Thirteen genes (*Cebpb*, *E2f1*, *Tcf4*, *Cyp24*, *Pck1*, *Acra4*, *Edn3*, *Kcnb1*, *Mc3r*, *Ntsr*, *Cd40*, *Plcg1* and *Rcad*) that probably lie in the distal imprinting region of mouse Chromosome 2 are not monoallelically expressed

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Summary

Seven imprinted genes are currently known in the mouse but none have been identified yet in the distal imprinting region of mouse Chromosome (Chr) 2, a region which shows striking linkage conservation with human chromosome 20q13. Both maternal duplication/paternal deficiency and its reciprocal for distal Chr 2 lead to mice with abnormal body shapes and behavioural abnormalities. We have tested a number of candidate genes, that are either likely or known to lie within the distal imprinting region, for monoallelic expression. These included 3 genes (*Cebpb*, *E2f1* and *Tcf4*) that express transcription factors, 2 genes (*Cyp24* and *Pck1*) that are involved in growth, 5 genes (*Acra4*, *Edn3*, *Kcnb1*, *Mc3r* and *Ntsr*) where a defect could lead to neurological and probably behavioural problems, and 3 genes (*Cd40*, *Plcg1* and *Rcad*) that are less obvious candidates but sequence information was available for designing primers to test their expression. On/off expression of each gene was tested by reverse transcription–polymerase chain reaction (RT–PCR) analysis of RNA extracted from tissues of mice with maternal duplication/paternal deficiency and its reciprocal for the distal region of Chr 2. None of the 13 genes is monoallelically expressed in the appropriate tissues before and shortly after birth which suggests that these genes are not imprinted later in development. This study has narrowed down the search for imprinted genes, and valuable information on which genes have been tested for on/off expression is provided. Since there is considerable evidence of conservation of imprinting between mouse and human, we would predict that the 13 genes are not imprinted in human. Five of the genes: *E2f1*, *Tcf4*, *Kcnb1*, *Cd40* and *Rcad*, have not yet been mapped in human. However, because of the striking linkage conservation observed between mouse Chr 2 and human chromosome 20, we would expect these genes to map on human chromosome 20q13.

1. Introduction

Genomic imprinting is the phenomenon whereby a germline marking results in the repression of one or other parental allele at some point in development. Previous genetic studies in mice using various Robertsonian and reciprocal translocations have defined 15 imprinting effects ranging from early and late embryonic lethalities and neonatal death to retarded growth, behavioural and morphological abnormalities suggesting that genes with important roles in growth and development are involved (Cattanach & Jones, 1994). The 15 imprinting effects are associated with 10 regions of the genome in 6 different autosomes (Beechey & Cattanach, 1994) and all the known imprinted genes (*Igf2*, *Igf2r*, *H19*, *Snrpn*, *Ins2* and *U2afbp-rs*), except *Ins1* (Davies *et al.* 1994) map to the defined imprinting regions. Several imprinted genes, *Igf2*, *H19* and *Ins2*, are clustered together within an imprinting region on distal Chr 7.

Two of the 15 imprinting effects have been associated with distal Chr 2 (Cattanach & Kirk, 1985). Maternal duplication/paternal deficiency of this region leads to mice with long flat-sided bodies and arched backs, which fail to suckle and are totally inactive at birth with very few surviving more than a few hours. Paternal duplication/maternal deficiency results in an opposite phenotype where the mice have short square bodies with broad flat backs, are distinctly hyperactive and generally survive for only a few days. Preliminary observations showing that the phenotype

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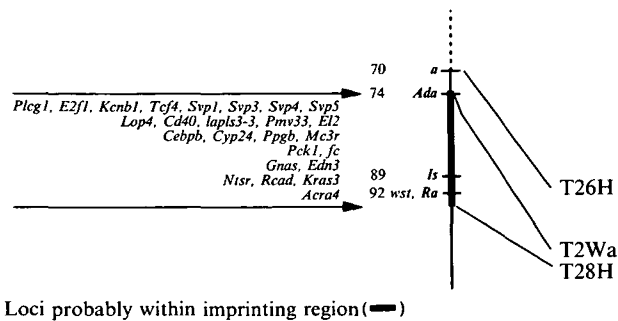

Fig. 1. Genetic map of distal Chr 2 showing the location of translocation breakpoints that define the imprinting region, and loci that are known or predicted to lie within the region. Anchor loci are shown in bold; the figures to the left of the genetic map are genetic distances in cM from the centromere (Hillyard *et al.* 1993). Mapping data were taken from Siracusa & Abbott, 1993; Copeland *et al.* 1993; Klocke *et al.* 1993; Matsunami *et al.* 1993; Abbott *et al.* 1994; Laurent *et al.* 1994; Li *et al.* 1994; Lock *et al.* 1994; Malas *et al.* 1994; Peters *et al.* 1994; Williamson *et al.* 1994 and Dutton *et al.*, personal communication.

associated with paternal duplication can be rescued by a maternally derived distal Chr 2 and *vice versa* for a maternal duplication suggests that at least two genes are imprinted in opposite directions (Beechey & Peters, 1994). From genetic studies the imprinting region is defined as lying between the breakpoints of two reciprocal translocations T(2;8)2Wa in band H3 and T(2;16)28H in band H4 and is estimated to be about 20 cM in genetic length and probably only 7·5 Mb in physical size (Cattanach *et al.* 1992; Peters *et al.* 1994). From the current data, *Ada* can be taken as the proximal, and *Ra* as the distal gene marker of the imprinting region on the linkage map (Peters *et al.* 1994). By comparing the available mapping data, a number of loci can be identified that are either likely or known to map within the imprinting region. These loci, excluding the simple sequence repeats, are shown in Fig. 1 (mapping data taken from Siracusa & Abbott, 1993; Copeland *et al.* 1993; Klocke *et al.* 1993; Matsunami *et al.* 1993; Abbott *et al.* 1994; Laurent *et al.* 1994; Li *et al.* 1994; Lock *et al.* 1994; Malas *et al.* 1994; Peters *et al.* 1994; Williamson *et al.* 1994 and Dutton *et al.*, personal communication).

