a single template DNA molecule and each circle represents a CpG dinucleotide. Filled circles designate methylated cytosines and open circles correspond to unmethylated cytosines. The position of the primers is indicated by arrows. The DNA methylation of KvDMR1 (C) was determined by Southern blotting. The patterns obtained in the transgenic mice are compared with those of wild-type SD7/SD7, Dom/Dom, SD7/Dom and Dom/SD7 mice. DNA was digested with *Apa*I with (+) or without (−) the addition of the methylation-sensitive restriction enzyme *Sma*I and was hybridized with a 0.7 kb *Kcnq1ot1* cDNA probe. An *Apa*I RFLP (*Apa*I*) was used to distinguish the transgene (Dom) from the endogenous locus (SD7) in the transgenic mice and the maternal from the paternal allele in the wild-type mice. The maternal-specific methylation of KvDMR1 and the paternal-specific methylation of the *Cdkn1c* DMR are reproduced on the 800 kb transgene.

Growth-deficient phenotype in mice with maternal inheritance of the 800 kb transgene

Mice with maternal duplication/paternal deficiency for the distal chromosome 7 are growth-deficient, whereas mice with paternal duplication/maternal deficiency leads to embryo growth enhancement (38). These phenotypes are caused partly by the presence, on distal chromosome 7, of the paternally expressed *Igf2* gene that encodes an embryonic mitogen. However, other imprinted genes of this chromosome region might also contribute to the phenotypes. Maternal transmission of the 800 kb transgene increases the expression of the maternally expressed imprinted genes of the IC2 domain (Fig. 2). The mRNA of two of these genes (*Tssc4* and *Ascl2*, in addition to the paternally expressed *Kcnq1ot1*) are increased also in the transgenic mice with paternal transmission, because the imprinting of these genes is not reproduced by the transgene. We therefore investigated the growth phenotype after maternal and paternal transmission of the transgene. We observed that at birth, the mice with maternal transmission were 18% growth-retarded when compared with their wild-type littermates (Fig. 5). No difference in birth weight was found between the mice with paternal transmission and wild-type mice (Fig. 5). This indicates that the presence of two active maternal alleles of *Phlda2*, *Slc22a18*, *Cdkn1c* and *Kcnq1* results in prenatal growth retardation. This phenotype is consistent with that observed after deletion of KvDMR1 (18).

DISCUSSION

It has been previously observed that two different ICs control a centromeric and a telomeric set of imprinted genes in the mouse distal chromosome 7 cluster. It was also demonstrated that additional *cis*-acting elements contribute to the imprinting control of these genes. However, it was unclear whether the two sets of genes shared imprinting control elements and needed to be located close to each other for proper function or were regulated independently and could be expressed normally even if removed from their normal chromosomal context. We demonstrated that the imprinted methylation of IC2 and the imprinted expression of the paternally expressed anti-sense *Kcnq1ot1* gene, together with that of four IC2-dependent maternally expressed genes, are reproduced ectopically on an 800 kb transgene that does not contain any IC1 gene or regulatory element. These results support the hypothesis that each of the two domains of the BWS locus contains all the information needed for its appropriate control (19).

BAC transgenes extending 50 kb up-stream and 260 kb down-stream of the *Cdkn1c* transcription unit displayed neither the maternal-specific methylation of KvDMR1 nor

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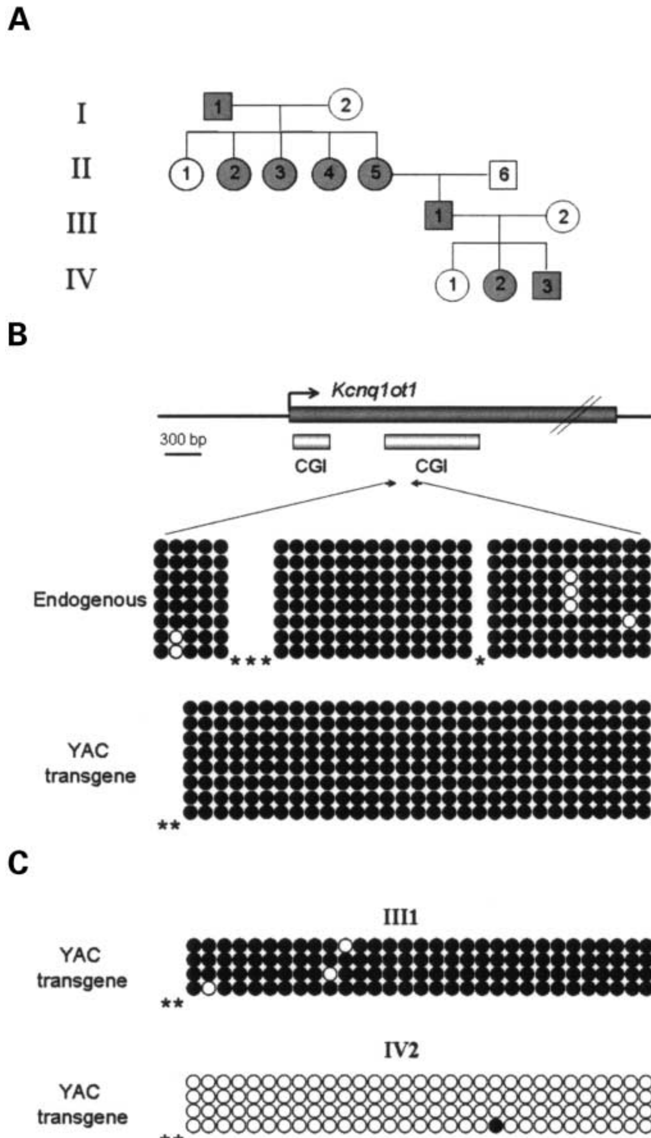

