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Imprinted expression of the *Igf2r* gene depends on an intronic CpG island

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Gametic imprinting is a developmental process that induces parental-specific expression or repression of autosomal and X-chromosome-linked genes^{1,2}. The mouse *Igf2r* gene (encoding the receptor for insulin-like growth factor type-2) is imprinted and is expressed from the maternal allele after embryonic implantation³. We previously proposed that methylation of region 2, a region rich in cytosine-guanine doublets (a 'CpG island') in the second intron of *Igf2r*, is the imprinting signal that maintains expression

of the maternal allele⁴. Here we use mouse transgenes to test the role of region 2 and the influence of chromosome location on *Igf2r* imprinting. Yeast artificial chromosome transgenes successfully reproduced the imprinted methylation and expression pattern of the endogenous *Igf2r* gene; deletion of region 2 from these transgenes caused a loss of imprinting and restored biallelic *Igf2r* expression. These results define a primary role for region 2 and a negligible role for chromosomal location in *Igf2r* imprinting; they also show that methylation imprints can maintain allelic expression. Short transgenes containing only region 2 and yeast artificial chromosome transgenes with an inactive *Igf2r* promoter do not attract parental-specific methylation. All transgenes showing paternal-specific repression of *Igf2r* produced an antisense RNA whose transcription was dependent on region 2. The production of an antisense RNA by the repressed parental allele is reminiscent of the imprinting of the *Igf2/H19* gene pair⁵ and may indicate that expression competition could play a general role in imprinting.

The current model of the imprinting mechanism suggests that an imprinting signal, inherited during male or female gametogenesis, acts on one gene or a cluster of genes to generate allele-specific expression in diploid cells. The demonstration that transgenes of the imprinted *H19* gene^{6,7} can maintain imprinted expression at other chromosomal locations suggests that imprinting signals are closely linked to the affected genes. However, it is also clear from deletions of the mouse *H19* gene and deletions in the human Prader-Willi/Angelman syndromes^{8–10} that long-range *cis* effects are active in imprinting. A role for long-range effects and/or the chromosome location was also indicated by the clustering of imprinted genes into regions that show parental-specific differences in replication timing and recombination frequency^{11,12}.

Although, a common imprint sequence motif has not been identified among the twenty-two genes known to be imprinted^{1,2}, we have proposed that C + G-rich sequences that resemble CpG islands¹³ and which are associated with many imprinted genes and are often subject to parental-specific methylation, could act as a common imprinting element without a requirement for direct

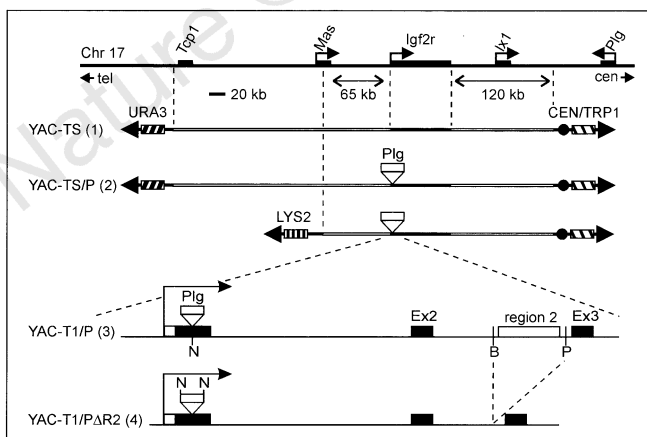


Figure 1 Cloning of YACs in *S. cerevisiae*. Top line shows the physical map (black boxes indicate genes) around the *Igf2r* locus²⁵ on mouse chromosome 17 and alignment of the YAC constructs YAC-TS, YAC-TS/P, YAC-T1/P and YAC-T1/PΔR2. YAC-TS (step 1) was modified by inserting a fragment of mouse Plasminogen cDNA (Plg) into the *NotI* site (N) in exon 1 of the *Igf2r* gene to give YAC-TS/P (step 2). This was truncated at the *Mas* locus to derive the 300-kb YAC-T1/P. YAC-T1/P was modified by deleting region 2 (steps 3 and 4), which gave YAC-T1/PΔR2. Correctly modified YAC clones were identified by standard Southern blot analysis and by PFGE. *Sall* fragments from 20–80 kb were unchanged in the YACs, and *EcoRI*, *HindIII*, *PstI* fragments (0.5–6.5 kb) were correctly modified around the *Igf2r* promoter and region 2 (data not shown).

