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Temporal and spatial regulation of *H19* imprinting in normal and uniparental mouse embryos

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SUMMARY

The mouse *H19* gene is imprinted so that the paternal copy is both methylated and repressed during fetal development. However, the CpG-rich promoter region encompassing the transcription start is not methylated in sperm; this region must therefore become methylated postzygotically. We first examined the timing of DNA methylation of this region and the corresponding expression of H19. Both parental copies are initially undermethylated in blastocysts and the paternal copy then becomes fully methylated in the embryo around implantation; this methylation is more protracted in the extraembryonic lineages, especially in the trophoblast. By contrast to the lineage-dependent methylation, we observed exclusive expression of the maternal copy in preimplantation embryos and in all the lineages of early postimplantation embryos although variability may exist in cultured embryos. This indicates that methylation of the CpG-rich promoter is not a prerequisite for the paternal repression. We then examined whether methylation and expression occurs appropriately in the absence of a maternal or a paternal genome. Both *H19* copies in androgenetic embryos are fully methylated while they are unmethylated in parthenogenetic embryos. This correlates with the lack of expression in androgenetic embryos but expression in parthenogenetic embryos. However, the androgenetic trophoblast was exceptional as it shows reduced methylation and expresses *H19*. These results suggest that promoter methylation is not the primary inactivation mechanism but is a stabilizing factor. Differential methylation in the more upstream region, which is established in the gametes, is a likely candidate for the gametic signal and may directly control *H19* activity.

Key words: *H19*, DNA methylation, imprinting, androgenetic, parthenogenetic, mouse, extraembryonic tissue, trophoblast

INTRODUCTION

The mouse *H19* gene is a developmentally regulated gene, originally identified as a fetal liver-specific cDNA which is repressed after birth and induced upon liver regeneration (Pachnis et al., 1984). During development, expression of this gene is first detectable in the trophectoderm of late blastocysts and, subsequently, it is expressed at high levels in a wide array of tissues of both endoderm and mesoderm origin (Poirier et al., 1991). Apparent lack of a conserved open reading frame in the mouse and human genes suggests that their final product may be an RNA (Pachnis et al., 1988; Brannan et al., 1990). Although the precise function of this gene is unknown, *H19* RNA has been shown to suppress tumor morphology in transfected tumor cells (Hao et al., 1993).

The *H19* gene maps to the distal end of mouse chromosome 7 (Bartolomei et al., 1991), which has been shown to contain imprinted genes by genetic tests (Searle and Beechey, 1990). Direct evidence for differential expression of the paternally and maternally derived *H19* genes came from an allele-specific RNase protection assay in interspecific hybrids (Bartolomei et al., 1991). In neonatal liver and skeletal muscle, only the copy inherited from the mother was expressed. This was also true for the midgestation embryo, as shown by an analysis of fetuses with maternal duplication for this chromosome segment (Ferguson-Smith et al., 1993). Nuclear run-on assay with the disomy cells further demonstrated that the gene is controlled by imprinting at the level of transcription (Ferguson-Smith et al., 1993).

Although parental imprinting of a subset of developmental genes, such as *H19*, is apparently important for normal development (Solter 1988; Surani 1991), the molecular nature of the imprinting mechanism is yet to be clarified. A number of studies including the targeted disruption of the DNA methyltransferase gene (Li et al., 1993) suggest that DNA methylation is one of the important modifications involved in imprinting.

We and others have previously observed that the inactive paternal *H19* copy is hypermethylated at its CpG-rich promoter and in the 5′ flanking region, while the active maternal copy is unmethylated in these regions (Ferguson-Smith et al., 1993; Brandeis et al., 1993; Bartolomei et al., 1993). In addition, the inactive promoter on the paternal chromosome was more resistant to nucleases in chromatin, suggesting that this copy assumes closed chromatin configuration (Ferguson-Smith et al., 1993; Bartolomei et al., 1993). Thus methylated CpGs and/or condensed chromatin structure are likely to prevent initiation complex formation at the paternally derived *H19* promoter.

It is assumed that the imprinting signal is imparted during gametogenesis since this is the only time when the two haploid genomes are physically separated. Tremblay et al. (1995) recently showed that the region 5′ to the *H19* promoter is methylated in sperm but not in oocytes and that such a difference in methylation can persist through preimplantation development at a subset of sites. However, the most CpG-rich region containing the transcription start is unmethylated in sperm DNA (Ferguson-Smith et al., 1993; Tremblay et al., 1995) just as for CpG islands (Bird, 1986); methylation of this region must therefore occur postfertilization.