Our aim is to identify the imprinted genes on the distal region of mouse Chr 2. Generally, studies of imprinted genes have shown that the product of one of the parental alleles is undetectable (for review see Cattanach & Jones, 1994). Our criterion for identifying an imprinted gene is based on looking for on/off expression of candidate genes in appropriate tissues taken from mice with maternal duplication/paternal deficiency and its reciprocal for distal Chr 2. Whereas observations so far indicate that a minimum of two genes must be imprinted to account for the phenotypes, the maximum number of genes that are imprinted is completely unknown both for distal Chr 2 and any other imprinted region. We have attempted to address this question by testing all possible known candidate genes on distal Chr 2. Such a candidate gene may be defined as a gene within the imprinting region that, based on either its expression pattern or known effects, could be responsible for the imprinting phenotype. The genes: *Cebpb, E2f1* and *Tcf4* (Fig. 1) code for transcription factors and are considered to be likely candidates because of their role in regulating the expression of a number of genes that may give rise to a range of different phenotypes. Imprinting of genes such as *Cyp24* and *Pck1* that are involved in growth may explain why the paternal duplication/deficient animals are slightly larger than the maternal duplication/deficient animals. Similarly the imprinting of genes such as *Acra4*, *Edn3*, *Kcnb1*, *Mc3r* and *Ntsr* that are expressed in the nervous system may explain the behavioural anomalies observed in the paternal duplication/deficient animals. Other genes, *Cd40*, *Plcg1* and *Rcad* that lie within the region are less obvious candidates. However, *Plcg1* and *Rcad* are expressed in brain where a defect could give rise to the phenotype. Of the remaining loci (*Svp1*, *Svp3*, *Svp4*, *Svp5*, *Lop4*, *fc*, *El2*, *Kras3*, *Pmv33*, *Iapls3-3* and *Gnas*) only *El2*, epilepsy 2, and *Gnas* appear to be good candidates. *El2* has not been cloned and so sequence was not available for designing primers to test expression and *Gnas* is unlikely to be monoallelically expressed as discussed later.

In this communication we describe expression studies to show that neither the maternal nor the paternal allele of 13 genes (*Cebpb*, *E2f1*, *Tcf4*, *Cyp24*, *Pck1*, *Acra4*, *Edn3*, *Kcnb1*, *Mc3r*, *Ntsr*, *Cd40*, *Plcg1* and *Rcad*) that are either likely or known to lie in the imprinting region are completely repressed.

2. Materials and Methods

(i) *Duplication-deficient mice for the distal region of mouse Chr 2*

Mice with maternal duplication/paternal deficiency and its reciprocal for the distal region of mouse Chr 2 can be generated by intercrossing heterozygotes for any reciprocal translocation that has a Chr 2 breakpoint proximal to the imprinting region. For this study, we used T(2;8)26H to generate duplication/deficient mice as shown in Fig. 2. In each mating one of the parents was homozygous for brachypodism, *bp*, a mutant causing shortening of the limbs (Grüneberg & Lee, 1973; Storm *et al.* 1994), which lies distal to the T26H breakpoint. Reciprocal crosses T26H+/++ × T26H*bp*/+*bp* were set up, from which it is expected that one-sixth of live-born offspring arising from normal adjacent-1 disjunction will be of genotype T26H+/++ and one-sixth will be of genotype T26H*bp*/+*bp*. The T26H+/++ offspring will carry a uniparental duplication of the distal Chr 2 translocated region (which includes the imprinting region)

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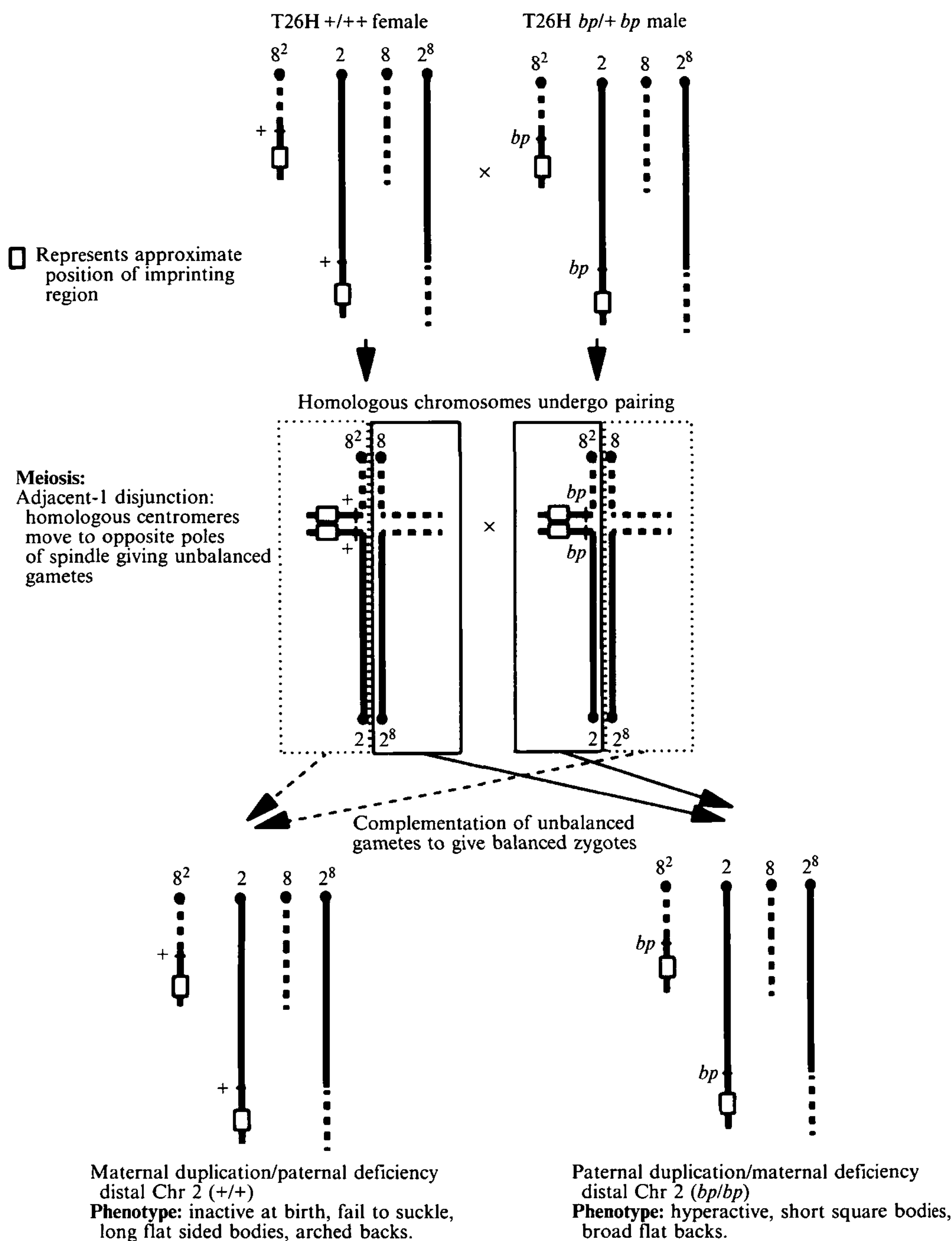