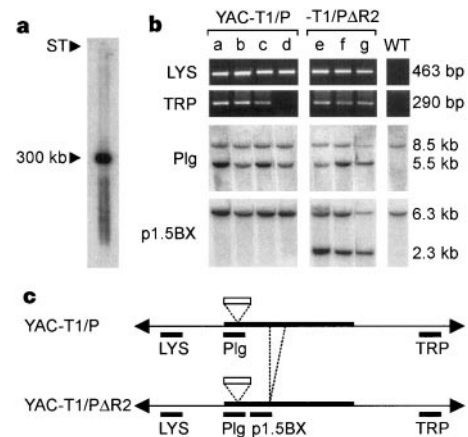


Figure 2 a, YAC DNA for microinjection (20 μl) separated by PFGE was hybridized with Plg cDNA; the 300-kb signal indicates intact YAC molecules (ST; slot). **b**, Analysis of transgene tail DNA of region-2-positive YAC-T1/P lines (a, b, c and d) and region-2-deleted YAC-T1/PΔR2 lines (e, f and g) by PCR of the YAC arms (LYS, TRP) and by blot-hybridization for *Igf2r* exon 1 (Plg) and region 2 (p1.5BX) to demonstrate the integrity of the YAC transgenes after integration into the mouse genome (WT, wild type). Line d lacked the TRP1 arm. The presence of the Plg-modified *Igf2r* exon 1 is shown by the 5.5-kb transgene-specific fragment; the 8.5-kb band is the endogenous Plasminogen gene. The 2.3-kb fragment identified by p1.5BX results from the deletion of region 2 from YAC-T1/PΔR2 transgenes (the endogenous region-2 fragment is 6.3 kb). **c**, The location of probes relative to the 300-kb YAC-T1/P and the 296-kb YAC-T1/PΔR2.

sequence homology¹⁴. The mouse *Igf2r* gene (for the insulin-like growth factor type-2 receptor, also known as the cation-independent mannose-6-phosphate receptor) contains in the second intron a 2-kilobase (kb) CpG island known as region 2, proposed to be an imprinting element⁴. It inherits a methylation mark from the female gamete which remains restricted to the maternal chromosome in diploid cells in the embryonic and adult stages. The remainder of a 130-kb region containing the 93-kb-long *Igf2r* gene is either equally methylated or, acquires allelic methylation late in development⁴. All imprinted genes examined so far are associated with patches of allele-specific methylation in adult tissues, but only three genes, *Igf2r*, *H19* and *Xist*, have been linked to germline-specific methylation imprints¹⁵. The significance of methylation in the imprinting mechanism has been demonstrated by the loss of allele-specific expression of *Igf2r*, *Igf2*, *H19* and *Xist* in mice deficient in genomic methylation following targeted mutation of the DNA methyltransferase^{16,17}.

The 450-kb yeast artificial chromosome construct YAC-TS, spanning the complete 93-kb *Igf2r* locus, was isolated from the ICRF C57BL6 YAC library¹⁸ using a Tcp1 (T complex protein 1, ref. 25) complementary DNA. YAC-TS was tagged (to disrupt the reading frame and provide a unique marker) by insertion of a Plg (plasminogen²⁵) fragment into the first exon of *Igf2r*. The YAC was shortened to 300 kb and shown to be collinear with the genomic map (data not shown) and renamed YAC-T1/P (Fig. 1). Four independent YAC-T1/P founder mice, lines a, b, c and d (Fig. 2b, c) were analysed for YAC-linked markers, bred with wild-type

C57BL6/CBAF1 mice, and expression of the YAC-T1/P-*Igf2r* allele was analysed after transmission through the male and female germ line. Imprinted *Igf2r* expression was studied in offspring hemizygous for the transgene in all major organs of adult mice and in day-13.5 embryos, by northern blot analysis (Fig. 3a) and RNase protection assay (Fig. 3b, c) using double-stranded and strand-specific probes based on the Plg tag inserted into the YAC-*Igf2r* allele. Northern analysis using a double-stranded Plg probe identified a 9-kb YAC-specific RNA in heart tissue following maternal transmission (heart is the principal site of *Igf2r* expression in adult mice³), and a marginally larger RNA following paternal transmission of the transgene (Fig. 3a), initially suggesting a lack of imprinting. However, analysis of the same RNA samples using the strand-specific riboprobe 1 (which specifically detects YAC-*Igf2r* messenger RNA) identified an RNA whose expression was seen only on maternal transmission (Fig. 3b, lanes M). Riboprobe 1 did not detect any mRNA from paternally inherited YAC transgenes (Fig. 3b, lanes P). The paternally expressed YAC-specific RNA (Fig. 3a, Plg) was shown, by using the strand-specific riboprobe 2, to be a transcript in reverse orientation to the *Igf2r* mRNA (referred to as an antisense RNA, or AS RNA). Riboprobe 2 identified the antisense RNA only in offspring with a paternally inherited YAC transgene (Fig. 3c, lanes P). All offspring with a maternally inherited YAC transgene lacked the antisense RNA (Fig. 3c, lanes M). The same antisense RNA is also expressed from the endogenous *Igf2r* locus at the exon 1/intron 1 boundary (Fig. 3c, lane 1)