In this report, we first address the question of when the hypermethylation of the paternally derived CpG-rich promoter is established and show that it occurs around implantation with different schedules in different lineages. The mode of the lineage-dependent methylation closely resembles that seen with the X-linked CpG islands upon X-chromosome inactivation and is consistent with the global undermethylation of the genomic DNA in the extraembryonic tissues (Sanford et al., 1985; Monk, 1990). By contrast, exclusive expression of the maternal copy was observed in preimplantation and postimplantation embryos. The imprinting and expression of *H19* was also examined in androgenones and parthenogenones. Appropriate parental-origin-dependent methylation and expression was also found in these postimplantation embryos. However, there was an exception to this rule since the paternal copy is activated in the trophoblast of androgenetic embryos, where this copy is undermethylated. Thus methylation of the parental *H19* promoter is subject to stage- and tissue-specific regulation and this methylation probably serves as an important maintenance mechanism.

MATERIALS AND METHODS

Mouse embryos

For DNA methylation studies, (C57BL/6J × CBA)F_1 females and males were crossed to obtain normal blastocysts and early postimplantation embryos. For expression studies, (C57BL/6J × CBA)F_1 × *Mus spretus* embryos were produced by natural crossing or by in vitro fertilization as described by Kay et al. (1991). After in vitro fertilization, the fertilized eggs were cultured in vitro and transferred to the oviducts of recipients at the 2-cell stage, or to the uteri of recipients at the early blastocyst stage. Parthenogenetic and androgenetic embryos were produced from unfertilized (C57BL/6J × CBA)F_1 eggs and fertilized (C57BL/6J × CBA)F_1 females × 129Sv males respectively, as described previously (Surani et al., 1988). Control embryos were of (C57BL/6J × CBA)F_1 × (C57BL/6J × CBA)F_1 genotype.

Methylation analysis by Southern blotting

Groups of approximately 1400 normal blastocysts, 150 and 50 normal postimplantation embryos collected at 6.5 and 7.5 day postcoitum (dpc) respectively, were used to isolate genomic DNA. When recovering the postimplantation embryos, care was taken to remove maternal tissue as completely as possible. The postimplantation embryos were carefully dissected into the embryonic portion, trophoblast (ectoplacental cone) and the rest of the extraembryonic portion with a fine glass needle, and each portion was pooled. Parthenogenetic, androgenetic and control embryos were recovered at 9.5 dpc, and the embryo proper, trophoblast and yolk sac were collected and pooled separately.

Genomic DNA was isolated from the material using the method described by Allen et al. (1987). The whole DNA sample from early embryos (blastocysts, 6.5 and 7.5 dpc embryos) and approximately 5 μg of the sample from parthenogenetic, androgenetic and control embryos were cut with restriction enzymes, run through 1.4 % agarose gel, and blotted onto Hybond-N^+. Labeling of the probe was performed by the random priming method of Feinberg and Vogelstein (1984). Hybridization was carried out at 65°C in a buffer containing 0.5 M sodium phosphate (pH 7.2), 7% SDS, 1 mM EDTA and the probe. The hybridized membrane was washed in 0.1× SSC, 0.1% SDS at 65°C and autoradiographed.

Methylation analysis by PCR (polymerase chain reaction)

Twenty blastocysts were collected at 3.5 dpc and ten of these were cultured overnight to allow development to the late blastocyst stage. Genomic DNA was isolated from these embryos as above except that yeast transfer RNA or glycogen was added as carrier. PCR methylation analysis was carried out as described by Kafri et al. (1992). DNA samples of 100 pg were incubated with or without *Hpa*II and then subjected to PCR for 35 cycles. Each PCR cycle consisted of 1 minute at 93°C, 1 minute at 60°C and 1 minute at 72°C. The primers used are: HP1 (5′ primer for site 1), 5′-TTGAAGGATCACTAGGAGCCAG-3′; HP2 (3′ primer for site 1), 5′-ATTGCTCTTAGCTTCTGTTGAAAG-3′; HP5 (5′ primer for site 3 and sites 3 plus 4), 5′-AGCAGGCTACGGGGCTATATG-3′; HP6 (3′ primer for site 3), 5′-CTCTGTCAACCAATCAGTACATG-3′; HP7 (5′ primer for site 4), 5′-CATGTACTGATTGGTTGACAGAG-3′; HP10 (3′ primer for site (3+)4), 5′-GTGCTTCGGGCCCTCTAGCC-3′. The PCR products were electrophoresed through 1% agarose/2% NuSieve GTG agarose (FMC BioProducts) gel, blotted onto Hybond-N^+ and hybridized with either HP1 (site 1) or HP7 (sites 3, 4 and 3+4), which had been end-labeled with ^{32}P. Under these conditions, the assay yields quite accurate results if the degree of methylation is less than 15%. However, the method does not distinguish, for example, between 50 and 100% methylation (Kafri et al., 1992).