Fig. 2. Intercross of T(2:8)26H heterozygotes to generate maternal and paternal duplication/deficient mice. The solid line refers to Chr 2 and the dashed line refers to Chr 8. The imprinting region lies within the rearranged Chr 8^2 and the normal Chr 2 in both parents. Unbalanced gametes derived from adjacent-1 disjunction in both parents can pair to give chromosomally balanced zygotes which are either maternal duplication/paternal deficient or paternal duplication/maternal deficient for the region of mouse Chr 2 distal to the translocation breakpoint (and *vice versa* for Chr 8). These can be detected when one parent is homozygous for a gene, *bp*, that causes limb shortening and marks the distal region of mouse Chr 2. Adjacent-2 disjunction (not shown) can give rise, at low frequency, to phenotypically normal progeny which are paternal duplication/maternal deficient for the proximal region of Chr 2. Maternal duplication/paternal deficiency for the proximal region of Chr 2 is lethal (Cattanach & Kirk, 1985).

and a corresponding deficiency from the other parent, whereas the T26H *bp*/+*bp* progeny will carry the reciprocal duplication/deficiency. The *bp* marker provided a means of easily identifying duplication/deficient mice from 17·5 d of gestation (e17·5) onwards. Although Fig. 2 shows only the paternal duplication/maternal deficient offspring marked with brachypodism, both types of imprinted offspring could be detected by brachypodism as the reciprocal cross was made. Unmarked duplication/deficient mice were taken when the imprinting phenotype was clearly visible.

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(ii) *Reverse transcription–polymerase chain reaction (RT–PCR)*

Total RNA was isolated from tissue using RNAzol B (Biogenesis) based on the methods of Chomczynski & Sacchi (1987). The final pellet was resuspended in diethylpyrocarbonate-treated water. Total RNA (approximately 3 μg) was reverse transcribed with 200 units of M-MLV reverse transcriptase (Life Technologies) and oligo $(dT)_{15}$ as recommended by the supplier of the enzyme. An aliquot of the reverse transcription products equivalent to 0·5μg of RNA was mixed with the following reagents in a total volume of 22·5μl for the PCR: 1×PCR buffer (100 μg/ml DNase-free BSA [Pharmacia], 50 mM-KCl, 2·5 mM-$MgCl_2$, 20 mM-Tris-HCl, pH 8·0), 100 μM of each dNTP (Pharmacia), 320 μM DTT (Sigma), 0·05% W-1 (Sigma), 0·4 μM of each primer and five units of *Taq* polymerase (Boehringer Mannheim). The samples were amplified by PCR for 30 cycles using a Techne PHC-3 thermocycler. Each cycle consisted of 1 min at 94 °C, 1 min at 55 °C and 1 min at 72 °C, and ended with one cycle of 9 min at 72 °C. The *Cebpb*, *E2f1*, *Edn3*, *Kcnb1*, *Ntsr* and *Plcg1* primers were amplified in the presence of 20% glycerol to enhance specificity of PCR amplification (Lu & Négre, 1993). Two μl of 6× loading buffer (0·25% bromophenol blue in 30% glycerol) was added to 10 μl of the PCR products. The whole sample was separated on a 2% agarose gel in 1×TBE and the products were visualised by ethidium bromide (EtBr) staining. The PCR primers used for each gene are shown in Table 1. For some genes where the primers did not amplify across an intron, DNA contamination was excluded by the absence of signal in the sample without reverse transcriptase which was subjected to PCR in parallel under the same conditions.

(iii) *Probes and hybridization conditions*

If the PCR products were not visible by EtBr staining, the DNA was transferred to Hybond N^+ (Amersham) with 0·4 M-NaOH by Southern blotting. The PCR products for labelling were gel purified using either MERmaid (Stratch Scientific Ltd) for DNA less than 500 bp or GENECLEAN (Stratech Scientific Ltd) for DNA greater than 500 bp. Gel purified DNA (25 ng) was radiolabelled with 25 μCi [α-^{32}P]dATP (3000 Ci/mmol, ICN Flow) using the Megaprime kit as recommended by the supplier except that gene specific primers (1 μM) were used instead of the random nonamers. The filters were hybridized as described by Church & Gilbert (1984).

3. Results

Expression of 13 genes that lie within or close to the distal imprinting region was investigated in mice with maternal duplication/paternal deficiency and its reciprocal for distal Chr 2 to ascertain whether both maternally and paternally derived genes are expressed during development. On/off expression of each gene was tested by RT-PCR analysis using the primers listed in Table 1. Expression was investigated in at least one tissue where the gene had previously been reported to be expressed. The results are as follows:

(i) *Genes coding for transcription factors*

Cebpb is an intronless gene for CCAAT/enhancer binding protein. This gene shows homology with two other C/EBP-related genes, and encodes a liver enriched transcription factor that plays a role in maintaining/establishing the state of terminal cell differentiation and in cytokine induction of the acute

Table 1. *Primers for detecting expression of genes that map to the distal imprinting region of mouse Chr 2*