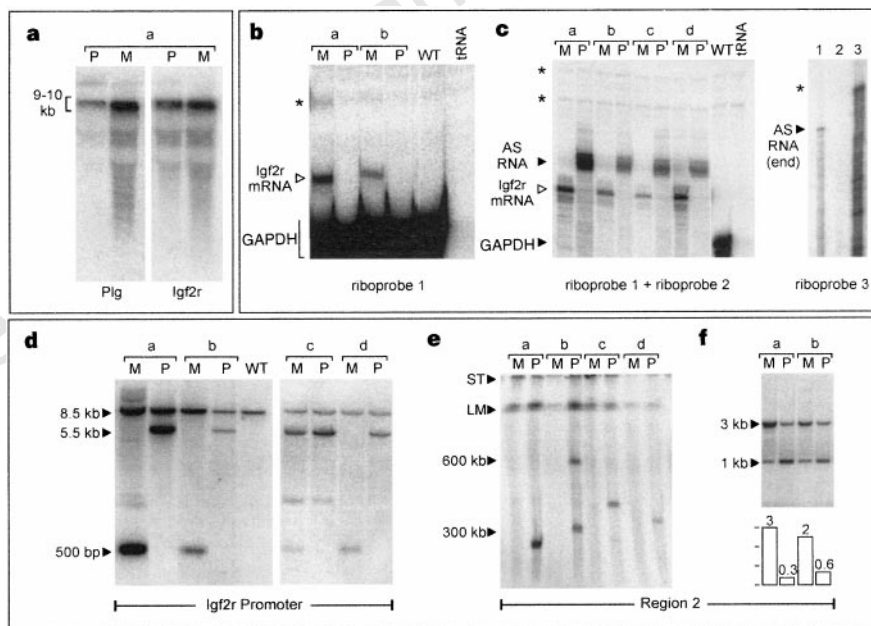


Figure 3 Parental-specific expression and methylation of YAC-T1/P transgenes. Four independent YAC-T1/P lines (a, b, c, d) were analysed after maternal (lane M) and paternal (lane P) transmission of the transgene. **a**, Heart RNA from line a, analysed by northern blot using a double-stranded Plg fragment (left) that identifies a transgene-specific signal, and the same filter rehybridized with a double-stranded probe spanning nt 500 to 1,500 of the 9-kb *Igf2r* cDNA (right) which identifies the endogenous plus transgene *Igf2r* signal. **b**, Heart RNA from line a and b transgenes, analysed by RNase protection and demonstrating maternal-specific expression of the YAC-*Igf2r* allele in adult heart. The probe, riboprobe 1, is specific for YAC-*Igf2r* mRNA and protected the expected 135-bp fragment following maternal transmission only. A GAPDH (glyceraldehyde-3-phosphate dehydrogenase) riboprobe (nt 794–698) was used as a loading control; an asterisk indicates the size of the input probe. **c**, RNase protection assay, demonstrating maternal YAC-*Igf2r* mRNA expression and paternal YAC-AS RNA expression in all four YAC-T1/P transgenes. YAC-*Igf2r* mRNA was assayed with riboprobe 1 and YAC-AS RNA with riboprobe 2: both are transgene-specific.