Expression analysis by RT(reverse transcriptase)-PCR

(C57BL/6J × CBA)F_1 × *Mus spretus* embryos produced as above were recovered for isolation of RNA. Approximately twenty preimplantation embryos or five postimplantation embryos were collected at each developmental stage. The postimplantation embryos were dissected into the three portions as described above and pooled separately. Yeast transfer RNA or glycogen as carrier was added to the pooled material and RNA was purified according to the method of Kay et al. (1993). The samples were treated with DNase I prior to RT-PCR to destroy any contaminating DNA as described previously (Sasaki et al., 1992).

RT-PCR was carried out using the GeneAmp RNA PCR Kit (Perkin Elmer Cetus) according to the manufacturers protocol. One fifth of the RNA samples was used for the first strand cDNA synthesis with the 3′ primer, OLG2 (5′-GTAGGGCATGTTGAACACTTTATG-3′). The whole reaction was then brought into the PCR mixture and amplified for 35 or 60 cycles of 95°C for 1 minute and 65°C for 1 minute. To minimize the heteroduplex formation by the allelic sequences, an extra final cycle was performed after adding an equal

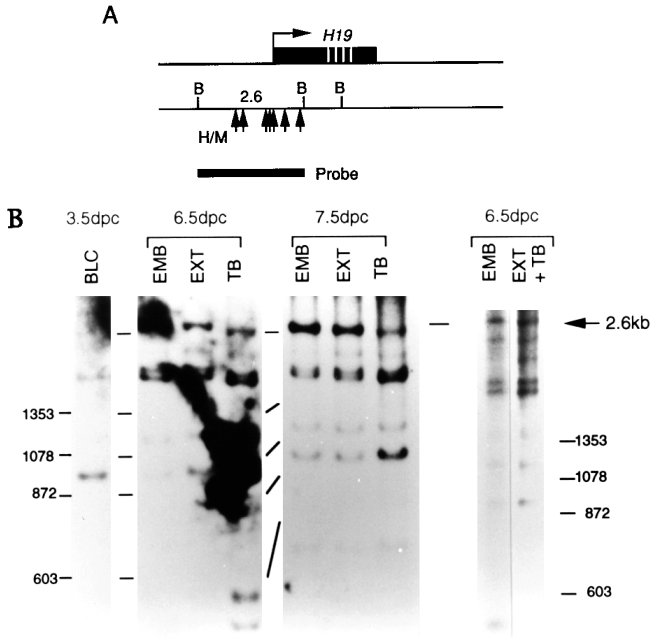

Fig. 1. Methylation analysis by Southern blotting of the *H19* gene in blastocysts and in different tissues of the conceptus after implantation. (A) Structure of the *H19* gene. The position of the transcription start site and the direction of transcription are indicated. The positions of *Hpa*II/*Msp*I (H/M) sites (upward arrows) are indicated only for the *Bam*HI (B) 2.6 kb fragment.
(B) Autoradiographs showing the methylation status of the *Bam*HI 2.6 kb region in early embryos. BLC, blastocyst; EMB, embryonic portion; EXT, extraembryonic portion excluding the trophoblast; TB, trophoblast. Samples are from conceptuses at 3.5, 6.5 and 7.5 dpc. An additional sample from 6.5 dpc confirms that methylation of *H19* has occurred in the embryo by this time and is ongoing in the extraembryonic and trophoblast tissues (EXT+TB).