	Sequence of primers		
Locus	Forward	Reverse	References
Cebpb	5′-AAGAAGGTGGAGCAGCTGTCG-3′	5′-CGCAGGAACATCTTTAAGGTG-3′	Cao *et al.* (1991)
E2f1	5′-GGGTTTGGTTGCTGCCACAT-3′	5′-TGTTCACCTTCATTCCCGGG-3′	Li *et al.* (1994)
Tcf4	5′-CTGGTTCTGGTCGAATGGGC-3′	5′-AGCTTGATGAACTGGATCTGC-3′	Sladek *et al.* (1990)
Cyp24	5′-AGCTGCCCCATTGACAAACGG-3′	5′-AATCTGGCCATACTTCTTGTG-3′	Ohyama *et al.* (1993)
Pck1	5′-CGCACCATGTATGTCATCCC-3′	5′-GCGCAAAGCATTTCTTCCCA-3′	Beale *et al.* (1985)
Acra4	5′-GACGAGAAGAACCAGATGAT-3′	5′-ATGTCAGGCCTCCAGATGAG-3′	Goldman *et al.* (1987)
Edn3	5′-ATTCGTGCCTTGCCCCCAG-3′	5′-ATCCAGATGATGTCCAGGTG-3′	Bloch *et al.* (1989)
Kcnb1	5′-GAGGGCGTCATCGACATGCG-3′	5′-AGCAGCTGACCCTCGTCATC-3′	Pak *et al.* (1991)
Mc3r	5′-CTGCGGCGTGATGTTCATCA-3′	5′-GTAGCAGATGCAGTAGGGAT-3′	Genbank no. X74983
Ntsr	5′-CTTCAAGGCCAAGACCCTCAT-3′	5′-GGTAGGGCAGCCAGCAGACCA-3′	Vita *et al.* (1993)
Cd40	5′-GTTTAAAGTCCCGGATGCGA-3′	5′-CTCAAGGCTATGCTGTCTGT-3′	Grimaldi *et al.* (1992)
Plcg1	5′-CACTGTGGCTATGTGCTGCAGC-3′	5′-GAAGTTCTGGTCACTAAACATG-3′	Stahl *et al.* (1988)
Rcad	5′-CCGGAATTCACCACAAGCACA-3′	5′-GTCCTCCTCGCCACCCCCAT-3′	Matsunami *et al.* (1993)

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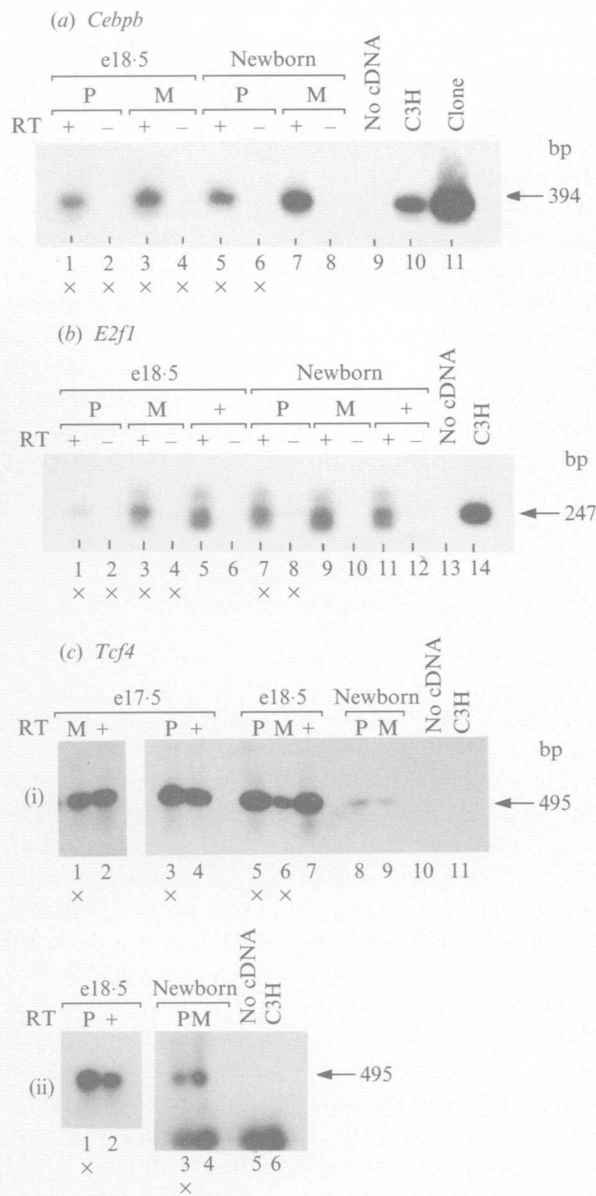

Fig. 3. RT–PCR showing expression of three transcription factor genes, *Cebpb*, *E2f1*, and *Tcf4* that map to the distal imprinting region of mouse Chr 2. RNA was from mice with maternal duplication/paternal deficiency (M) and its reciprocal for distal Chr 2 (P) and from siblings that appeared to be phenotypically normal (+) at a variety of ages from e17·5 to birth. The symbol '×' refers to samples taken from progeny that were identified by homozygosity for the *bp* marker gene. +/− RT refers to the presence and absence of reverse transcriptase. No amplification was observed in any of the samples lacking reverse transcriptase, indicating the absence of DNA contamination in the RNA samples. (*a*) Southern blot of *Cebpb* PCR products derived from kidney. The blot was probed with 394 bp product derived from C3H/HeH genomic DNA. Lane 11 is the product derived from amplification of an expression plasmid containing C/EBPB cDNA. (*b*) Southern blot of *E2f1* PCR products, derived from brain. The blot was probed with 247 bp product derived from C3H/HeH. (*c*) Southern blot of *Tcf4* PCR products derived from kidney (i) and from liver (ii). The blot was probed with 495 bp product derived from rat cDNA in pF7. C3H/HeH genomic DNA and 'no cDNA' were included as positive and negative controls, respectively.

phase response. Primers were designed from the mouse sequence (Cao *et al.* 1991) where the reverse primer was from the 3′ untranslated region of the gene in order to make the product specific to *Cebpb*. RT-PCR analysis was carried out in the presence and absence of reverse transcriptase (+/−RT). The PCR products were Southern blotted and probed with 394 bp product derived from C3H/HeH genomic DNA. A single band of 394 bp was amplified in +RT samples derived from lung (Fig 3*a*) and liver (results not shown) at e18·5 and at birth. The absence of bands in the samples lacking reverse transcriptase indicated that the amplification had not arisen from DNA contamination in the RNA samples. C3H/HeH genomic DNA, 'no cDNA' and plasmid containing C/EBPB cDNA (kindly supplied by S. L. Mcknight, Cao *et al.* 1991) were included as controls. The biallelic expression of *Cebpb* in lung and liver suggests that this gene is not imprinted.

E2f1 is the gene for transcription factor E2f-1. This factor has been implicated in controlling the activation of genes associated with cell proliferation. Direct evidence has shown that sustained unregulated expression of E2f-1 can lead to the loss of cell proliferation control (Singh *et al.* 1994). We investigated *E2f1* expression in the brain of duplication/deficient animals using primers corresponding to nucleotides 1518–1537 and the reverse sequence of nucleotides 1746–1765 from the 3′ untranslated region of the mouse sequence (Li *et al.* 1994). The 247 bp PCR products could only be detected after probing with the 247 bp product derived from C3H/HeH (Fig. 3*b*). Neither the maternal nor the paternal allele was repressed at e18·5 and at birth thus suggesting that this gene is not imprinted.

