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Developmental control of allelic methylation in the imprinted mouse *Igf2* and *H19* genes

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SUMMARY

The Insulin-like growth factor 2 (*Igf2*) and *H19* genes are reciprocally imprinted and closely linked. *Igf2* encodes a fetal growth-factor and is predominantly expressed from the paternal allele, while *H19* is expressed from the maternal allele and encodes a transcript which may down-regulate cellular proliferation. One of the epigenetic modifications thought to be involved in parental imprinting is DNA methylation. Here we analyse methylation in two regions of the *Igf2* gene, one approx. 3 kb upstream of the gene and one in the 3′ part of the gene. Both regions are more methylated on the expressed paternal chromosome. Genomic sequencing of individual chromosomes in the first region shows this parent-specific methylation to be highly mosaic; interestingly, individual sperm chromosomes carry different methylation patterns into the egg. In the more 3′ region, which is fully methylated in sperm, the level of methylation on the paternal allele is highly tissue-specific and is correlated with expression of the gene in fetal tissues. Hence, the paternal allele is highly methylated in fetal liver (high expression) but is undermethylated in fetal brain (virtually no expression). Adult choroid plexus, a brain tissue in which *Igf2* is expressed from both alleles and *H19* is not expressed, represents an apparent loss of imprinting. Here, both *Igf2* and *H19* adopt a paternal type methylation pattern on both parental chromosomes. Analysis of early-passage androgenetic and parthenogenetic embryonic stem (ES) cells shows that the methylation patterns of *Igf2* and *H19* on maternal and paternal chromosomes are very similar. Androgenetic and parthenogenetic teratomas derived from these ES cells show the appropriate paternal and maternal patterns, respectively, of allelic methylation in both genes. Our results suggest that allelic methylation patterns in *Igf2* and *H19* arise early in embryogenesis and change progressively during development. Some of these developmental changes are apparently under tissue-specific control.

Key words: imprinting, mouse embryo, DNA methylation, bisulphite sequencing, embryonic stem cells, choroid plexus

INTRODUCTION

Parental imprinting is an important genetic mechanism by which some genes are repressed or expressed depending exclusively on their inheritance from mother or father. Such imprinted genes have crucial roles in mammalian development, in particular in the control of fetal growth and viability (Cattanach and Beechey, 1990; Surani et al., 1993). An imbalance of parental chromosomes in the embryo, or aberrant imprinting of these genes is implicated in an increasing number of genetic disorders (Reik, 1989; Nicholls, 1993) and may be involved in carcinogenesis (Feinberg, 1993). A number of imprinted genes have been identified in the mouse. These include the gene for the major fetal growth factor insulin-like growth factor 2 (*Igf2*) (DeChiara et al., 1991), the *Snrpn* (Small nuclear ribonucleoprotein particle n) gene (Leff et al 1992; Cattanach et al., 1992), the *U2afbp-rs* gene (Hatada et al., 1993; Hayashisaki et al., 1994) and the *insulin* genes (Giddings et al., 1994), all of which are maternally imprinted (repressed), and those for the *Igf2* /mannose-6-phosphate receptor and *H19*, which are paternally imprinted (Barlow et al., 1991; Bartolomei et al., 1991). The *IGF2* and *H19* genes are also imprinted in the human (Rainier et al., 1993; Zhang and Tycko, 1992) whereas imprinting of the *IGF2*-receptor gene might not be conserved (Kalscheuer et al., 1993).

The molecular basis of parental imprinting is unknown. However, it is thought that DNA methylation might be one of the molecular components involved, since parent-specific methylation patterns have been detected both in imprinted transgenes and in some of the endogenous imprinted genes (Sasaki et al., 1992; Surani et al., 1993; Stöger et al., 1993; Ferguson-Smith et al., 1993; Brandeis et al., 1993; Bartolomei et al., 1993; Zhang et al., 1993; Schneid et al., 1993; Reik and Allen, 1994). Indeed, methylation must be involved in some way in imprinting, perhaps in its somatic maintenance, since the expression of imprinted genes is altered in mutant mouse embryos that are deficient in the methyltransferase enzyme (Li et al., 1993).

We are particularly interested in the imprinting of the mouse *Igf2* gene and in epigenetic modifications in and around this gene (Sasaki et al., 1992). Remarkably, while the paternally imprinted *H19* and *Igf2*-receptor genes show methylation of CpG-rich regions on the repressed allele (Stöger et al., 1993; Ferguson-Smith et al., 1993; Brandeis et al., 1993; Bartolomei

et al., 1993) as well as reduced chromatin accessibility (Ferguson-Smith et al., 1993; Bartolomei et al., 1993), the repressed maternal copy of *Igf2* is largely devoid of major modifications (Sasaki et al., 1992). However, there is a region upstream of the gene which was more methylated on the expressed paternal chromosome (Sasaki et al., 1992; Brandeis et al., 1993).

Here we present a detailed molecular and developmental analysis of parent-specific methylation in the *Igf2* gene. We applied bisulphite genomic sequencing to characterise individual chromosomes and show a mosaic pattern of methylation. We also identified a second region of allelic methylation in the 3′ part of the gene where methylation of the paternal allele is regulated in a tissue-specific fashion which is correlated with expression. Since there is apparent removal or loss of imprinting in the choroid plexus, where *Igf2* is biallelically expressed (DeChiara et al., 1991), we analysed allele-specific methylation of both *Igf2* and *H19* in this brain tissue. As a first step to understand how and when allelic methylation patterns arise in the early embryo we examined monoparental androgenetic and parthenogenetic ES cells and their differentiated derivatives for allelic methylation in both genes.

MATERIALS AND METHODS

Mice

MatDi7 embryos were produced by inter-crossing mice heterozygous for the T9H translocation (Searle and Beechey, 1990). Normal littermates were used as control embryos. F_2 animals, bearing *M. spretus* and *M. m. domesticus* alleles, were identified using RFLPs in the *Igf2* and *H19* genes.

ES cells and teratomas

ES cells were cultured in ES medium (1:1 DMEM: Hams F12. 10% FCS with supplements of sodium pyruvate, 20 mM L-glutamine, nucleosides, non-essential amino acids, sodium bicarbonate, β2-mercapto-ethanol) (Robertson, 1987) containing 10^3 units/ml recombinant LIF (ESGRO, BRL) in the absence of feeder cells. The derivation of the ES cell-lines (normal ES cells GeoIII, parthenogenetic PK1, and androgenetic AK1 cells) is described elsewhere (Allen et al., 1994). Methylation analyses were performed on early-passage ES cells (< 9 passages). To obtain teratomas, ES cells were trypsinised and aggregated in ES medium; teratomas were obtained 4 weeks after transferring the aggregates under the kidney capsule of isogenic adult mice.

Southern and northern analysis

Genomic DNAs were digested and electrophoresed on 0.8-1.2% agarose gels in 1× TBE buffer, alkaline blotted onto Hybond N^+ membrane (Amersham), and UV cross-linked (Stratalinker, Stratagene). Radiolabeled fragments 1-6, subcloned from cosmid IGF4 (Rotwein and Hall, 1990), were used as probes. Total RNAs were prepared according to Chomczynski and Sacchi (1987), electrophoresed on a 1% formaldehyde gel and transferred to Hybond N^+ membrane; hybridisation was with radiolabeled *Igf2*, *H19* and *Gapdh* cDNA probes. Hybridisation and washing of northern and Southern blots was performed as described by Church and Gilbert (1984). Densitometry of band intensities was performed on a Chromoscan 3 (Joyce Loebl) apparatus. Overall methylation in DNAs from embryonic and adult tissues, ES cells, and derived teratoma, was performed by hybridising with a Line-1 repeat sequence-specific probe.