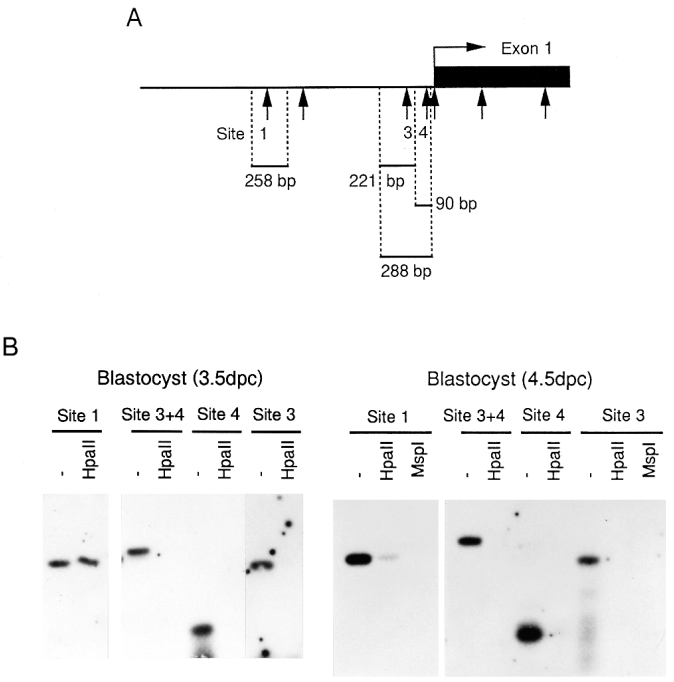

Fig. 2. Confirmation of the unmethylated state in blastocysts by a PCR methylation assay. (A) Map of the *H19* promoter region. The positions of *Hpa*II/*Msp*I sites are indicated by upward arrows. Only sites 1, 3 and 4 were analyzed. The sizes of the PCR products, which appear only when the internal site(s) is methylated, are also indicated. (B) Autoradiographs showing that the CpG-rich promoter region is completely unmethylated (sites 3 and 4) at both 3.5 and 4.5 dpc. Site 1 showed 50-100% methylation at 3.5 dpc and less methylation at 4.5 dpc.

volume of the PCR mixture containing the buffer, primers, dNTPs and the polymerase. The primers used are as follows: OLG1 (5′ primer for genomic DNA), 5′-GTGAAGCTGAAAGAACAGATGGTG-3′; OLG7 (5′ primer for RNA), 5′-TGCTCCAAGGTGAAGCTGAAAG-3′; OLG2 (3′ primer for both DNA and RNA, see above for sequence). The PCR products were purified and concentrated by Microcon 100 (Amicon) and a proportion of each sample was cut with *Bgl*I, electrophoresed through 1.7% agarose gel and blotted onto a piece of Hybond-N^+ (Amersham). The membrane was hybridized with OLG1, which had been end-labeled with ^{32}P at the 5′ terminus, and then washed and autoradiographed. Radioactivity of each band was measured on a Bio-Image Analyzer BAS2000 (Fuji).

In situ hybridization analysis

Androgenetic embryos were dissected out at 9.5 dpc in ice-cold phosphate-buffered saline. Since androgenetic embryos were delayed in development, control embryos were dissected out at 8.5 dpc and similarly treated in order to obtain material at a comparable developmental stage. The mesometrium was removed and the whole decidua was fixed in 4% paraformaldehyde at 4°C overnight.

A 2 kb fragment from mouse *H19* cDNA (Poirier et al., 1991) was subcloned into the *Eco*RI site of pBluescript II KS+. The anti-sense RNA probe was generated by transcription with T7 polymerase from *Kpn*I-linearized recombinant plasmid while the sense probe was generated with T3 polymerase from *Xba*I-linearized template. Similarly, a 680 bp *Hin*fI-*Pst*I fragment from human *Igf2* cDNA was subcloned into the pGEM-3 vector. Transcription with SP6 polymerase from non-linearized recombinant plasmid generated the anti-sense RNA probe while T7 polymerase generated the sense probe. All the probes were labeled with ^{35}S-UTP.

In situ hybridization was carried out as described in Wilkinson and Green (1990). Briefly, sections were treated with proteinase K (10 μg/ml) and hybridized with the probe overnight at 60°C. The sections were then subjected to high stringency wash at 67°C for 30 minutes (*H19* probe) or 55°C for 45 minutes (*Igf2* probe). After dehydration, slides were coated with Ilford K5 emulsion and exposed at 4°C for 4 days. Developed slides were stained with hematoxylin and mounted in DPX. Microscopic examination was carried out under bright and dark fields and photographs were taken with PanF film.