Lanes 1–3, RNase protection in wild-type RNA using riboprobe 3 (specific for the wild-type allele); lane 1, embryonic day 14 (end, endogenous AS RNA); lane 2, tRNA; lane 3, input riboprobe 3. **d**, Methylation of the YAC-*Igf2r* promoter. *Eco*RI- and *Not*I-digested DNA was hybridized with the Plg probe: a 5.5-kb fragment results if both *Not*I sites flanking the Plasminogen insert are methylated; the 0.5-kb band results if both these sites are unmethylated; the 8.5-kb fragment is the endogenous Plasminogen gene. **e**, Methylation of YAC region 2 assessed by *Mlu*I digestion, PFGE, and hybridization with the Plg probe. A single *Mlu*I site⁴ contained within region 2 is cut exclusively on paternal transmission of the transgene, as indicated by fragments (300–600 kb) entering the gel (LM, limiting mobility; ST, slots). **f**, DNA was digested with *Pvu*II and *Hpa*II and hybridized with a 1-kb *Mul*I-*Pvu*II fragment from region 2. The 3-kb band corresponds to methylated region 2, and the 1-kb band to unmethylated region 2. Signals from the endogenous and transgene loci are superimposed. The histogram below shows a quantification of the methylated (3 kb) to unmethylated (1 kb) signal using ImageQuant Software (Molecular Dynamics).

Table 1 Expression of YAC transgenes after maternal and paternal transmission

Transgene type	Transgene name	Copy number	Expression after maternal transmission	No. of offspring examined	Expression after paternal transmission	No. of offspring examined
YAC-T1/P	a	4-6	Igf2r mRNA	6	AS RNA	10
	b	2	Igf2r mRNA	19	AS RNA	11
	c	4	Igf2r mRNA [or AS RNA]	11 [12]	AS RNA	16
	d	2	Igf2r mRNA	2	AS RNA	6
YAC-T1/PΔR2	e	2	Igf2r mRNA	11	Igf2r mRNA	18
	f	4	Igf2r mRNA	3	Igf2r mRNA	3
	g	20	Igf2r mRNA	3	Igf2r mRNA	5

Expression of four YAC-T1/P and three YAC-T1/PΔR2 transgenic lines was examined following maternal or paternal transmission of the transgene. Imprinted expression of the YAC-Igf2r allele was maintained only in transgenes containing region 2 (YAC-T1/P lines a, b and d; the anomalous behaviour of line c is described in the text). YAC-T1/PΔR2 transgenes deleted for region 2 (lines e, f and g) expressed *Igf2r* mRNA biallelically. The copy number was estimated by the ratio of the PhosphorImager signal intensities of the Plg-tagged *Igf2r* exon 1 on the transgene to that of the endogenous Plasminogen locus.

specifically from the paternal chromosome (our unpublished data) and its involvement in repressing the paternal *Igf2r* allele is being assessed.

All the YAC transgenes analysed showed the expected³ tissue-specific expression pattern for *Igf2r* (data not shown). Three independent transgenes, lines a, b and d, showed the imprinted pattern of exclusive maternal-specific expression of *Igf2r* mRNA and exclusive paternal-specific expression of antisense RNA (Table 1) in embryonic and adult tissues. Furthermore, pedigrees from these three lines showed that imprinting was consistently reversed after passage through the germ line of the opposite sex in successive generations (data not shown). Lines a and b contained intact copies of YAC-T1/P, whereas line d lost the TRP1 arm but retained imprinted expression of *Igf2r* (Figs 2 and 3b, c). Males from line c did not pass the transgene to sons ($n = 56$), suggesting that it has been integrated into the X chromosome, but imprinting was preserved after parental transmission to daughters ($n = 26$), as shown in Fig. 3c (lane P). Maternal line c transmission ($n = 36$) resulted in male and female offspring which expressed only *Igf2r* mRNA (Fig. 3c, lane M), but also gave offspring expressing either the antisense RNA or both antisense RNA and *Igf2r* mRNA (data not shown), suggesting that there is a position effect at this integration site.

The 300-kb YAC-T1/P was modified to delete a 4-kb fragment containing all the CpG-rich sequences from region 2, and renamed YAC-T1/PΔR2. Region 2 is methylated at 28 CpGs spanning a 2-kb region⁴. Three transgenic YAC-T1/PΔR2 lines (e, f and g) containing intact copies were obtained (Table 1, and Fig. 2b). All three lines expressed Plg-tagged *Igf2r* mRNA following both maternal and paternal inheritance (Fig. 4a, b). Expression of *Igf2r* mRNA was comparable following maternal or paternal inheritance within any one line, and similar to that of *Igf2r* mRNA expressed from the YAC-T1/P transgenes that contained region 2. But, in contrast to the mice containing the region 2-positive YAC-T1/P transgene (Fig. 3), no antisense RNA was observed following paternal inheritance of the region-2-deleted YAC-T1/PΔR2 transgenes (Fig. 4a, b). The finding that deletion of region 2 restored paternal *Igf2r* mRNA expression but did not change maternal expression shows that region 2 is an imprinting element that appears to act only as a *cis*-repressor of the *Igf2r* promoter. Furthermore, whereas region 2 is not necessary for *Igf2r* mRNA expression, the lack of antisense RNA from the YAC-T1/PΔR2 transgenes indicates that region 2 is required for expression of this transcript and that a promoter and/or enhancer is contained in the 4-kb deletion. The negative correlation between antisense RNA production and region-2 methylation indicates that methylation has an inhibitory effect on this element.