DNA sequencing

Sequences were obtained from double-stranded plasmid templates, subcloned from cosmid IGF4 (Rotwein and Hall, 1990). Sequenase kit and enzyme (USB), and both vector (pBluescript KS+; Stratagene) and internal primers were used. Restriction sites polymorphic between *M. m. domesticus* and *M. spretus* were identified by restriction analysis or by DNA sequencing.

Bisulphite sequencing and PCR amplification

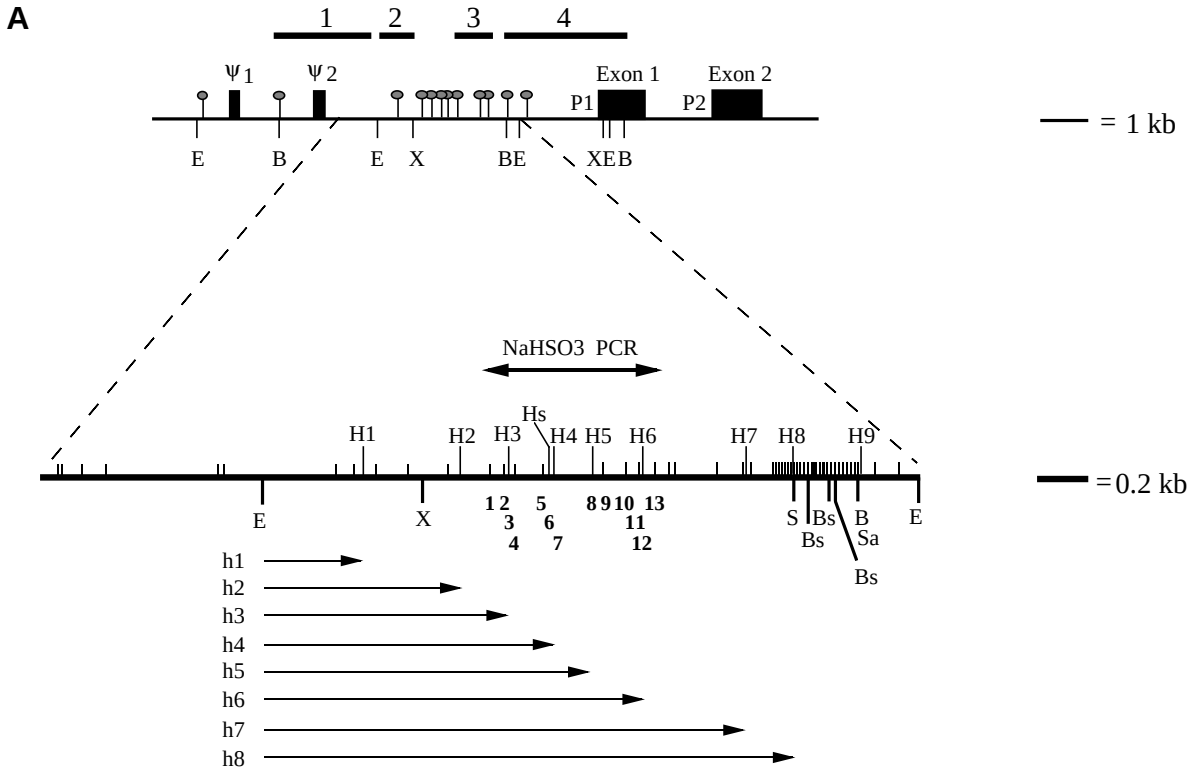
2 μg of genomic DNA, digested with *Eco*RI or *Bam*HI, were alkaline-denatured directly prior to bisulphite treatment. Treatment was performed in 1.2 ml of 3.5 M $NaHSO_3$; 1 mM hydroquinone; pH 5.0, at 0°C for 24 hours. DNA was then extracted using the Geneclean II kit (Stratech Scientific Ltd., London), dissolved in 100 μl of H_2O, desulfonated by adding 11 μl of 2 N NaOH, ethanol precipitated, dissolved in 100 μl of H_2O and stored at –20°C (Feil et al., 1994). PCR amplifications were performed at annealing temperatures of 52-53°C and for 33-40 cycles. PCR products were digested with *Eco*RI+*Xba*I (restriction sites within the primers) and ligated into M13mp18 phage (Stratagene). Single-stranded DNAs from individual phages were sequenced using the M13 mp18 '-40 primer' and internal sequencing oligonucleotides.

RESULTS

Methylation sequencing on individual maternal and paternal chromosomes in the *Igf2* upstream and promoter regions

Previously we identified a differentially methylated region upstream of the *Igf2* gene (Sasaki et al., 1992). We compared E15 embryos with maternal duplication (and paternal deficiency) of distal chromosome 7 (MatDi7) with normal E15 embryos to distinguish the parental alleles (Fig 1). Analysis of this region with various probes revealed that differential methylation occurred mainly at three *Hpa*II sites (H3-H5) that were more methylated on paternal than on maternal chromosomes (Fig 1B and not shown). Sites more 5′ to this region were highly methylated, whereas sites more 3′ (H6, H7) were almost entirely unmethylated, and so were *Sma*I, *Bss*HII and *Sac*II restriction sites found in the adjacent CpG island (Fig. 1A,B and not shown). DNAs from various embryonic and adult tissues all had a similar pattern of methylation in this region, with the exception of sperm (and testis) DNA which was more highly methylated (Fig. 1B).

Since Southern blot analysis revealed a complex and mosaic pattern of allele-specific methylation upstream of the *Igf2* gene, we decided to examine this region in more detail, applying a protocol ('bisulphite sequencing'; Frommer et al., 1992) that enables one to detect any methylated CpG dinucleotide in genomic DNA. We also examined other regions in the gene with this method, in particular the extensive CpG island associated with promoter 2, because enzyme analysis detected only a fraction of the methylatable CpGs in this region. The genomic sequencing protocol, which we modified (Feil et al., 1994), is based on chemical modification of genomic DNA induced by bisulphite, such that cytosine residues are converted to uracil by deamination, but 5-methylcytosine remains unchanged. The modified genomic DNA is then PCR amplified in the region of interest. Sequences of cloned PCR products provide 'methylation maps' of single DNA molecules and therefore of individual parental chromosomes. The differentially methylated upstream region was analysed by bisul-