Tcf4 (formerly known as *Hnf4*) encodes the liver enriched transcription factor 4 that is expressed in liver, kidney and intestine (Sladek *et al.* 1990). This factor binds to sites required for the transcription of genes for transthyretin, apolipoprotein CIII, α1 antitrypsin and pyruvate kinase. PCR primers were designed from peptide 4 and peptide 2 of the rat cDNA sequence (Sladek *et al.* 1990). The PCR products were detected by probing with a 495 bp product derived from rat cDNA of pf7, which was kindly supplied by J. E. Darnell (Sladek *et al.* 1990). A 495 bp product was amplified from cDNA and no amplification was observed in genomic DNA probably because the presence of introns made the product too large to amplify in the conditions of this experiment. Neither the maternal nor the paternal allele of *Tcf4* was completely repressed in the kidney and liver (Fig. 3*c*(i) and (ii), respectively) of duplication/deficient mice at e17·5, e18·5 and at birth. The reduced intensity of bands in samples derived from kidney of 18·5 day maternal duplication/deficient embryo (lane 6) and in samples derived from kidney of newborn mice (lanes 8 and 9) was due to RNA loading as determined when *Hprt* primers were included as a coamplification

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control (results not shown). The lower band present in lanes 3–6 is due to the probe hybridizing to the primers.

(ii) *Other genes involved in growth*

In addition to the involvement of *E2f1* in growth, two other genes, *Cyp24* and *Pck1*, known to map to the region are also involved in growth and are therefore deemed likely candidates for the observed imprinting effects. Vitamin D 24-hydroxylase is encoded by *Cyp24* and contributes to the control of cellular growth, development and homeostasis. This enzyme is responsible for inactivating vitamin D metabolites through the C-24 oxidation pathway. In order to study expression of this gene, PCR primers were designed from exon 1 and exon 2 of the rat sequence (Ohyama *et al.* 1993). A PCR product of the expected size, 264 bp, was amplified from cDNA and a larger band was obtained with C3H/HeH genomic DNA. The band derived from cDNA was present in all kidney samples taken from duplication/deficient mice at e17·5, e18·5 and at birth (Fig. 4*a*) thus suggesting that this gene is also not imprinted.

Pck1 is the gene for phosphoenolpyruvate carboxykinase 1, a key regulatory enzyme of gluconeogenesis

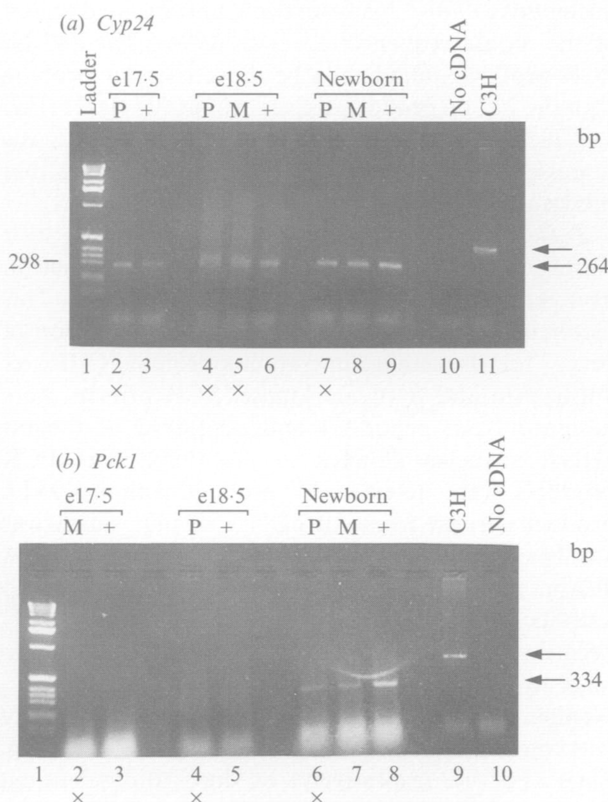

Fig. 4. RT-PCR showing expression of two genes, *Cyp24* and *Pck1* that are involved in growth. The abbreviations are given in the legend of Fig. 3. (*a*) EtBr stained gel of *Cyp24* PCR products derived from kidney. (*b*) EtBr stained gel of *Pck1* PCR products derived from liver. Size marker, 1-kb ladder (Life Technologies).

that is not expressed in the liver until birth (Garcia Ruiz *et al.* 1978). *Pck1* expression was investigated in liver with primers from exon 4 and exon 5 of the rat sequence (Beale *et al.* 1985). Since the genomic PCR product was significantly larger than the product derived from cDNA (Fig. 4*b*), control samples without reverse transcriptase were not included. The primers amplified a 334 bp product in the liver of duplication/deficient mice at birth. There was no amplification in prenatal samples thus showing that the enzyme had not been induced as a result of an imprinting effect. Northern analysis using 334 bp cDNA probe showed no evidence of monoallelic expression in the liver of newborn duplication/deficient mice (data not shown). These results suggest that *Pck1* is not imprinted in the liver.

(iii) *Genes with neurological functions*

Five genes were tested for monoallelic expression as follows: (1) *Acra4* is the gene for the fourth variant of the α subunit in the neuronal acetyl choline receptor which mediates synaptic activity in areas of the central nervous system. Gene specific primers were designed from exon 3 and exon 4 by comparing the sequences of rat (Goldman *et al.* 1987) and chicken (Nef *et al.* 1988) and choosing well conserved regions. Primers were designed across introns so that products derived from cDNA could be distinguished from those derived from contaminating genomic DNA. A single band of the expected size, 140 bp, and smaller than that obtained with genomic DNA, was amplified in all samples derived from the brain of maternal and paternal duplication animals at 18·5 d of gestation (e18·5) and at birth (Fig. 5*a*). The product was more easily visible after probing with the 2·2 kb *Hind* III rat cDNA, taken from pSP65, a kind gift from J.-L. Guénet. These results suggest that *Acra4* is not imprinted in the brain.