To determine which part of the 4-kb element containing region 2 stimulates maternal-specific methylation, we generated a series of short transgenes centred around region 2 containing 1 kb (RII transgenes, five founder mice), 3 kb (P4 transgenes, five founders) or 14 kb of DNA (H/X-14 transgenes, two founders) (Fig. 5a). Methylation status was analysed following maternal and paternal

inheritance for three lines (two lines for H/X-14) for successive generations. Figure 5b-d shows that whereas all the region-2 short transgenes were methylated to various degrees, none showed any parental differences. The 1-kb RII transgene which contained the central repeat-rich part of region 2, previously proposed to be involved in attracting the gametic methylation imprint¹⁴, displayed a random methylation pattern in each litter (Fig. 5b). Offspring did not inherit the methylation status of the parent, instead hypomethylated and hypermethylated transgenes were found in each litter. Similarly, the P4 transgene was transmitted in a hypo- and hypermethylated form by both parents (Fig. 5c). However, P4 transgenes differed because their methylated status was stably inherited by the next generation. The 14-kb H/X-14 transgenes were relatively undermethylated, and this status was stably inherited in successive generations. The endogenous region 2 prevented analysis of these transgenes in oocytes or preimplantation embryos using a polymerase chain reaction (PCR) strategy¹⁹. However, the high-copy-number line-2 H/X-14 transgene (60-80 copies)

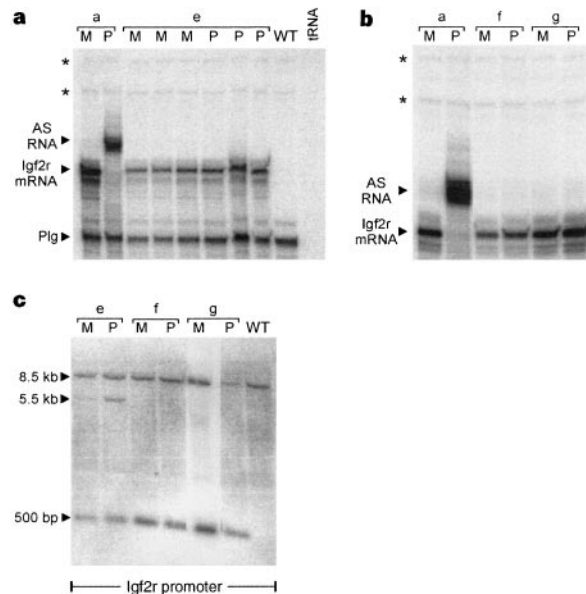


Figure 4 a and b, expression of the region-2-deleted YAC-T1/PΔR2 transgenes. Three independent lines (e, f, and g) analysed after maternal (lanes M) and paternal (lanes P) transmission of the transgene. **a, b**, Biallelic, non-imprinted expression of YAC-Igf2r mRNA in lines e, f and g transgenes in embryonic-day (E) 13.5 embryos using riboprobe 1 and riboprobe 2. Fetal liver endogenous Plg is used as a loading control. The imprinted YAC-T1/P line a was included as a control for riboprobe 2; asterisk indicates input probe. **c**, Methylation of the *Igf2r* promoter on the YAC-T1/PΔR2 transgene. Adult spleen DNA of transgenic mice was analysed as for Fig. 3d. A 0.5-kb fragment is seen after maternal and paternal inheritance in all three lines, indicating a lack of methylation regardless of the parental origin of the transgene. Line e is an exception (see text).

was analysed by DNA-blot hybridization and showed a low level of methylation at the *MluI* and *SfuI* sites in blastocysts after maternal inheritance (Fig. 5e).