Figure 4. Life-cycle of *KvDMR1* methylation on the 800 kb transgene. (**A**) Pedigree of the mice analysed. Filled symbols indicate mice carrying the 800 kb transgene and open symbols represent wild-type animals. (**B**) DNA methylation of KvDMR1 in the female gametes, as determined by bisulphite sequencing. Unfertilized oocytes were collected from adult SD7/SD7 females inheriting the transgene from the father (II2–II5). The transgene was distinguished from the endogenous alleles by the presence of a single nucleotide polymorphism and a CGGCCGTGAAACGAGGAC insertion/deletion polymorphism. Polymorphic CpGs are indicated by asterisks. The extension of the KvDMR1 CpG island (CGI) is shown. (**C**) Demethylation of the transgenic KvDMR1 in mice with paternal transmission of the 800 kb transgene. KvDMR1 methylation was analyzed by bisulphite sequencing on DNA extracted from the tails of a mouse with maternal inheritance (III1) and one with paternal inheritance (IV2) of the 800 kb transgene. On the 800 kb transgene, KvDMR1 methylation is correctly acquired in the female gametes and normally reprogrammed when passed through the male germ-line.

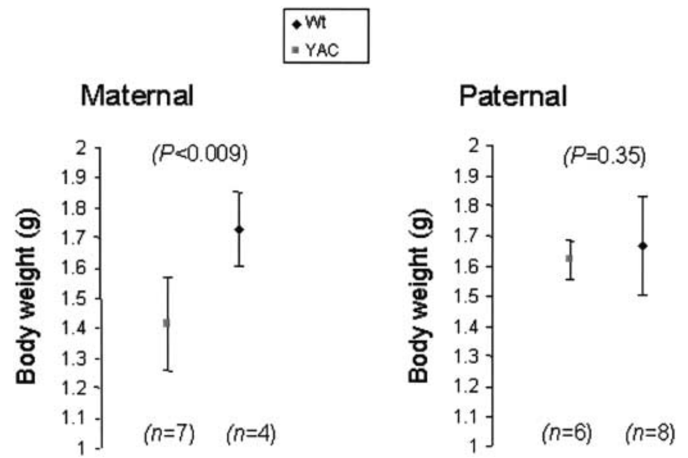

Figure 5. Phenotype of the mice carrying the 800 kb transgene. The histograms show the body weights at birth of the mice with maternal and paternal inheritance of the 800 kb transgene and their wild-type littermates. Data are expressed as mean ± SEM. Statistical significance using Student's *t*-test is indicated as *P*-value. Mice with maternal transmission of the transgene are 18% growth-retarded when compared with their wild-type littermates.

the maternal-specific expression of *Cdkn1c* (28). In contrast, appropriate imprinted methylation of KvDMR1 and appropriate imprinted expression and methylation of *Cdkn1c* were observed on our 800 kb transgene. In addition, when present on this larger transgene, KvDMR1 methylation was normally established in oocytes and erased if passed through the male germ-line. Therefore, key regulatory elements for the imprinting of the IC2 domain must be located in the 400 kb centromeric to the BAC transgenes (Fig. 1). This region is also required for the placenta-specific expression of *Cdkn1c*, because this was absent from the BAC transgenes. These results are consistent with the observation that a targeted translocation between *Cdkn1c* and KvDMR1 results in loss of imprinting and inappropriate expression of telomeric genes, but not of those centromeric of the breakpoint (39).