RESULTS

Undermethylation of the paternally derived promoter in blastocysts and subsequent lineage-dependent methylation

The CpG-rich promoter region of the paternally derived *H19* copy, which is undermethylated in sperm, must become methylated after fertilization. We have previously shown that this copy is already considerably methylated at 8.5-10.5 dpc both in the embryonic and extraembryonic tissues (Ferguson-Smith et al., 1993). To determine the timing of methylation of this copy more precisely, we analyzed DNA from blastocysts and early postimplantation embryos using a methylation-sensitive restriction endonuclease, *Hpa*II. Fig. 1A depicts the structure

and restriction map of the mouse *H19* gene. The *Bam*HI fragment of 2.6 kb contains the differentially methylated sites and the three *Hpa*II sites, which are closely located around the transcription start, represent the CpG-rich promoter.

When DNA from blastocysts collected at 3.5 dpc was double digested with *Hpa*II and *Bam*HI and probed with the whole *Bam*HI fragment, no 2.6 kb band was seen (Fig. 1B), indicating that neither of the parental copies is fully methylated. The faint band of about 1.8 kb arose from cutting at one of the three *Hpa*II sites within the CpG-rich promoter, suggesting that this promoter is unmethylated. This was confirmed by a PCR methylation assay which showed that two *Hpa*II sites (sites 3 and 4, Fig. 2A) within this region are completely unmethylated in both 3.5 and 4.5 dpc blastocysts (Fig. 2B). By contrast, one *Hpa*II site (site 1) located approximately 800 bp 5′ to the transcription start was nearly fully methylated at 3.5 dpc, and at least partially methylated at 4.5 dpc (Fig. 2B). This site is identical with site Hpa6 or H6 in Tremblay et al. (1995) and has been shown to be methylated on the paternal chromosome in blastocysts.

When DNAs from postimplantation embryos at 6.5 and 7.5 dpc were analyzed, the 2.6 kb *Bam*HI band appeared in all three different portions of the egg cylinders, i.e. the embryonic portion, trophoblast (ectoplacental cone) and the rest of the extraembryonic portion (Fig. 1B). This indicates that a proportion of DNA had become methylated at all sites within this fragment shortly after implantation. The methylated fraction is most probably of paternal origin since methylation of the paternal *H19* copy, but not the maternal copy, has been clearly demonstrated in later stage embryos, neonates and adults (Ferguson-Smith et al., 1993; Bartolomei et al., 1993) (see also later for data on androgenetic and parthenogenetic embryos in Fig. 5).

An interesting observation that we made during the study was that the progression of methylation differs between different portions of the egg cylinders. For example, the paternal copy was already almost fully methylated at 6.5 dpc in the embryonic portion since the pattern was indistinguishable from those of later stage embryos, but this copy was less methylated in the trophoblast since the signal of the uncut 2.6 kb band was weaker and those of the cutting products were stronger, when compared with those of the embryonic portion. This was also true at 7.5 dpc, but some sub-bands in the low molecular weight region were not so intense as at 6.5 dpc. The extraembryonic tissue other than the trophoblast showed a pattern similar to that of the embryonic portion but some sub-bands appeared to be stronger at 6.5 dpc. Thus, the hypermethylation of the paternal *H19* gene occurs quickly in the