Thus, short sequences around region 2 are insufficient to recapitulate the allelic methylation of the endogenous locus. These results contrast with those from CpG islands from non-imprinted genes which preserve their hypomethylated status as transgenes independent of transcription^{20,21}. Maintenance of methylation on region 2 may require other elements on the 300-kb YAC and expression from the *Igf2r* promoter may play a part. In support of this, two independent YAC transgenes inadvertently derived with an inactive *Igf2r* promoter (these transgenes failed to express *Igf2r* mRNA after insertion of the P_{lg} tag in the opposite transcription orientation to *Igf2r*; data not shown) were

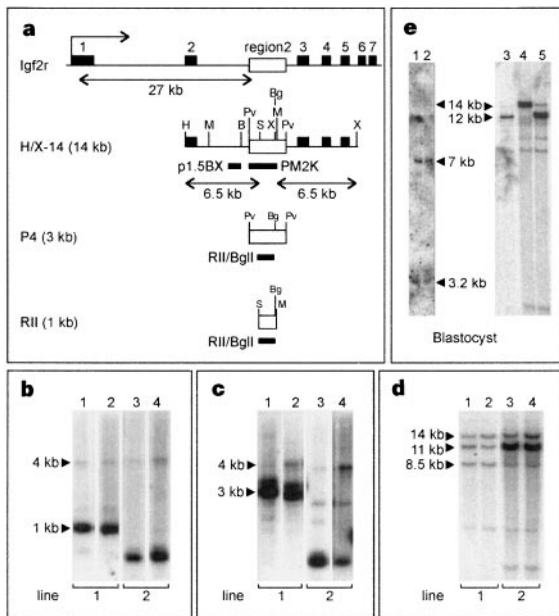


Figure 5 Methylation of region 2 on short transgenes. **a**, The mouse *Igf2r* locus and short transgenes carrying region 2. H/X-14 (a 14-kb *HindIII/XhoI/XhoI* fragment spanning exons 2–5); P4 (a 3-kb *PvuII–PvuII* fragment containing only region 2); RII (a 1-kb *SfuI–MluI* fragment from the central part of region 2 that contains direct repeats¹⁴). H, *HindIII*; S, *SfuI*; M, *MluI*; P, *PacI*; B, *BamHI*; Bg, *BglII*, P_v, *PvuII*, X, *XhoI*. **b**, Tail DNA digested with *BglII* and *HpaII* and blots hybridized with pRII/BgII. The transgene-specific 1-kb band is hemizygous and is not cut by *HpaII* in line 1, but is cut in line 2. Maternal transmission, lanes 1 and 3; paternal transmission, lanes 2 and 4. The intensity of the 4.0-kb endogenous band is reduced by 50% by *HpaII*. **c**, P4 transgenes analysed as in **b**. The transgene-specific 3-kb band is not cut by *HpaII* in line 1, but is cut in line 2. Maternal transmission, lanes 1 and 3; paternal transmission, lanes 2 and 4. **d**, H/X-14 transgenes (line 1: 2–3 copies, line 2: 60–80 copies). The transgene-specific 14-kb fragment is cut to 11 kb when *MluI* is unmethylated. Maternal transmission and paternal transmission blots were hybridized with p1.5BX. The 8.5-kb endogenous band is the flanking *BamHI* fragment upstream of region 2. The single tissue analysed in **b–d** is representative of a range of adult organs and E13.5 embryonic tissue. **e**, Methylation in blastocysts of line-2 H/X-14 transgene after maternal inheritance. In blastocysts, an additional *MluI* site upstream of region 2 in intron 2 is unmethylated on both parental alleles, so a 7-kb fragment will result if the *MluI* site inside region 2 is also unmethylated. Lanes 1 and 2, duplicate samples of 150 blastocysts digested with *BamHI* and *MluI* hybridized with probe PM2K. Only a 7-kb *MluI–MluI* fragment is seen, indicating hypomethylation at the region 2 internal *MluI* site. This fragment is reduced to 3.2 kb by *BamHI* (the *BamHI* digest was incomplete). Lane 3, 230 blastocysts digested with *BamHI* and *SfuI* hybridized with probe PM2K. The 14-kb fragment is cut to 11 kb when *SfuI* is unmethylated. Lanes 4 and 5, control digest with 0.1 μg adult spleen DNA from a maternally inherited H/X-14 transgene. The *SfuI* site on the 14-kb *BamHI* transgene-specific fragment remains hypomethylated in this tissue.

not methylated at region 2 after maternal or paternal inheritance and, in keeping with the results shown in Fig. 3, expressed the antisense RNA following maternal and paternal inheritance.