(2) *Edn3* is the gene for endothelin 3, a vasoconstrictive peptide that has two other related members. Messenger RNA derived from *Edn3* has been found in the fetal human hypothalamus suggesting it may have a role in neurotransmission. Gene-specific PCR primers were designed from the presumptive exon 1 of the gene by comparing rabbit (Ohkubo *et al.* 1990) and human (Bloch *et al.* 1989) sequences and choosing well conserved regions as described by Malas *et al.* (1994). A single band of 300 bp was amplified in all samples containing reverse transcriptase (Fig. 5*b*). Bands were not seen in samples without reverse transcriptase even after probing with the 300 bp cDNA product derived from a newborn that appeared phenotypically normal (Fig. 5*b*ii). These results suggest that *Edn3* is not imprinted in the lung of duplication/deficient animals at e18·5 and at birth. Furthermore, neither the maternal nor the paternal alleles were repressed in brain samples from

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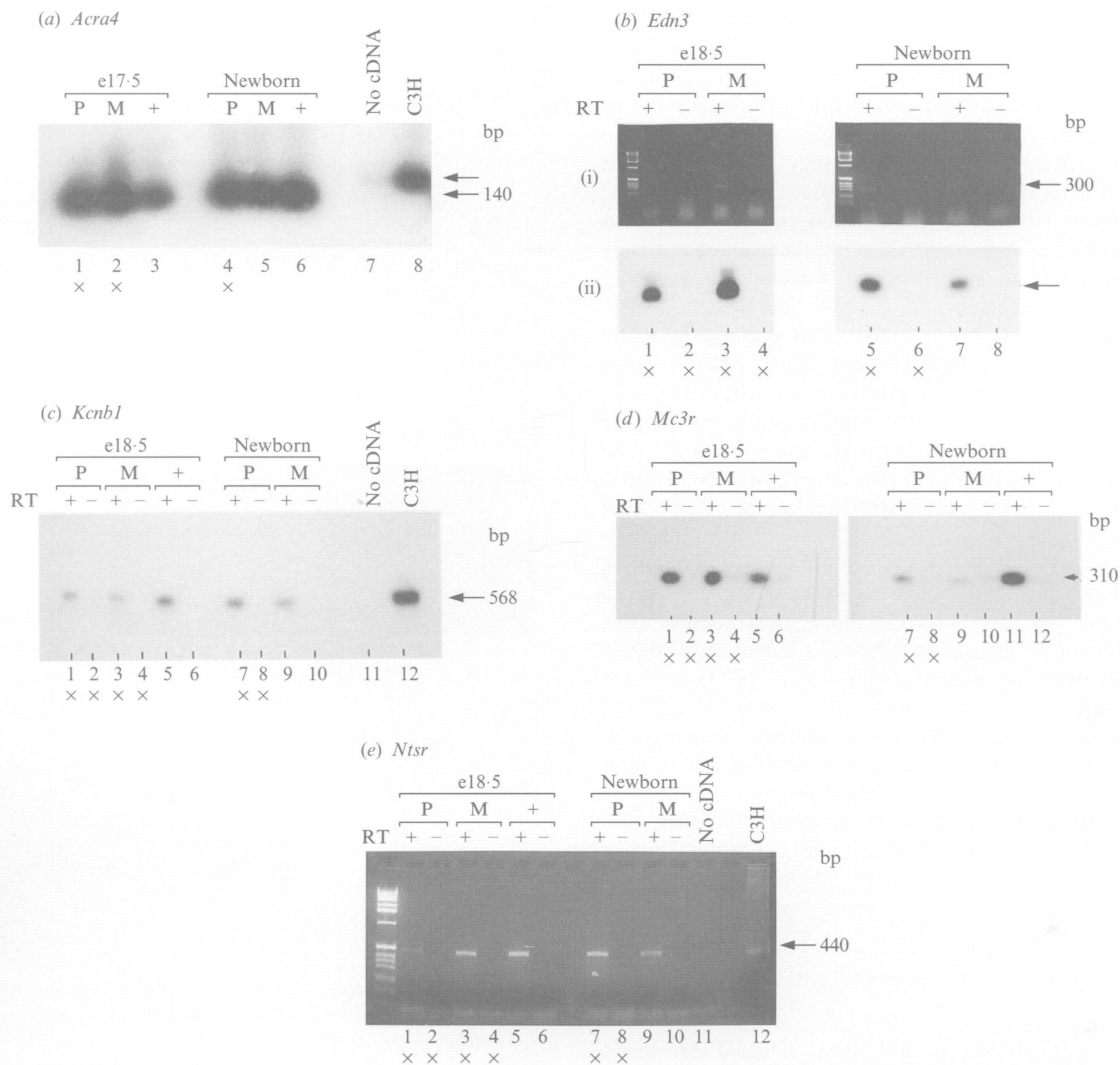

Fig. 5. RT–PCR showing expression of five genes: *Acra4*, *Edn3*, *Kcnb1*, *Mc3r* and *Ntsr* in brain of duplication-deficient animals. The abbreviations are given in the legend of Fig. 3. (*a*) Southern blot of *Acra4* PCR products derived from brain. The blot was probed with 2·2 kb rat cDNA. (*b*) EtBr stained gel (i) and Southern blot, probed with 300 bp product derived from C3H/HeH genomic DNA (ii), of *Edn3* PCR products, derived from lung. (*c*) Southern blot of *Kcnb1* PCR products derived from brain. The blot had been probed with 568 bp product derived from C3H/HeH genomic DNA. (*d*) Southern blot of *Mc3r* PCR products derived from brain and probed with 310 bp product derived from C3H/HeH genomic DNA. (*e*) EtBr stained gel of *Ntsr* PCR products derived from brain.

duplication/deficient animals at e18·5 and at birth (results not shown).

(3) *Kcnb1* encodes the *Shab*-related delayed rectifier type potassium channel that plays an important part in control of excitability in nerve and muscle cells. Malfunction of the delayed rectifier might evoke diseased states of the heart, muscle tissue or brain by causing abnormal firing rhythms, neurotransmitter release and/or action potential durations (Albrecht *et al.* 1993). *Kcnb1* expression was investigated in brain with primers corresponding to nucleotides 1729–1748 and the reverse sequence of nucleotides 2278–2297 of the mouse sequence (Pak *et al.* 1991). Because the PCR product from mRNA and genomic DNA were similar in size (Fig. 5*c*), a PCR reaction without reverse transcriptase was included as a control for the presence of contaminating DNA. A single band of 568 bp was amplified in lanes 1, 3, 5, 7 and 9, thus demonstrating that neither the maternal nor the paternal alleles of *Kcnb1* are completely repressed in brain samples at e18·5 and at birth.