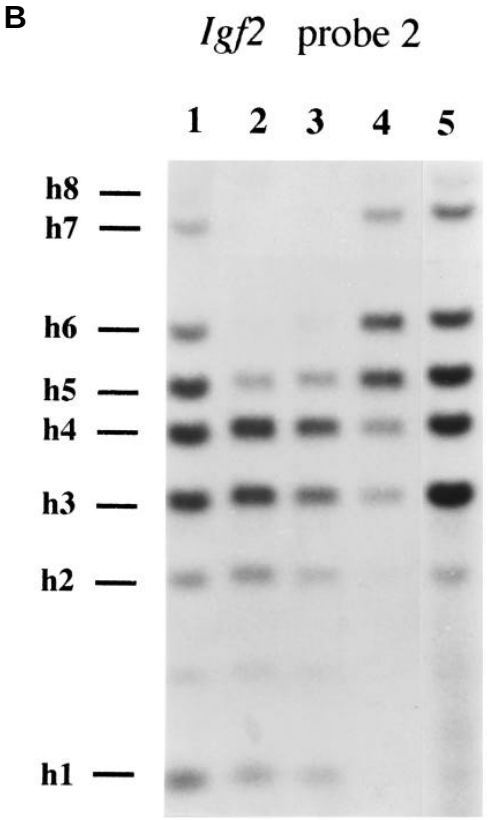


Fig. 1. The *Igf2* upstream region is allele-specifically methylated. (A) Map of the 5′ portion (exons 1 and 2) and the upstream region of the mouse *Igf2* gene showing *Eco*RI (E), *Bam*HI (B), *Xba*I (X), *Bss*HII (Bs), *Sac*II (Sa), *Sma*I (S) and H*pa*II (🍍 in upper, H in lower panel) restriction sites. Hs is a *Hpa*II site found only in *M. spretus*, P1 and P2 are promoters 1 and 2 respectively, and Ψ1 and Ψ2 are pseudo-exons 1 and 2, corresponding to exons 2 and 3 in the human *IGF2* gene (Rotwein and Hall, 1990). With genomic probes 1, 2, 3 and 4, the upstream region was analysed: 1 is a 2.2 kb *Bam*HI-*Eco*RI fragment; 2 is a 720 bp *Eco*RI-*Xba*I fragment; 3 is an 800 bp *Hin*dIII-*Sma*I fragment and 4 a 2.6 kb *Bam*HI fragment. The sequenced, 4.4 kb region is shown in the middle part. Vertical bars represent CpG dinucleotides; thirteen CpGs (numbered 1-13) were studied by genomic sequencing on bisulphite-modified genomic DNAs (Fig. 2). Below are shown the restriction fragments h1-h8 detected by probe 2 in *Eco*RI+*Hpa*II digested genomic DNAs. (B) Genomic DNAs from a normal E15 embryo (lane 1), two E15 MatDi7 embryos (lanes 2 and 3), vas deferens sperm (lane 4) and adult liver (lane 5) were digested with *Eco*RI+*Hpa*II. Hybridisation was with probe 2; this probe also hybridises to the H1-H3 fragment, which is visible between bands h1 and h2 in lanes 1, 2 and 3.

phite sequencing in an E18 (*M. m. domesticus* × *M. spretus*) F_1 embryo and in *M. m. domesticus* sperm DNA (Fig. 2). Sequence polymorphisms between the two species enabled us to distinquish between products amplified from maternal and from paternal chromosomes. An 800 bp fragment (b2d2E) was amplified that contains 13 CpGs, five of which are within previously tested *Hpa*II sites (H3, H4, H5 and H6 in both species, and Hs in *M. spretus* only, see Fig. 1A). A total of 36 clones was sequenced (Fig. 2A), the CpG methylation maps thus obtained are shown in Fig. 2B.

Internal controls were used to assess the efficiency and fidelity of the bisulphite treatment and subsequent PCR amplification. First, virtually all cytosine residues located outside the CpG dinucleotides had been deaminated (see Fig. 2A for an example). Second, CpGs that had previously been shown to be largely unmethylated (H6) or largely methylated (H3 in sperm) were found to be deaminated or unchanged, respectively, in the majority of clones (Fig. 2B). Third, the two sequence poly-