About half of the patients affected by BWS lack imprinted methylation of KvDMR1 (10,11). This is associated with activation of *KCNQ1OT1* on the normally silent paternal allele and is believed to lead to down-regulation of *CDKN1C* and other maternally expressed genes of the IC2 domain (13,25,26). No genetic defect has been so far associated with these epigenetic abnormalities. We have recently shown that BWS patients with imprinting defects in the IC1 domain had microdeletions in the *H19* DMR (16). The results obtained in transgenic mice suggest that some of the patients with KvDMR1 hypomethylation may have a mutation in the centromeric imprinting control element of the IC2 domain. A few BWS patients have a balanced translocation with breakpoints on the maternal chromosome 11p15.5 (40). Interestingly, the majority of these chromosome rearrangements interrupt the *KCNQ1* primary transcript. Because the KCNQ1 protein is unlikely to have a role in BWS, it is possible that its RNA is involved in the control of the expression of other genes. Its function could be exerted, for example, by inhibiting the expression of the anti-sense *Kcnq1ot1* gene on the maternal chromosome. Therefore, the *Kcnq1* promoter is a candidate for the centromeric imprinting control element of the IC2 domain.

We have recently shown that the genes regulated by IC2 that are imprinted exclusively in the placenta do not depend on DNA methylation for the differential expression of their

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parental alleles (35). Instead, placental imprinting relies on repressive histone methylation on the paternal chromosome. It is likely that this type of imprinting mechanism is less stable than that dependent on DNA methylation. The repressive chromatin conformation of the paternal *Tssc4* and *Ascl2* alleles may be disrupted and/or activating histone modifications may be acquired on the 800 kb transgene, as suggested by the absence of DNA methylation on the paternal *Tssc4* DMR. This could result from position effects from nearby loci and explain why the imprinted expression of these genes is not reproduced on the transgene. It cannot be excluded, however, that the imprinting of *Tssc4* and *Ascl2* requires additional centromeric control elements and these are absent on the transgene. The lack of differential methylation of the *Tssc4* DMR on the transgene indicates that this region is not involved in the control of the telomeric part of the IC2 domain and is consistent with the observation that this is a secondary DMR (34).

The distal chromosome 7 imprinting cluster is well conserved during evolution. Linkage between the IC1 domain- and IC2 domain-genes is already evident in chicken and zebrafish (www.ensembl.org) and precedes the appearance of imprinting. What could be the cause of such linkage conservation if the two domains have separate control mechanisms? The growth phenotype that we observed after maternal transmission of the transgene could provide a possible explanation. Our results clearly show that the maternally expressed genes of the IC2 domain (likely *Cdkn1c*) inhibit the growth of the embryo. This is consistent with the phenotype of human individuals with maternal duplication of chromosome 11p15.5 (41) and contrasts with the growth-promoting property of the IC1 domain (mediated by the paternally expressed *Igf2* gene). Genomic imprinting is believed to have evolved in mammals from a conflict between maternal and paternal genomes for the allocation of maternal resources to the offspring (42). The opposite functions of the two domains on embryo growth could have facilitated the acquisition and/or conservation of imprinting in the cluster.

MATERIALS AND METHODS

Modification of the YAC transgene and generation of mice carrying the 800 kb transgene

YAC W408A5 derived from the WI/MIT820 mouse YAC library (strain C57BL/6) and was previously characterised (30). The YAC vector was further manipulated by homologous recombination. It was first retrofitted with two copies of a neomycin-selection cassette into the left arm (43). The Herpes virus *TK* gene was then removed from the YAC vector, because high TK expression was reported to cause sterility in male mice (44). In order to introduce the YAC DNA into the mouse cells, yeast spheroplasts were fused with ES cells, as previously described (43). Using such procedure, we derived ES cell clones containing the YAC. An intact full-length transgene was detected in an ES cell clone using polymorphic markers between the YAC sequence (C57BL/6 genotype) and the endogenous locus (129Sv genotype). Chimeric mice were generated by injection of the YAC-containing ES cells into C57BL/6 blastocysts, which were implanted into pseudopregnant mice. Germ-line transmission of the 800 kb transgene was demonstrated for several chimeras. The transgenic mice were then crossed with the SD7 mouse line (*M. spretus* distal chromosome 7 on *M. domesticus* background) to provide a source of polymorphisms to distinguish expression and methylation of all the genes present on the transgene (*M. domesticus* C57/Bl6) from that of the endogenous locus. Mice were typed for the presence of the transgene by Southern blotting. Genomic DNA was extracted from tails according to standard techniques and digested with *Bam*HI. The probe used for hybridisation was obtained by amplification of the neomycin-resistance cassette (primers: NEO for 5′-GTCGAGCAGTGTGGTTTTGC-3′ and NEO rev 5′-CGAACAAACGACCCAACACC-3′; PCR conditions: 2 min at 95°C followed by 94°C for 30 s, 60°C for 30 s and 72°C for 30 s for 30 cycles followed by 72°C for 5 min) followed by cloning in pCG 2.1 vector (Topo-TA cloning kit, Invitrogen) and digestion with *Eco*RI.