(4) *Mc3r* is an intronless gene that encodes the melanocortin 3 receptor which recognizes core hepta-peptide sequence of melanocortins that have immuno-modulatory and neurotrophic properties (Gantz *et al.* 1993). To study the expression of *Mc3r*, primers which show no homology with other members of the receptor gene family were designed from the mouse

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sequence (Genbank accession number X74983; Desarnaud *et al.*, unpublished). On/off expression of *Mc3r* was investigated in brain of duplication/deficient mice at e18·5 and at birth. A single band of 310 bp was amplified in all samples containing reverse transcriptase thus suggesting that this gene is not imprinted (Fig. 5*d*). The band amplified in the newborn maternal duplication/paternal deficient animal (Fig. 5*d*, lane 9) was weaker than the bands obtained from the newborn paternal duplication/maternal deficient and normal animals (Fig. 5*d*, lanes 7 and 11, respectively). This was due to RNA loading as determined when *Hprt* primers were included as a control (results not shown).

(5) *Ntsr* is the gene for neurotensin receptor. This gene has been assigned to the H region of Chr 2, to the long arm of human chromosome 20 (Laurent *et al.* 1994) and recently below the breakpoint of T2Wa on the distal region of Chr 2 (Dutton *et al.* unpublished). Neurotensin acts as a neuromodulator of dopamine transmission and so is likely to have a role in dopamine-associated behavioural neurodegenerative and neuropsychiatric disorders. Primers were designed by comparing human (Vita *et al.* 1993) and rat (Tanaka *et al.* 1990) sequences and choosing well conserved regions. Primers corresponding to nucleotides 891–911 and the reverse sequence of nucleotides 1310–1330 (Vita *et al.* 1993) were used to study the expression of *Ntsr*. Fig. 5*e* shows amplification of a single band of 440 bp in all samples containing reverse transcriptase thus suggesting that this gene is not imprinted.

(iv) *Other genes that map to the distal region of Chr 2*

Cd40 is a single copy gene for an integral membrane glycoprotein found on the surface of B lymphocytes. Antibodies specific for CD40 augment proliferation of activated B lymphocytes, prevent B cell apoptosis, and prolong maintenance of normal B lymphocytes in culture. The primers, designed from exon 7 and exon 9 of the mouse sequence (Grimaldi *et al.* 1992), amplified a product in C3H/HeH genomic DNA that appeared to be of similar size to the 307 bp cDNA band. In order to use these primers for analysing *Cd40* expression in lung of duplication/deficient animals, RT-PCR analysis was carried out in the presence and absence of reverse transcriptase. The PCR products were Southern blotted and probed with the 307 bp cDNA product derived from a newborn that appeared phenotypically normal (Fig 6*a*). A single band of 307 bp was present in all samples containing reverse transcriptase and no amplification was present in any of the samples lacking the enzyme. Thus neither the maternal nor the paternal alleles appeared to be completely repressed in the lung of duplication/deficient animals at e18·5 and at birth. The bands amplified at e18·5 (Fig. 6*a*, lanes 1 and 3) appeared to

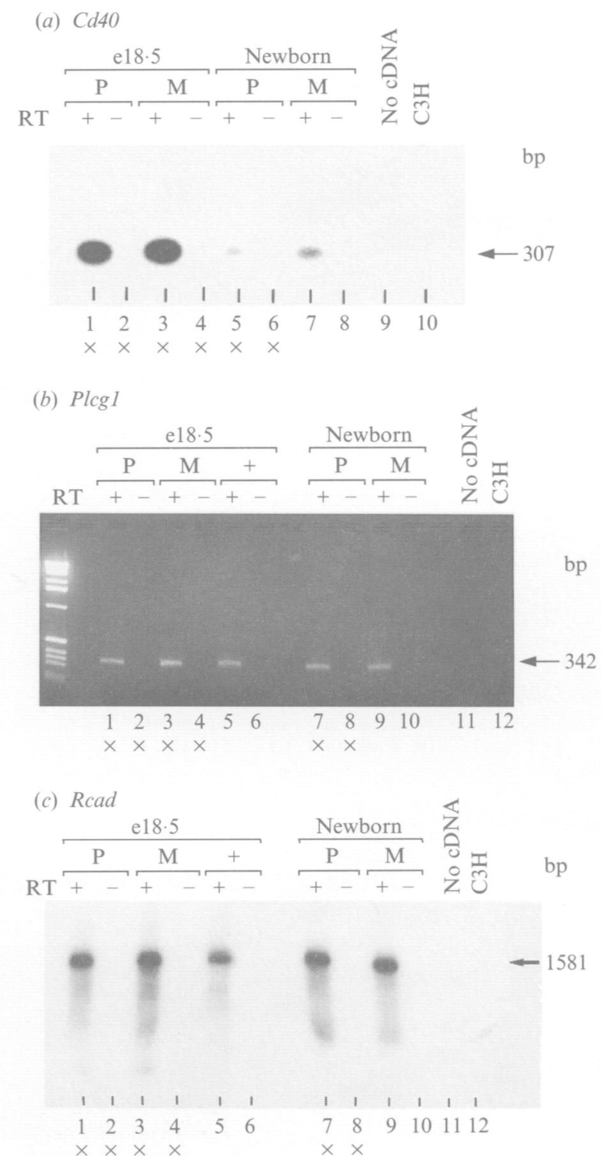

Fig. 6. RT–PCR showing expression of three genes: *Cd40*, *Plcg1* and *Rcad* that are doubtful candidates for being imprinted. The abbreviations are given in the legend of Fig. 3. (*a*) Southern blot of *Cd40* PCR products derived from lung. These products were probed with 307 bp cDNA product derived from a normal at birth. (*b*) EtBr stained gel of *Plcg1* PCR products derived from brain. (*c*) Southern blot of *Rcad* PCR products derived from brain. These products were probed with 1581 bp product derived from brain cDNA of a phenotypically normal embryo at 18·5 d of gestation.

be more intense than those from newborn (Fig. 6*a*, lanes 5 and 7). This appeared to be due to the level of RNA as determined when *Hprt* primers were included as a coamplification control (results not shown).