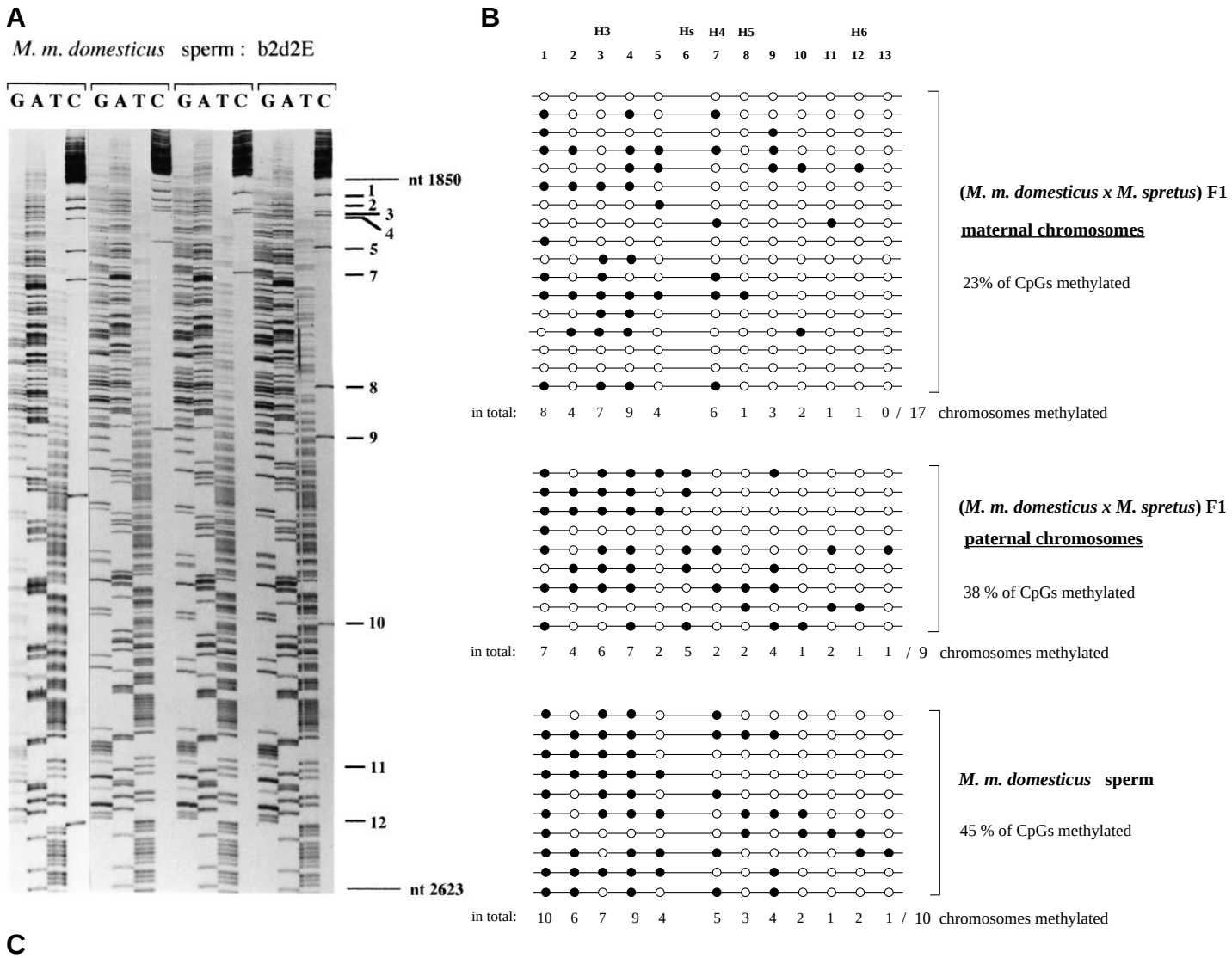


Fig. 2. Methylation sequencing on individual maternal and paternal chromosomes in the *Igf2* upstream and promoter 2 regions. (A) Vas deferens sperm DNA was digested with *Eco*RI, denatured, and chemically modified with $NaHSO_3$. PCR amplification from the lower strand was then performed with primers b2 (ggatctagaAGTTAAAGTTAGTGGATAGGTGT) and d2E (ccggaattccAACTCTTCCCTACCCCTTAAACC); the amplified b2d2E fragment (nt 1850-2623) comprises CpGs 1-5, 7-12 (see Fig. 1). DNA sequences of four individual cloned PCR products are presented. Virtually all cytosine residues in non-CpGs have been converted into thymidine, remaining cytosine residues correspond to methylated CpGs. However, note that one C halfway up the sequence ladder shown on the left, is not within a CpG dinucleotide; this may be a result of incomplete chemical treatment or may represent a PCR artefact. (B) *Igf2* methylation maps of sperm and maternal and paternal chromosomes in (*M. m. domesticus* × *M. spretus*)F_1 embryos. The methylation status of 13 CpGs was determined by sequencing b2d2E PCR products, amplified from bisulphite-modified embryo and sperm DNAs. CpGs 3, 6, 7, 8, and 12 are within *Hpa*II sites H3, Hs (paternal *M. spretus* chromosomes only), H4, H5, and H6, respectively. Polymorphisms between the two species (all G/A transitions) at positions nt 2230, 2239, and 2620 allowed the distinction of PCR products amplified from maternal and paternal chromosomes. A total of 17 embryonic maternal, 9 embryonic paternal, and 10 sperm amplified b2 d2E fragments were sequenced. Chromosomes are shown as horizontal lines, unmethylated CpGs as open circles, and methylated CpGs as black circles. Maternal chromosomes have a smaller fraction of the CpGs methylated (23%) than paternal (38%) and sperm (45%) chromosomes. (C) Map of the promoter 2-exon 2 region showing the 373 bp fragment (line underneath map) amplified from $NaHSO_3$-treated DNAs. Amplification from the upper strand was performed with primers 599 (ctagaattcGGTGTGGGTGTAAGGTTAGTGGGG) and 808 (agtaggatcCTACCTACAATAAAAACCACCTCCTC). In total, 10 normal and 10 MatDi7 chromosomes were studied. Vertical bars indicate CpGs; only the 5′ part of exon 2 is shown. The *Sma*I (S), *Xho*I (X) and *Sac*II (Sa) restriction sites have been shown previously by Southern blotting to be unmethylated on both parental alleles (Sasaki et al., 1992).

morphisms that were used to differentiate between products amplified from maternal *M. m. domesticus* and paternal *M. spretus* chromosomes are 400 bp apart, and none of the PCR amplification products was found to result from amplification partially from a maternal and partially from a paternal chromosome (crossover during PCR amplification). Finally, genomic DNAs showed no contamination with plasmid DNA, since *Eco*RII sites (CC(A/T)GG; which in *E. coli* are methylated on the 3′ cytosine residue by Dcm methylase) were found unmethylated in all clones sequenced.

In agreement with the previous restriction enzyme analysis, a gradient of methylation was found in this region from almost complete methylation of CpG 1 to virtually no methylation of CpG 13. Overall, maternal chromosomes were less methylated than paternal and sperm chromosomes. However, there was not a single CpG residue that was unmethylated on all maternal, and methylated on all paternal chromosomes. The most pronounced differences occurred in CpGs 1 to 4 (CpG 3 is within *Hpa*II site H3) with roughly twice as much methylation on paternal as compared to maternal chromosomes. Surprisingly, although almost all individual chromosomes exhibit different patterns of methylation, which do not appear to be very orderly, on a population basis the patterns are highly consistent with observations on the extent of methylation from genomic Southern blots, and are also reproducible between different samples. This analysis also revealed that paternal chromosomes carried by individual spermatozoa exhibit a variety of different methylation patterns. It therefore seems unlikely that gametic methylation in this *Igf2* upstream region provides the primary signal for parental imprinting.

Bisulphite analysis was also carried out on a stretch of 50 CpGs in the CpG-rich promoter 2-exon 2 region of the *Igf2* gene. Since there were no known polymorphisms between *M. m. domesticus* and *M. spretus* in this region, DNAs from MatDi7 and normal embryos were chemically treated and amplified (Fig. 2C). In all 20 PCR products sequenced (10 from MatDi7 and 10 from normal embryos) none of the 50 CpGs in the amplified fragment were found to be methylated, indicating that both parental alleles are entirely unmethylated in the promoter 2-exon 2 region.

Paternal allelic methylation in the 3′ part of the *Igf2* gene is correlated with expression

In addition to the upstream region and to promoter 2, we decided to perform a more detailed search for allelic differences further downstream in the *Igf2* gene. This region had not previously been studied in detail (Sasaki et al., 1992). *Hpa*II, *Sac*II and *Sma*I restriction sites in the exon 4 to exon 6 region were therefore examined in the disomy system and in interspecific hybrids between *M. m. domesticus* and *M. spretus*.

Three *Hpa*II restriction sites, one within intron 4 directly upstream of exon 5, one within exon 5, and one located within the coding sequences of exon 6, were found to be more methylated on the expressed paternal than on the repressed maternal allele (Fig. 3). We initially compared MatDi7 and normal embryos (lanes 5 and 6). Hybridisation of *Bam*HI+*Hpa*II digested DNAs with probe 6, a fragment comprising intron 5 and exon 6 sequences, revealed that in MatDi7 embryos these three *Hpa*II sites are digested completely (lane 5) and therefore unmethylated on the maternal allele. In normal embryos, however, we found the three *Hpa*II sites to be methylated to about 30% (lane 6; determined by scanning, see legend to Fig. 3), indicating that in the embryo these sites are methylated to approximately 60% on the paternal allele. The same observation was made for a *Sac*II site located close to the most 3′ of the three *Hpa*II sites, which is almost completely unmethylated in MatDi7 embryos and methylated to about 40% in normal embryos (not shown). We confirmed this finding in interspecific hybrids by using a polymorphic *Hpa*II restriction site located within intron 4, which is present in *M. m. domesticus* and absent in *M. spretus* (lanes 3 and 4). This polymorphic *Hpa*II site is within a *Sma*I site (see Fig. 3). We did not detect differential digestion of the S*ma*I site comparing the reciprocal interspecific hybrids, and the *Sma*I site was found to be methylated to about 30% on both parental alleles. Analysing various DNAs with probe 5, which comprises intron 4 sequences, we found that this was also true for a *Sac*II and a *Hpa*II site located close to the *Sma*I site (not shown). However, hybridisation of *Bam*HI+*Hpa*II digested adult liver DNAs from the reciprocal interspecific hybrids with probe 6 revealed striking differences. When the *M.m. domesticus* specific *Hpa*II site was paternally derived, an uncut 2.1 kb fragment (and a partial product of 1.5 kb) was visible (Fig. 3, lane 4). On the paternal chromosome therefore, the three *Hpa*II sites were partially methylated. By contrast, when the same polymorphic *Hpa*II site was maternally derived, the 2.1 kb and 1.5 kb bands were completely absent, indicating no methylation of the three *Hpa*II sites on the maternal chromosome (lane 3). All *Hpa*II sites in the 2.4 kb *Bam*HI fragment were completely methylated in sperm DNA (lane 2).

Various fetal tissues from E16 (*M. m. domesticus* × *M. spretus*) F_1 embryos were examined for methylation in this region and levels of methylation were found to correlate with known levels of expression of the *Igf2* gene. Fig. 3 shows fetal brain and fetal liver (lanes 7 and 8), where *Igf2* is expressed in a minority or majority, respectively, of cells in the tissue (Lee et al., 1990; DeChiara et al., 1991). In fetal brain, as in MatDi7 embryos, the three *Hpa*II sites are almost entirely unmethylated (lane 7), while in fetal liver the three sites are methylated to about 50% (lane 8), suggesting that the paternal allele is almost entirely methylated. Thus, although maternal chromosomes are always unmethylated in this region, paternal chromosomes are methylated in fetal tissues that express *Igf2*, but unmethylated in tissues that do not express the gene.