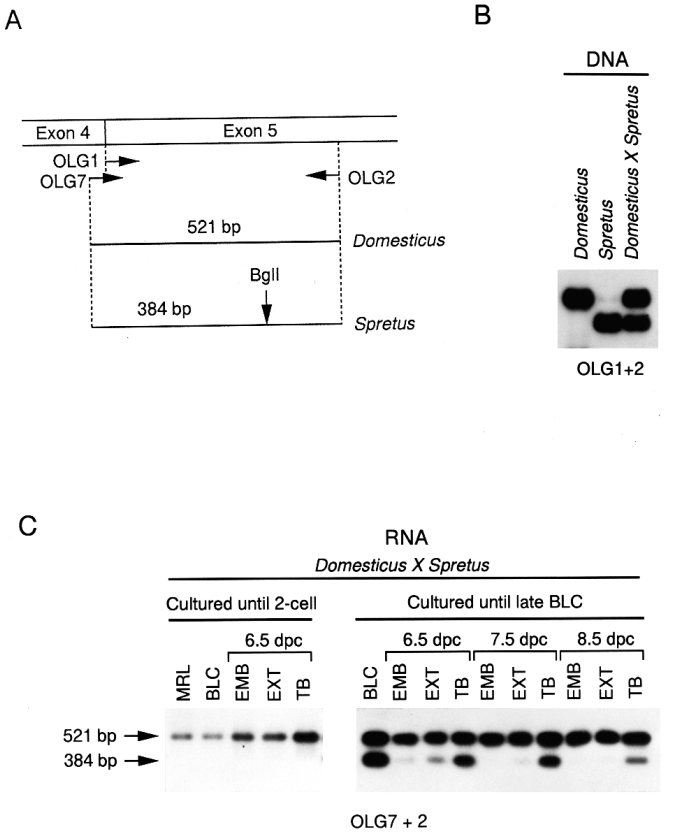

Fig. 3. RT-PCR assay to determine expression of maternal and paternal alleles of *H19* gene in blastocysts and in different tissues of the conceptus from 6.5-8.5 dpc. (A) Species-specific *Bgl*I polymorphisms in *H19* transcripts. *Mus spretus* contains a *Bgl*I site within the exon 5 sequence while *Mus musculus domesticus* does not. Positions of the PCR primers are indicated by horizontal arrows (OLG1, 2 and 7). OLG7, which was used in the RT-PCR assay, contains the exon 4-5 junction in its middle in order to prevent cross hybridization with the genomic DNA. Size of the RT-PCR product detected by ^{32}P-end-labeled OLG1 following *Bgl*I digestion is indicated. (B) Confirmation of the *Bgl*I polymorphisms in genomic DNA. (C) Analysis of paternal and maternal *H19* expression by RT-PCR followed by *Bgl*I digestion. The lefthand panel represents the monoallelic expression of *H19* in preimplantation embryos and all the portions of early postimplantation embryos. However, in two batches of embryos cultured for a prolonged period (righthand panel), biallelic expression was observed (the blastocyst to 7.5 dpc data from one batch and the 8.5 dpc data from the second). Note the steady decline in the expression of the paternal copy in the trophoblast. The transcripts from the paternal *H19* copy is represented by a 521 bp band and that from the maternal copy by a 384 bp band. Different portions of the embryo are indicated as in Fig. 1.

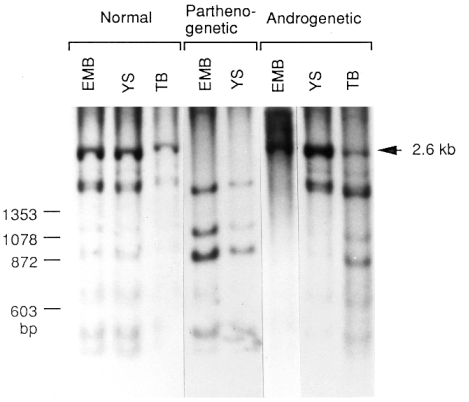

Fig. 4. DNA methylation analysis in normal, parthenogenetic and androgenetic conceptuses. The methylation status of the 2.6 kb *Bam*HI region was examined in conceptuses all of which were recovered at 9.5 dpc, as described for Fig. 1. EMB, embryo proper; YS, yolk sac; TB, trophoblast. We were unable to recover enough parthenogenetic trophoblast because of its poor development.

embryonic lineages but slowly and progressively in the extraembryonic lineages, notably in the trophoblast.

Exclusive expression of the maternal copy in early embryos

The methylation data described above prompted us to examine expression of parental alleles in early stage embryos in order to determine if these observations correlated with expression of *H19*. To do this, we first looked for a polymorphism that can be used as a genetic marker to distinguish between the paternally and maternally transmitted copies. Sequencing of PCR-amplified genomic DNA from different *Mus* species identified one such polymorphism that can be detected by restriction digestion. As illustrated in Fig. 2A, *Mus spretus* contains a *Bgl*I site in exon 5 whereas this restriction site is not present in ordinary laboratory mice (*Mus musculus domesticus*) due to a single nucleotide change. The presence or absence of this restriction site in these species was confirmed by *Bgl*I digestion of the PCR products amplified from their genomic DNA, as shown in Fig. 2B.