Plcg1 is the gene for phospholipase C, an enzyme of key importance in the production of second messenger molecule which controls cellular activity. Primers were designed by comparing bovine (Stahl *et al.* 1988) and rat (Suh *et al.* 1988) sequences and choosing well conserved regions. Primers corresponding to nucleotides 3174–3196 and the reverse sequence of nucleo-

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tides 3495–3516 (Stahl *et al.* 1988) were used for studying expression of *Plcg1* in brain. A single band of 342 bp was amplified in all samples containing reverse transcriptase (Fig. 6*b*) thus suggesting that this gene is not imprinted.

Rcad is the gene for R-cadherin and is one member of a family of homophilic cell–cell adhesion molecules that are crucial not only for physical cell–cell associations but also for selective cell–cell adhesion (Takeichi, 1991). *Rcad* expression was investigated in the brain with primers corresponding to nucleotides 1213–1233 and the reverse sequence of nucleotides 2774–2793 of the mouse sequence (Matsunami *et al.* 1993). Fig. 6*c* shows that a single band of 1581 bp was amplified following 2 min extension time at 72 °C in all samples containing reverse transcriptase thus suggesting that this gene is not imprinted.

We can therefore conclude that 13 genes that map within or close to the imprinting region of mouse Chr 2 do not appear to be monoallelically expressed in the appropriate tissues before and after birth. These results suggest that none of these genes are imprinted.

4. Discussion

In this present study RT-PCR has been used to determine whether genes that map to the distal imprinting region of mouse Chr 2 are monoallelically expressed and thus imprinted. The expression of two genes, *Ada* and *Ppgb*, that map to the imprinting region (Williamson *et al.* 1994) has previously been tested and neither was monoallelically expressed (Peters & Ball, 1989; Williamson *et al.*, 1994, respectively). In addition three other loci, *ls*, *Ra* and *wst*, that lie within or very close to the region and are classified by visible phenotype, do not show evidence of imprinting (Peters *et al.* 1994). Our data demonstrate that a further 13 genes that are either likely or known to map to the region are not monoallelically expressed and thus are probably nor imprinted.

By comparing the distal imprinting region of Chr 2 with the homologous region of human chromosome 20q13, we would also expect the mouse homologues of PTPRB (Brown-Shimer *et al.* 1990), PI3 (Molhuizen *et al.* 1994) and ZNF8 (Lania *et al.* 1990) to lie within the imprinting region. The gene PTPRB is a potentially good candidate for imprinting as it encodes for a protein tyrosine phosphatase that has been implicated in the control of cell growth, proliferation and neoplastic transformation. Although the mouse homologue of PTPRB has not yet been mapped, the mouse sequence (Miyasaka & Li, 1992) was available for the design of primers. This gene did not appear to be monoallelically expressed in the brain, kidney and liver of duplication/deficient mice before and after birth (Williamson *et al.* unpublished). The gene PI3 encodes for an epidermal serine proteinase inhibitor that is only expressed in epidermis following induction by inflammatory responses and epidermal injury and thus is probably a doubtful candidate for imprinting. The gene ZNF8 encodes for a protein containing a potential zinc finger-like nucleic acid binding protein of unknown function. Its mouse homologue, when identified, could be a good candidate for imprinting as a zinc finger gene, *Znf127*, that maps to central Chr 7, has recently been shown to be tissue specifically imprinted (Jones *et al.* in preparation). The distal imprinting region of mouse Chr 2 is likely to contain many genes, any one of which could be responsible for the imprinting effect. In fact 7·5 Mb is 0·25% of the mouse genome, assuming that the size of the genome is 3000 Mb. If there are 50000–200000 genes in the genome (Lovett, 1994), then 125–500 genes might be expected to lie within the imprinting region.

There are experimental limits to our approach in that we do not have an imprinted gene on distal mouse Chr 2 to use as a positive control for testing whether our templates derived from maternal and paternal duplication animals will reveal an imprinting difference. However, RT–PCR has been used successfully to demonstrate maternal repression of *Snrpn* and *Znf127* in the brain of mice with duplication of central Chr 7 (personal communications from J. Barr and J. Jones, respectively), and to confirm monoallelic expression of *Igf2* in parthenogenotes (J. Jones, personal communication). Although it is unlikely that our PCR assay will detect subtle differences in expression, it should detect the general on/off imprinting phenomenon. There is the possibility that an early imprinting effect would have been missed because the maternal duplication/paternal deficient and paternal duplication/maternal deficient offspring could not be detected until later in development. A tissue-specific imprinting effect could have been missed due to a limit on the number of tissues that were examined. Nevertheless, we feel that the main target organs have been studied for each gene.

Gnas maps within the distal imprinting region of Chr 2 as shown in Fig. 1. There is clinical and biochemical evidence suggesting that its human homologue (GNAS1) that maps to chromosome 20q13, is imprinted (Davies & Hughes, 1993). This is based on studies of patients with Albrights Hereditary Osteodystrophy (AHO) that have null mutations within GNAS1. Maternal transmission of the defective allele gives rise to AHO somatic features together with resistance to parathormone and other hormones that act via cAMP whereas paternal transmission of the defective allele gives rise to AHO somatic features alone. These findings can be explained by an unusual imprinting effect acting only in hormone responsive cells. There is no evidence that GNAS1 is imprinted at the level of whole tissues for expression of both parental alleles has been seen in all tissues examined (Campbell *et al.* 1994), and furthermore patients with null mutations have about 50% red cell Gs activity implying no preferential allelic origin. By analogy we would predict that the mouse homologue, *Gnas*, is

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also imprinted cell-specifically and therefore an imprinting effect would not be detectable by the RT-PCR assay used in this study. Even if *Gnas* is imprinted in the mouse, we should expect to find at least one other imprinted gene on the distal region of Chr 2, based on the preliminary findings of Beechey & Peters (1994).

Although IGF2R is not imprinted in human (for review see Bartolomei, 1994) which contrasts with the imprinting pattern in the mouse, there are three other murine genes: *Igf2*, *H19* and *Snrpn* whose homologues do appear to be imprinted in both species. If, in general, the phenomenon of imprinting is conserved between mouse and man as suggested from the results so far, then we would predict that the thirteen genes tested for monoallelic expression in this paper are probably not imprinted in human. Five genes, *E2f1*, *Tcf4*, *Kcnb1*, *Cd40* and *Rcad* do not appear to have been mapped in human. These genes are expected to lie on human chromosome 20q13 because of the linkage conservation observed between mouse Chr 2 and human chromosome 20.

Note added in proof. While this paper was in press, *ls* was found to be a dysfunctional allele of *Edn3* from gene targetting experiments (Baynash *et al.* 1994, *Cell* **79**, 1277–1285).

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