Allelic methylation and expression of *Igf2* and *H19* is altered in adult choroid plexus

Our results so far indicate that there are CpG dinucleotides upstream and within the *Igf2* gene that are allele-specifically methylated in the embryo with more methylation on the paternal (expressed) allele than on the maternal allele. Likewise, in the neighbouring and reciprocally imprinted *H19* gene there are CpGs upstream of the gene, in the promoter and the transcribed sequences, and in downstream sequences, that are more methylated on the paternal (repressed) allele than on the maternal allele, both in embryos and in adult tissues (Ferguson-Smith et al., 1993; Brandeis et al., 1993; Bartolomei et al., 1993). The functional significance of these allelic methylation patterns in parental imprinting is at present unknown. To examine this relationship, we studied allelic methylation of *Igf2* and *H19* in situations where imprinting of

these genes is altered. In choroid plexus epithelium in the brain, *Igf2* expression is from both alleles during development (Dechiara et al., 1991) and continues in the adult animal (Fig. 4A), whereas *H19* is not expressed in adult choroid plexus from either allele (Fig. 4A). We therefore examined methylation in adult hindbrain choroid plexus.

Analysis of the *Igf2* upstream region in choroid plexus revealed high levels of methylation (Fig. 4B). In this tissue, *Hpa*II sites H1-H6 were almost completely methylated (absence of bands h1-h6 in lane 3). Adult brain, by contrast, showed a pattern identical to that of all other adult tissues (Fig. 4B). The 3′ part of *Igf2* was also highly methylated in choroid plexus DNA, while it was almost unmethylated in the rest of the adult brain (Fig. 4C).

Similarly, *Hpa*II sites in the body of the *H19* gene, which in the embryo and in adult tissues including the brain, are methylated exclusively on the paternal chromosome (Ferguson-Smith et al., 1993; Brandeis et al., 1993; Bartolomei et al., 1993), were found to be completely methylated on both alleles in choroid plexus (Fig. 4D). Similarly to adult liver and brain, the completely methylated region in choroid plexus DNA extended to 3.5 kb downstream of the gene, where a *Hpa*II site was completely digested (see 6.5 kb fragment in lanes 1 and 2). However, at least one *Hpa*II restriction site in the *H19* promoter region was found to be approx. 60% digested in choroid plexus (as evidenced by the appearance of a 1.6 kb fragment in the *Hin*dIII+*Hpa*II digest in lane 5), corresponding to a similar degree of methylation in the promoter region to that found in other adult tissues (see lane 6 for liver DNA). Two *Hha*I sites located in the promoter were also digested to approx. 50% in choroid plexus DNA (data not shown). In conclusion, in adult choroid plexus, *Hpa*II sites in the body of the *H19* gene and in approx. 3.5 kb of downstream sequences have become methylated on both alleles, while *Hpa*II and *Hha*I sites in the promoter region remain about half methylated.

No differences were detected in overall levels of methylation between total brain and choroid plexus (data not shown). From our analysis it appears that sequences in the *Igf2* and *H19* genes that are methylated on the paternal, but not on the maternal, allele in the embryo also become methylated on the maternal

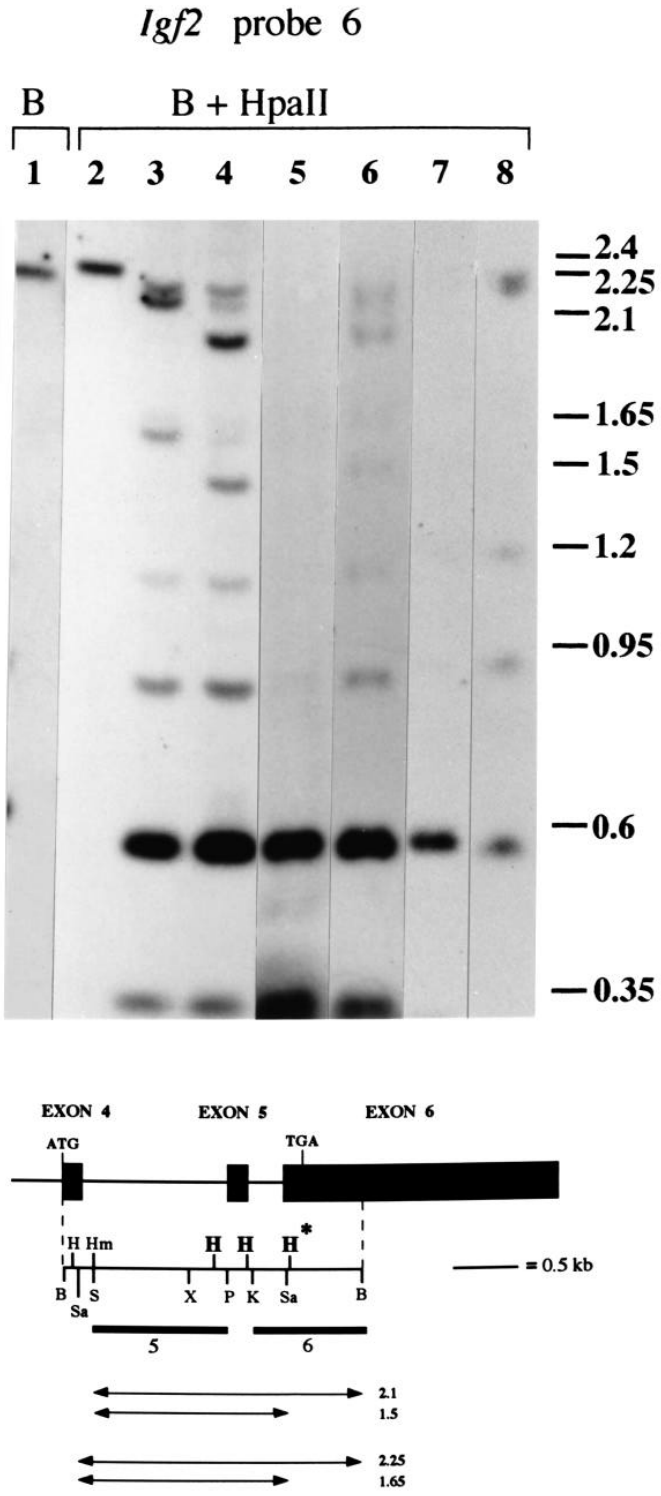


Fig. 3. Allele-specific methylation in the 3′ part of the *Igf2* gene. *Bam*HI (lane 1) and *Bam*HI+*Hpa*II (lanes 2-8) digested DNAs were hybridised with probe 6, a 900 bp *Kpn*I-*Bam*HI fragment comprising intron 5 and the 5′ portion of exon 6. Lanes: 1, *M. m. domesticus* adult liver; 2, *M. m. domesticus* vas deferens sperm; 3, (*M. m. domesticus* × *M. spretus*)F_1 adult liver; 4, ((*M. m. domesticus* × *M. spretus*)F_1 × *M. m. domesticus*)F_2 adult liver; 5, E15 MatDi7 embryo; 6,normal *M. m. domesticus* E15 embryo; 7, (*M.m. domesticus* × *M. spretus*)F_1 E16 brain; 8, (*M.m.domesticus x M. spretus*)F_1 E16 liver. Fragment sizes are in kb. The map below shows the 3′ portion of *Igf2*, with the 2.4 kb *Bam*HI fragment in which allele-specific methylation was detected. Coding exons 4, 5 and 6 (the 5′ portion) are shown as thick horizontal bars with start and stop codons indicated. *Sac*II (Sa), *Sma*I (S), *Xho*I (X), *Pvu*II (P), *Kpn*I (K), and *Hpa*II (H in both mouse species, Hm in *M.m. domesticus* only) restriction sites are shown. *Hpa*II sites in bold (**H**) were found to be more methylated on the paternal than on the maternal allele. * = *Hpa*II site that is also allele-specifically methylated in the human *IGF2* gene (Schneid et al., 1993). Four of the fragments that hybridise to probe 6 in the adult liver DNAs from reciprocal hybrids are indicated below the map. The *M. m. domesticus* specific 1.5 kb and 2.1 kb *Hpa*II fragments are absent when this allele is inherited from mother (lane 3) since the three downstream *Hpa*II sites (3 × **H**) are completely digested on the maternal allele. Likewise, the *M. spretus* specific 2.25 kb and 1.65 kb bands are absent when the *M. spretus* allele is inherited from mother (lane 4). The methylation index was determined by dividing the intensities of uncut fragments (of 0.95 kb and larger) by the combined intensities of all fragments: lanes 3 and 4 (adult liver), 0.40; lane 5 (MatDi7 embryo), 0.08; lane 6 (normal embryo), 0.30; lane 7 (fetal liver), 0.51; lane 8 (fetal brain), 0.10.