Maternally inherited YAC-T1/P transgenes (lines a, b and d) expressed *Igf2r* mRNA from an unmethylated promoter but were methylated at region 2 and did not express antisense RNA. Paternally inherited transgenes showed the opposite pattern: expression of antisense RNA and lack of methylation at region 2, plus methylation of the *Igf2r* promoter and absence of *Igf2r* mRNA (Fig. 3d–f). This transgenic methylation/expression pattern mimics the behaviour of the endogenous locus, where methylation of region 2 is acquired in the female germ line but *Igf2r* promoter methylation is acquired later in embryonic development⁴. Most transgenes behaved like the endogenous locus, but offspring from YAC-T1/P line c and YAC-T1/PΔR2 line e expressed *Igf2r* mRNA after maternal transmission (Figs 3d, lane c(M) and 4c, lane e(M)), despite sparse methylation at the *Igf2r* promoter. We suggest that this reflects stochastic methylation in a minority of cells and that other factors such as chromosomal position can influence methylation of the *Igf2r* promoter, despite the fact that the YAC transgenes carry 65 kb of 5'-flanking DNA.

The mouse *Igf2r* gene lies in a 2.0-megabase region on chromosome 17 which shows a parental bias in replication timing²². As *Igf2r* can retain imprinted expression at different autosomal locations, these long-range effects are not dominant, and/or they may arise from the imprinted gene itself. The imprinted *H19* gene lies in a domain on chromosome 7 that also shows a replication-timing bias, and *H19* also retains imprinted behaviour at other chromosomal sites^{6,7}. Analysis of *Igf2r* and *H19* highlights the importance of short-range elements in the imprinting process, which is striking in view of the known clustering of imprinted genes which implicates long-range control elements. The *Igf2r* maternal germline methylation imprint on region 2 correlates with maternal repression of the antisense RNA and maternal expression of *Igf2r* mRNA. In contrast, the *H19* paternal germline methylation imprint correlates with paternal repression of the *H19* allele and with paternal expression of the flanking *Igf2* and insulin genes^{6–8}. Despite these differences, imprinting of the *Igf2r*/AS-RNA gene pair may be similar to that of *Igf2* and *H19*. Our result showing that repression of the *Igf2r* promoter can be relieved by deletion of region 2 has parallels with the de-repression of *Igf2* and insulin following deletion of the *H19* gene⁸. These results from two distinct imprinted loci support the concept of expression competition⁵ as a unifying mechanism in imprinting and indicate that the view of imprinting as one active and one silent parental allele may need revision. □

Methods

YAC modifications. A plasminogen cDNA fragment (nucleotides 2,105–2,572) was inserted into the *NotI* site in *Igf2r* exon 1 by homologous transformation of YAC-TS in *Saccharomyces cerevisiae* AB1380. A fragmentation vector²³ containing nucleotides 134–614 of the mouse *Mas* gene removed the 5' part of *Mas* and truncated the YAC to 300 kb (YAC-T1/P). *Mas* is thought to be imprinted in a developmental and tissue-specific manner²⁴, but we have shown that *Mas* is not imprinted in laboratory mice²⁵. Region 2 was deleted in two steps: a 4-kb *BamHI–PacI* fragment containing region 2 was replaced by the yeast *URA3* gene, then *URA3* was removed by homologous recombination with a 2.3-kb linear fragment containing region-2 flanking sequences. YAC-T1/PΔR2 thus contained a 4-kb deletion eliminating all the CpG-rich sequences of region 2 but leaving splice junctions of the flanking second and third *Igf2r* exons.

Transgenic mice. YAC DNA was isolated for microinjection by preparative pulsed-field gel electrophoresis (PFGE) using 0.5% MP agarose (Boehringer Mannheim) and DNA concentrated by standard electrophoresis into 4% Nusieve GTG agarose (FMC Corporation, Philadelphia, PA, USA). Agarose was removed by Gelase (Epicenter). The size and concentration of the isolated YAC

DNA was checked (Fig. 2a), dialysed (0.2 mM EDTA, 7.5 mM Tris-Cl, pH 7.5, 100 mM NaCl) by floating filter (Millipore VMWP04700), diluted and injected at 1 ng μl^{-1} as described²⁶. 503 zygotes (C57Bl6/CBA-F1) were injected with YAC-T1/P, seventeen pups developed, five were transgenic and four transmitted the transgene. Microinjection of 1,006 zygotes with YAC-T1/PAR2 DNA yielded 99 offspring, of which nine were transgenic; three lines were bred. YAC transgenes were genotyped by PCR. The acentric YAC vector arm was identified using the *LYS2* gene (463 bp, primer pair TTGGACAATGGCGAGGAT and ACGCTGTGTTTCAGTGGTACC). The centric YAC arm was identified using the *TRP1* gene (290 bp; primer pair TAAGTATTGTTGTGCACTTGCCTGC and GTATTTATATACTAAGCTGCCGGCGG). The integrity of the integrated YACs was assayed by probes p15 (5-kb *EcoRI* fragment containing the *Igf2r* promoter and exon 1), p1.5BX (a 1.5-kb *BAMHI-XbaI* fragment outside region 2 but within intron 2). Standard techniques were used for the experiments shown in Fig. 5. Other probes used were: PM2K (a 2-kb *PvuII-MluI* fragment within region 2), RII/BglI (a 0.8-kb *SfiI-BglI* fragment within region 2).