RNA analysis

For all expression analyses, we recovered fetuses at E13.5 and extracted RNA from placenta, yolk sac and body using the Trizol reagent (Invitrogen). One microgram total RNA was treated with RNase-free DNase (Promega), and first-strand cDNA was synthesised using the Superscript II Reverse Transcriptase (Invitrogen) and random hexamers as primers, according to the protocol of the manufacturer. cDNA was amplified by hot-stop PCR by adding [α-32P]dGTP before the last cycle (45). Primers, PCR conditions and polymorphisms used for the analysis of each gene are described in Table 1. PCR products were digested with restriction enzymes and separated by electrophoresis on a non-denaturing polyacrylamide gel. For the analysis of *Phlda2*, γ-ATP was used for labelling one of the primers by polyncleotide T4 kinase (New England Biolabs) and the PCR products were directly run on a denaturing 8 M urea–6% polyacrylamide gel. The intensity of the bands was quantified by using a PhoshorImager and ImageQuant software by Molecular Dynamics. All RT–PCR assays were carried out in duplicate in the absence of reverse transcriptase to rule out effects from contaminating genomic DNA. The overall expression of the IC2 genes was determined by SYBR Green I real-time reverse transcription–PCR amplification (Applied Biosystems). Reactions were run on an ABI PRISM 7500 Sequence detector. The cycling conditions comprised a 50°C step for 2 min, a second stage step to 95°C for 10 min, followed by 40 cycles consisting of 15 s at 95°C and 1 min at 60°C followed by a stage of 15 s at 95°C, 1 min at 60° C and 15 s at 95°C. The concentration of the primers was 300 nM. Two independent cDNA preparations from each RNA sample were analysed in triplicate. All primer sequences are available upon request.

DNA methylation analysis

KvDMR1 methylation was analysed in embryonic tissues and placenta of E13.5 conceptuses by Southern blotting. Genomic DNA was digested with *Apa*I alone or in combination with the methylation-sensitive *Sma*I and hybridised with a *Kcnq1ot1*

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Table 1. Polymorphisms and PCR conditions for allele-specific expression analysis[a]

	Primers	PCR conditions	Dom/Spretus polymorphisms	Polyacrylamide gel 29:1
Ascl2	For 5′-TTAGGGGGCTACTGAGCATC-3′ Rev 5′-AAGTCCTGATGCTGCAAGGT-3′	Annealing at 58°C, 1 mM $MgCl_2$	*Bst*NI	12%
Tssc4	For 5′-GCTCCCCAAACCAGTGCCCC-3′ Rev 5′-AAAGGCCTTCGAGGTCCCCTG-3′	Annealing at 64°C, 1 mM $MgCl_2$	*Alu*I	7%
Kcnq1	For 5′-GATCACCACCCTGTACATTGG-3′ Rev 5′-CCAGGACTCATCCCATTATCC-3′	Annealing at 55°C, 1.3 mM $MgCl_2$	*Pvu*II	5%
Kcnq1ot1	For 5′-TTGCCTGAGGATGGCTGT-3′ Rev 5′-CTTTCCGCTGTAACCTTTCTG-3′	Annealing at 57°C, 1.7 mM $MgCl_2$	*Mwo*I	7%
Cdkn1c	For 5′-TTCAGATCTGACCTCAGACCC-3′ Rev 5′GACCGGCTCAGTTCCCAGCTCAT-3′	Annealing at 60°C, 1.5 mM $MgCl_2$	*Ava*I	8%
Slc22a18	For 5′-TGTCTGCCTGGGATGTCTG-3′ Rev 5′-GGCCGCCAGGAAGGAGAG-3′	Annealing at 61°C, 1 mM $MgCl_2$	*Hpa*II	7%
Phlda2	For 5′-GTATCAGCGCTCTGAGTCTG-3′ Rev 5′-ACACGGAATGGTGGGTTGGA-3′	Annealing at 57°C, 1 mM $MgCl_2$	6 bp insertion/deletion	6% 8 M urea

[a]The RFLPs have been previously described (15,31,39). The 6 bp insertion/deletion in the *Phlda2* gene was identified by sequencing the *M. domesticus* and *M. spretus* alleles.

cDNA clone (IMAGE 1265245) as a probe. Methylation of KvDMR1 in unfertilized oocytes and adult tails, methylation of the *Cdkn1c* DMR in neonatal kidney and E13.5 placenta and methylation of the *Tssc4* DMR in E13.5 placenta were analysed by sodium bisulphite-sequencing by following the conditions described by Cerrato *et al.* (46) and the primers reported by Yatsuki *et al.* (34) and Engemann *et al.* (36). DNA sequencing was obtained by PRIMM and TIGEM-IGB.

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