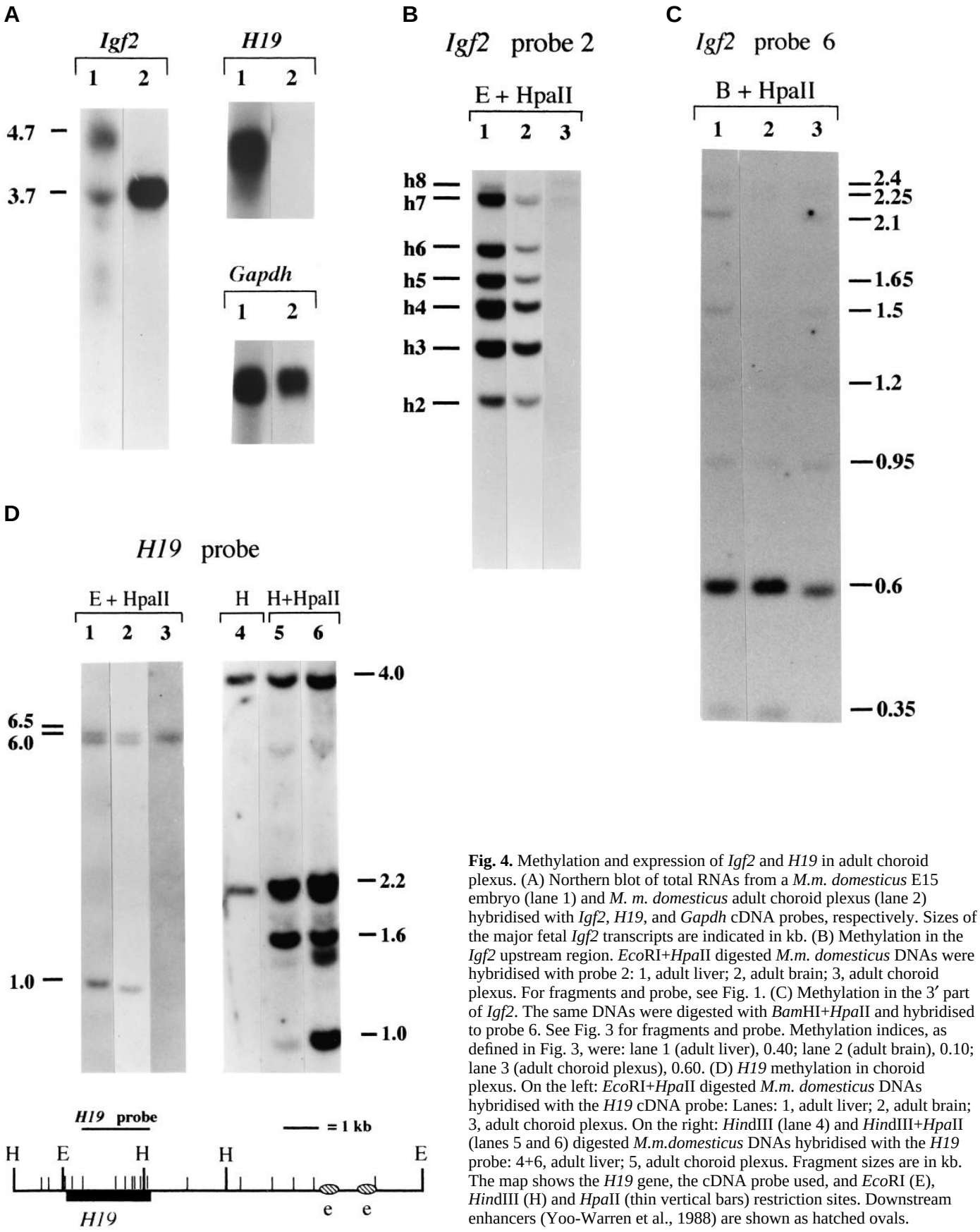


Fig. 4. Methylation and expression of *Igf2* and *H19* in adult choroid plexus. (A) Northern blot of total RNAs from a *M.m. domesticus* E15 embryo (lane 1) and *M. m. domesticus* adult choroid plexus (lane 2) hybridised with *Igf2*, *H19*, and *Gapdh* cDNA probes, respectively. Sizes of the major fetal *Igf2* transcripts are indicated in kb. (B) Methylation in the *Igf2* upstream region. *Eco*RI+*Hpa*II digested *M.m. domesticus* DNAs were hybridised with probe 2: 1, adult liver; 2, adult brain; 3, adult choroid plexus. For fragments and probe, see Fig. 1. (C) Methylation in the 3′ part of *Igf2*. The same DNAs were digested with *Bam*HI+*Hpa*II and hybridised to probe 6. See Fig. 3 for fragments and probe. Methylation indices, as defined in Fig. 3, were: lane 1 (adult liver), 0.40; lane 2 (adult brain), 0.10; lane 3 (adult choroid plexus), 0.60. (D) *H19* methylation in choroid plexus. On the left: *Eco*RI+*Hpa*II digested *M.m. domesticus* DNAs hybridised with the *H19* cDNA probe: Lanes: 1, adult liver; 2, adult brain; 3, adult choroid plexus. On the right: *Hin*dIII (lane 4) and *Hin*dIII+*Hpa*II (lanes 5 and 6) digested *M.m.domesticus* DNAs hybridised with the *H19* probe: 4+6, adult liver; 5, adult choroid plexus. Fragment sizes are in kb. The map shows the *H19* gene, the cDNA probe used, and *Eco*RI (E), *Hin*dIII (H) and *Hpa*II (thin vertical bars) restriction sites. Downstream enhancers (Yoo-Warren et al., 1988) are shown as hatched ovals.

allele in choroid plexus. Both parental alleles therefore seem to have adopted a largely paternal methylation pattern in this tissue.

Analysis of methylation in the *Igf2* and *H19* genes in monoparental embryonic stem cells and teratomas

As a first step to address the question of when allelic methylation in the *Igf2* and *H19* genes arises during development, we analysed monoparental embryonic stem (ES) cells, as well as their in vivo differentiated derivatives. We made use of early passage (<9) parthenogenetic (diploid with two maternal genomes) and androgenetic (diploid with two paternal genomes) embryonic stem cells (Allen et al., 1994). These stem cells have been thoroughly tested in chimaeras and in differentiation assays. They faithfully retain their parental imprints; thus, androgenetic (AG) ES cells begin to transcribe high levels of *Igf2* message upon in vitro differentiation, whereas, under identical conditions, in parthenogenetic ES (PG ES) cells the *Igf2* gene remains repressed. By contrast, high levels of *H19* transcript appear upon differentiation of PG ES cells (Allen et al., 1994).