Blastocyst DNA. Blastocysts from hormone-primed²⁶ transgenic females were washed in M2 medium, embedded in 0.5% low-melting-point agarose, incubated for 24 h at 50 °C in 0.5 M EDTA, pH 8, 1% sodium lauryl sarkosine, 0.5 mg ml⁻¹ proteinase K, and digested with restriction enzymes.

Expression analysis. For northern blots and RNase protection assay, 5–10 mg total RNA were used²⁷. Riboprobe 1 (175 bp), 5' [nucleotides 117–82 of mouse *Igf2r* exon 1, joined to nucleotides 2,572–2,472 of mouse Plasminogen terminal exon]3', protected a 135-bp fragment specific for tagged YAC-*Igf2r* mRNA (Figs 3, 4). Riboprobe 2 (190 bp), 5' (nt 117–82 of mouse *Igf2r* joined to nt 2,105–2,238 of Plasminogen]3', protected a 142-bp fragment specific to tagged YAC-*Igf2r* AS RNA (and a fragment of 35 bp not resolved in the gels in Figs 3 and 4). Riboprobe 3 (243 bp) is a *SaII-SmaI* fragment containing 20 bp of exon 1 and 194 bp of intron 1, and protects a 214-bp fragment specific for wild-type AS RNA (Fig. 3c). In Fig. 4a, endogenous *Plg* mRNA, expressed in fetal liver, was also detected by riboprobe 1 as a 100-bp fragment. Products were analysed on 8% polyacrylamide/46% urea gels.

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Activation of ATP P2X receptors elicits glutamate release from sensory neuron synapses

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Painful stimuli to the skin initiate action potentials in the peripheral terminals of dorsal root ganglion (DRG) neurons. These action potentials propagate to DRG central terminals in the dorsal horn of the spinal cord, evoking release of excitatory transmitters such as glutamate onto postsynaptic dorsal horn neurons. P2X receptors, a family of ligand-gated ion channels^{1,2} activated by the endogenous ligand ATP, are highly expressed by DRG neurons^{3–5}. Immunoreactivity to P2X receptors has been identified in the dorsal horn superficial laminae associated with nociceptive DRG central terminals⁵, suggesting the presence of presynaptic P2X receptors. Here we have used a DRG–dorsal horn co-culture system to show that P2X receptors are localized at presynaptic sites on DRG neurons; that activation of these receptors results in increased frequency of spontaneous glutamate release; and that activation of P2X receptors at or near presynaptic DRG nerve terminals elicits action potentials that cause evoked glutamate release. Thus activation of P2X receptors at DRG central terminals can modify sensory signal throughput, and might even initiate sensory signals at central synapses without direct peripheral input. This putative central modulation and generation of sensory signals may be associated with physiological and pathological pain sensation, making presynaptic P2X receptors a possible target for pain therapy.

As occurs *in vivo*, glutamatergic synapses were formed between DRG and dorsal horn neurons in micro-island co-cultures, as determined by paired cell recording of evoked excitatory postsynaptic currents (EPSCs; not shown). By recording the response of dorsal horn neurons to focal application of ATP, we tested whether activation of P2X receptors on DRG neurons induced glutamate release. To limit ATP action to P2X receptors at or near DRG nerve terminals, DRG cell bodies and their proximal neurites were removed using a suction pipette before recording from a dorsal horn neuron (Fig. 1a). In other cases, when DRGs were grown as explants with dissociated dorsal horn neurons, DRG explants were removed.

By using a micro-island preparation similar to that shown in Fig. 1a (right), recordings were made from dorsal horn neurons while 200 μM ATP was focally puffed onto the dorsal horn neurites with their associated DRG nerve terminals for 100 ms. All experiments were conducted in the presence of 500 nM tetrodotoxin (TTX), which completely blocked the generation of action potentials by