We then assayed the RNA from early embryos for allelic expression by RT-PCR followed by *Bgl*I digestion. The lefthand part of Fig. 2C shows that only the maternal, domesticus copy of *H19* is expressed in morulae, blastocysts, and all the three portions of 6.5 dpc embryos, all of which had been produced by in vitro fertilization followed by oviduct transfer at the 2-cell stage. This monoallelic expression was confirmed in four later experiments, one with embryos prepared in the same way, two with embryos transferred at the blastocyst stage and one with embryos developed completely in vivo. The fact that blastocysts and trophoblast cells express only the maternal copy indicates that methylation of the promoter region surrounding the transcription start is not the primary inactivation mechanism.

In the course of this study, however, we observed a very different picture with two batches of in vitro fertilized embryos, which had been cultured in vitro up to the blastocyst stage. The results are shown in Fig. 3C, right. Here, blastocysts and both 6.5 and 7.5 dpc embryos were from one batch and 8.5 dpc embryos were from the other. Strikingly, both parental copies were nearly equally expressed in fully expanded blastocysts at 4.5 dpc. After implantation, the trophoblast lineage continued to express both parental copies but the activity of the paternal copy relative to the maternal copy declined gradually. By contrast, in the embryonic portion, paternal *H19* expression was very low from the earliest stages of postimplantation development. The extraembryonic portion excluding the trophoblast showed a pattern intermediate between the above two portions. Thus there was generally an inverse correlation between the levels of methylation and expression. We first thought that this biallelic expression in specific tissues was due to the prolonged in vitro culture during the preimplantation period. However, as described above, only maternal expression was observed with the embryos prepared in the same way in two later experiments. Also, all these experiments were done with pooled materials and therefore the biallelic expression is not due to 'imprinting polymorphisms' among the embryos. Therefore the exact cause of the paternal activation is at present unknown. Our current interpretation is that the repression of