Three early-passage ES cell-lines (normal, PG, AG) were examined for *Igf2* as well as *H19* methylation. In the *Igf2* upstream region, the three ES cell-lines had a very similar degree of methylation of sites H1-H7 (Fig. 5A) comparable to that in embryonic and adult tissues (see Fig. 1B). Upon differentiation in vivo of the PG and AG ES cells into teratomas, striking methylation changes occurred in the upstream region. PG cells attained a much lower level of DNA methylation on differentiation, comparable to that in Matdi7 embryos. By contrast, an increase in methylation was observed in differentiated AG cells (Fig. 5A), which express the *Igf2* gene at a high level (not shown).

Very similar results were obtained when the allele-specific methylation in the 3′ part of the *Igf2* gene was examined. Although the ES cell-lines did not reveal different levels of DNA methylation, parent-specific changes in methylation occurred upon differentiation into teratomas (Fig. 5B). Differentiated AG cells (lane 5) showed a pattern of methylation comparable to that of the paternal allele in the embryo (see Fig. 3, lane 6), while in the PG teratoma (lane 4) this region became almost completely unmethylated, as in the MatDi7 embryos (compare with Fig. 3, lane 5).

In the *H19* gene, we initially examined three methylation sensitive *Hae*II restriction sites, located approx. 1 kb upstream of the promoter, in the body of the gene (in exon 3), and approx. 6 kb downstream of the gene (Fig. 5C). The first two sites are in the paternally methylated region and their allele-specific methylation is representative of other methylatable sites in this region (Ferguson-Smith et al., 1993; Brandeis et al., 1993; Bartolomei et al., 1993; our unpublished results). Hybridisation with a *H19* probe again revealed a similar pattern in AG and PG ES cells with the 5′ site being undermethylated and the exon 3 site methylated to about 70%. Upon in vivo differentiation, methylation of both sites increased strikingly in AG cells (lane 5), whereas in differentiated PG cells, methylation of the two *Hae*II sites decreased (lane 4). We also examined methylation of *Hpa*II sites located upstream of *H19* and within the body of the gene, as well as a *Hha*I site located within the promoter region, and found that they behaved exactly like the *Hae*II sites (not shown).

As no differences were detected in methylation levels of control genes between the different ES cell lines and between the teratomas (not shown), it follows that the parent-specific changes attained in the teratomas were specific for the imprinted genes examined.

DISCUSSION

In this study we report on a structural and developmental analysis of allelic methylation in the imprinted *Igf2* and *H19* genes. We examined the paternally methylated region upstream of the *Igf2* gene, a new region of paternal methylation in the 3′ part of the *Igf2* gene, and the paternally methylated *H19* gene. Most of these paternal methylation patterns appear to arise in the early embryo, and change progressively during development. Some of the developmental changes are significantly determined by tissue differentiation. First, methylation of the paternal allele of *Igf2* in the 3′ region correlates directly with expression, suggesting the presence of a silencer element that is suppressible by tissue-specific epigenetic modification. Second, biallelic expression of *Igf2* in adult choroid plexus in the brain is associated with a largely paternal pattern of methylation of the two genes on both parental chromosomes.

Methylation on individual parental chromosomes

The bisulphite sequencing analysis of the paternally methylated region 3 kb upstream of the *Igf2* gene in the fetus revealed a number of interesting features. First, in this differentially methylated region of approx. 500 bp just 5′ to a CpG island, there is a gradient of methylation with almost complete methylation at the most 5′ border and very little methylation at the most 3′ end. Second, a number of CpG dinucleotides around *Hpa*II site H3 (see Figs 1A and 2B) are on average twice as methylated on paternal chromosomes as compared to maternal ones. Third, not a single CpG dinucleotide is invariably methylated on paternal, and unmethylated on maternal, chromosomes. Fourth, in contrast to two recent bisulphite sequencing studies in *Neurospora crassa* (Selker et al., 1993) and on a *Petunia hybrida* transgene (Meyer et al., 1994), we did not detect nonsymmetrical cytosine methylation in the mouse *Igf2* upstream region. The methylation pattern that emerges from the bisulphite sequencing analysis is highly mosaic, however, on a population basis it is highly predictable and consistent with Southern blotting results. The degree of mosaicism does not reflect methylation differences between tissues in the fetus since all individual tissues analysed showed a very similar methylation pattern in this region. Interestingly, a highly mosaic pattern of methylation was also found in the single chromosome analysis in *Neurospora* (Selker et al., 1993), which may suggest a far more dynamic balance of demethylation and de novo methylation in the early embryo than hitherto suspected (see also Jost, 1993; Kafri et al., 1993). That patterns of methylation in this region of *Igf2* may be progressively altered and non-clonal, at least in the early embryo, is also suggested from our finding that early-passage normal and monoparental ES cell-lines had similar levels of mosaic methylation in this region, comparable to those found in the embryo (see below).

The bisulphite sequence analysis of individual spermatozoa

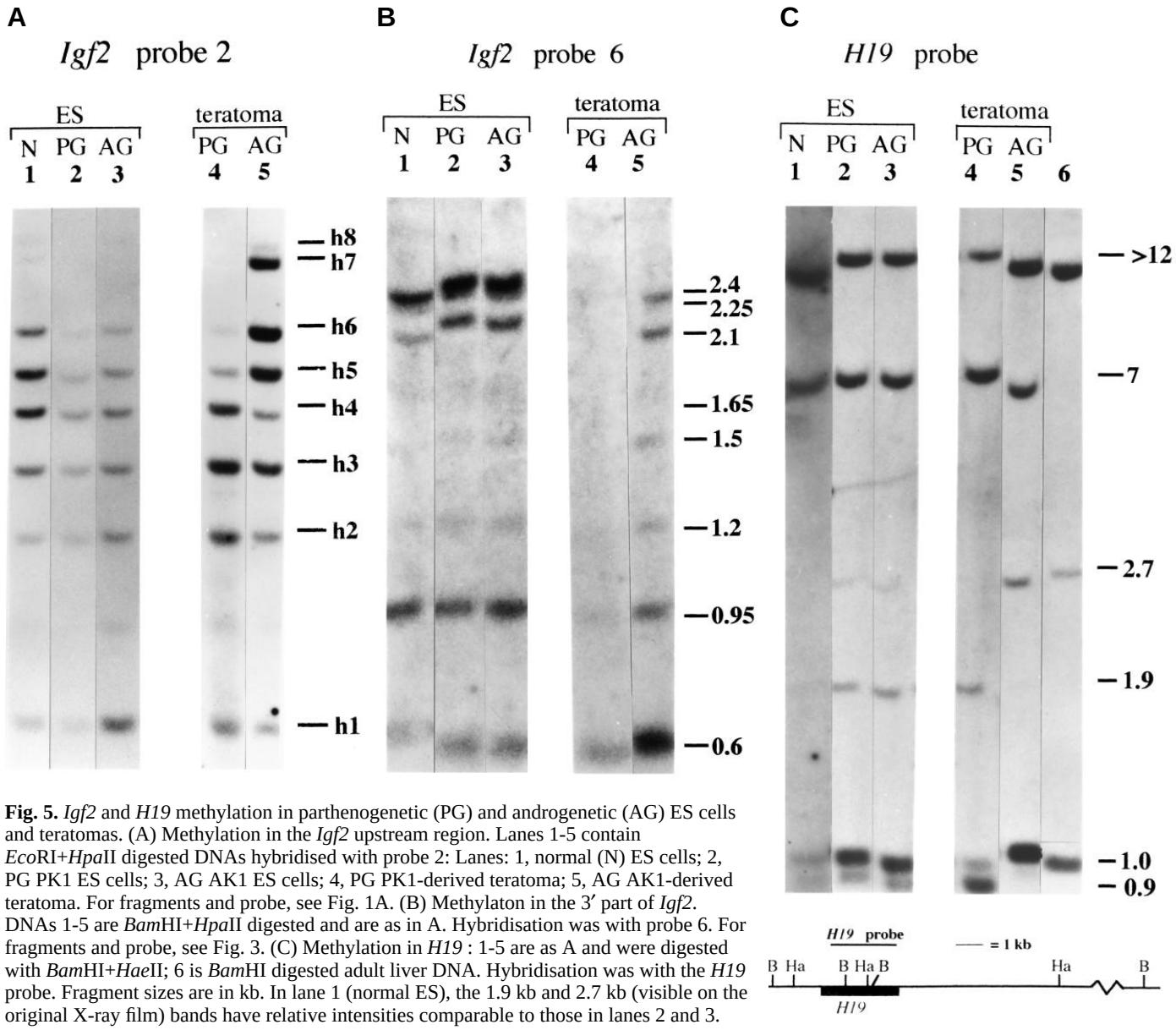


Fig. 5. *Igf2* and *H19* methylation in parthenogenetic (PG) and androgenetic (AG) ES cells and teratomas. (A) Methylation in the *Igf2* upstream region. Lanes 1-5 contain *Eco*RI+*Hpa*II digested DNAs hybridised with probe 2: Lanes: 1, normal (N) ES cells; 2, PG PK1 ES cells; 3, AG AK1 ES cells; 4, PG PK1-derived teratoma; 5, AG AK1-derived teratoma. For fragments and probe, see Fig. 1A. (B) Methylaton in the 3′ part of *Igf2*. DNAs 1-5 are *Bam*HI+*Hpa*II digested and are as in A. Hybridisation was with probe 6. For fragments and probe, see Fig. 3. (C) Methylation in *H19* : 1-5 are as A and were digested with *Bam*HI+*Hae*II; 6 is *Bam*HI digested adult liver DNA. Hybridisation was with the *H19* probe. Fragment sizes are in kb. In lane 1 (normal ES), the 1.9 kb and 2.7 kb (visible on the original X-ray film) bands have relative intensities comparable to those in lanes 2 and 3. The map underneath shows the *H19* gene and *Bam*HI (B) and *Hae*II (Ha) restriction sites.

further suggests that paternal methylation of specific CpGs in this region is unlikely to provide the primary imprinting signal. This is because almost all sperm chromosomes differ in their methylation patterns, and hence do not carry a predictable methylation pattern into the oocyte. For example, *Hpa*II site H3 (corresponds to CpG 3), which is paternally methylated as early as the blastocyst stage (Brandeis et al., 1993), is unmethylated in 30% of all spermatozoa (Fig. 2B, compare with Fig. 1B). If it were to serve as a paternal signal, it must become methylated upon fertilisation of oocytes with these sperm. However, the same site is also methylated in mature oocytes (Brandeis et al., 1993), hence loss of methylation from the maternal but not the paternal site during early embryogenesis would imply that signals other than methylation (at this site) are required.

Bisulphite analysis of 50 CpG dinucleotides in the CpG-rich region around promoter 2 extends our previous observation that the *Igf2* promoters are unmethylated on the repressed maternal chromosome. This is in contrast to both the *Igf2r* and *H19* genes, where CpG islands around the promoter become methylated on the repressed chromosomes.

Developmental and tissue-specific control of allelic methylation

Identification and analysis of a second region of differential methylation in the intron 4-exon 6 portion of *Igf2* revealed particularly interesting patterns of allelic methylation. While this region was virtually unmethylated on the maternal allele, methylation on the paternal allele was found to be tissue-specific, and to correlate with *Igf2* expression. Hence, in fetal brain in which only a minority of cells express *Igf2* (Lee et al., 1990; DeChiara et al., 1991), the paternal allele, like the maternal one, is almost completely unmethylated. By contrast, in fetal liver where a high proportion of cells express *Igf2* (Lee

et al., 1990; DeChiara et al., 1991) the paternal allele is almost completely methylated. While allelic differences are still present in adult liver (where *Igf2* is not expressed), the level of methylation of the paternal allele was lower than in fetal liver. Hence, a combination of availibility of transcription factors, density of methylation, and the possible involvement of other epigenetic modifications in this region, may influence transcription of the paternal allele. The fact that in the embryo, methylation in this region correlates directly with expression, may indicate the presence of a silencer element that is suppressible by tissue-specific epigenetic modification. Allelic methylation is also found in the corresponding sequences in the human *IGF2* gene (Schneid et al., 1993), which argues for the functional importance of this region. Indeed, particular alleles of this part of the *IGF2* gene have recently been implicated in aspects of the Beckwith-Wiedemann overgrowth syndrome in the human (Reik et al., 1994).

Allelic methylation in the 3′ part of *Igf2* is not unlike that found in intron 1 of the *Igf2r* gene in that (intronic) sequences within the gene are involved, the expressed allele is the more methylated one, and is methylated in the germ line (Stöger et al., 1993; Brandeis et al., 1993). Methylation of sequences in both genes is indeed required for expression, since embryos that lack the enzyme methyltransferase fail to express *Igf2* and *Igf2r* (Li et al., 1993).

Further support for allelic methylation in *Igf2* and *H19* being of functional importance, at least for the somatic maintenance of imprinting, comes from our observation that in choroid plexus, where *Igf2* is bi-allelically expressed, both copies are now highly methylated in the upstream region as well as in the 3′ region. Similarly, both parental copies of *H19* (which are not expressed) also become methylated in choroid plexus, quite unlike all other adult tissues including brain in which the gene is not expressed, but in which allelic methylation differences persist. In choroid plexus therefore, both genes now adopt a largely paternal pattern of methylation.

Previous studies have suggested that allelic methylation upstream of *Igf2*, and in the *H19* gene, is not present in parental germ cells, but arises in the early embryo (Brandeis et al., 1993), whereas methylation of the maternal *Igf2r* intron region is already present in the mature oocyte (Stöger et al., 1993; Brandeis et al., 1993). This impression is strengthened by our analysis of early-passage androgenetic and parthenogenetic ES cells and their differentiated derivatives in teratomas. For this analysis we used monoparental stem cells which have retained their *Igf2* and *H19* imprints, as shown by assays in vitro and in chimaeras (Allen et al., 1994). All regions analysed had a similar level of methylation in normal, parthenogenetic, and androgenetic stem cells, whereas in androgenetic and parthenogenetic teratomas the paternal and maternal patterns, respectively, of allelic methylation had clearly been established. The observation that *Igf2* and *Igf2r* are expressed in both androgenetic and parthenogenetic blastocysts (Latham et al., 1994) further supports the view that allelic methylation patterns are not yet fully established at these early stages. A recent study that also looked at methylation in monoparental stem cells found that there were no differences in the region upstream of *Igf2*, but some differences were detected in *H19* (Szabó and Mann, 1994). It is possible that some changes in methylation occur in stem cells as a result of particular culture conditions and passage number (Szabó and Mann, 1994). Analysis using ES cells should therefore always be complemented with direct experiments in the early embryo. Indeed, direct analysis of allelic methylation of *H19* in blastocysts also suggests that allele-specific methylation is not completely established at this stage (Brandeis et al., 1993).

In conclusion, our analysis suggests that differentiation and tissue-specific factors influence the final somatic patterns of allelic methylation of the imprinted *Igf2* and *H19* genes in fetal and adult tissues. Different elements in these genes can behave differently (as shown for the 3′ region of *Igf2*) and may thus have different roles in the imprinting process, from reversal and initiation in germ cells, to somatic maintenance and potential loss.

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