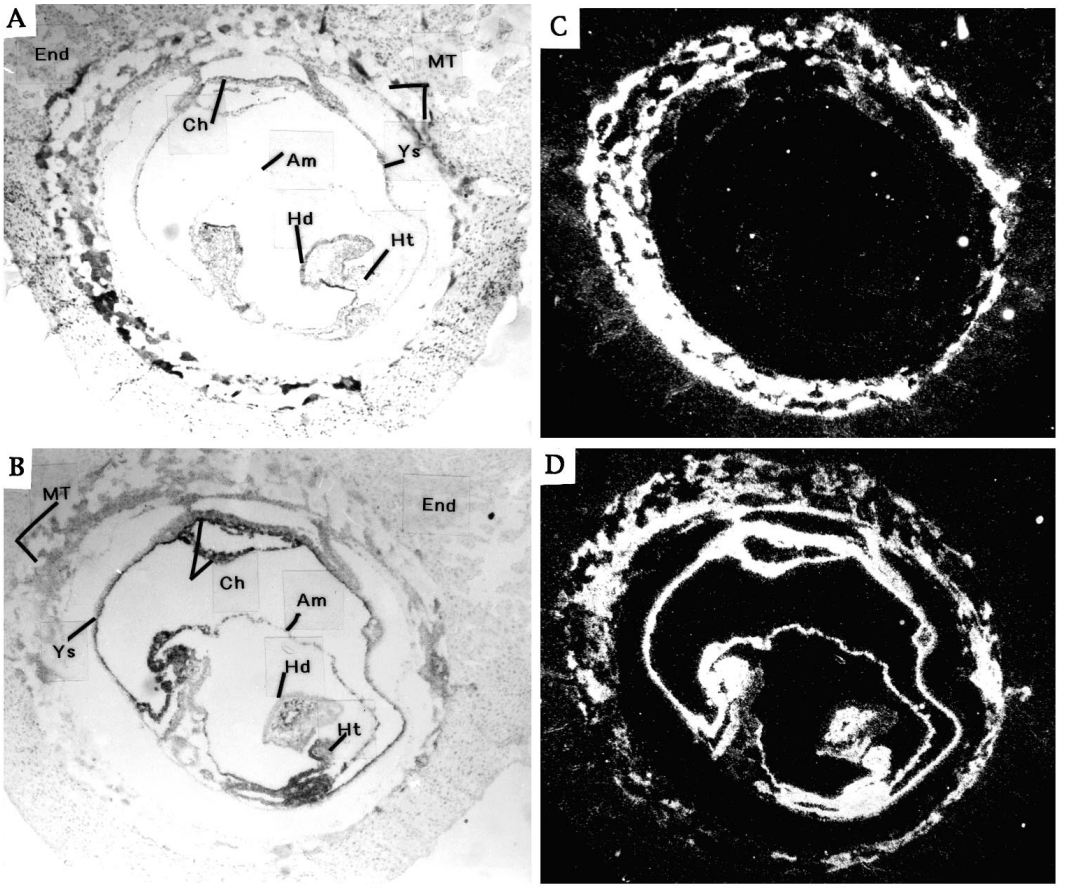

Fig. 5. Expression of *H19* and *Igf2* genes in adjacent sections from an androgenetic conceptus at about the 4-somite stage. Hybridization was carried out with an anti-sense *H19* probe (C) or with an anti-sense *Igf2* probe (D). The embryos were observed under bright field (A,B) or dark field (C,D). Control experiments with sense probes yielded very low background signal (data not shown). Expression of *Igf2* is observed in the embryo and the extraembryonic tissues (D). By contrast, *H19* expression was not detectable in the embryo but was observed in the trophoblast. Control embryos at the equivalent stage express both *Igf2* and *H19* (data not shown). Hd, head; Ht, heart; Am, Amnion; Ys, yolk sac; Ch, chorion; Mt, mural trophoblast; End, endometrium.

the paternal copy may be unstable/variable when embryos are cultured in vitro for a prolonged period.

Methylation and expression in androgenetic and parthenogenetic embryos

In order to establish if imprinting can occur appropriately when one of the parental genomes is absent, we examined methylation and expression of *H19* in diploid androgenetic and parthenogenetic embryos that lack the maternal and paternal genomes, respectively. Genomic DNA was isolated from different portions of normal, parthenogenetic and androgenetic embryos, all collected at 9.5 dpc, and double digested with *Hpa*II and *Bam*HI. As shown in Fig. 4, the embryonic portion of androgenetic embryos exhibited only the 2.6-kb *Bam*HI band, indicating that the two *H19* copies are fully methylated. By contrast, the trophoblast of these embryos were clearly less methylated since multiple sub-bands resulting from *Hpa*II digestion were present and the 2.6 kb band was faint. The yolk sac showed a pattern intermediate between the above two tissues. In parthenogenetic embryos, both embryo proper and yolk sac gave only the low molecular weight sub-bands, indicating that the two maternal *H19* genes are both undermethylated. We were unable to obtain enough trophoblast material from parthenogenetic embryos because this tissue develops particularly poorly. DNAs from different portions of the normal embryo show a pattern similar to those of the 7.5 dpc embryo (Fig. 1B) but the methylation profile of the trophoblast is no more significantly different from those of the other two tissues at this stage (9.5 dpc). These results show that methylation of *H19* occurs appropriately in both androgenetic and parthenogenetic embryos.

We went on to examine tissue-specific *H19* expression by in situ hybridization to determine how this correlated with the methylation changes described above. Since androgenetic embryos lack the maternal genome, it allows identification of any cells that may express the paternal copy in the absence of signals from the maternal copy. In this study, we also analyzed expression of another imprinted gene, the insulin-like growth factor II gene (*Igf2*), which served as a control. This gene is imprinted reciprocally to the *H19* gene, with the paternally derived copy being active and the maternal copy being inactive (DeChiara et al., 1991; Ferguson-Smith et al., 1991).

In situ hybridization with the anti-sense probes showed that in normal control embryos at about 4-somite stage (collected at 8.5 dpc) both *H19* and *Igf2* are widely expressed throughout the conceptus. Intense labeling was detected in all the extraembryonic and embryonic tissues except the neuroepithelium where the expression of both genes was close to the background level (not shown). Our results are similar to the tissue-specific expression of both *H19* (Poirier et al. 1991) and *Igf2* (Stylianopoulou et al. 1988) reported previously. It is interesting to note that the *H19* transcript was visible in the cytoplasm of the giant trophoblast cells with very little labeling in the nucleus (not shown). By contrast, androgenetic embryos at a comparable developmental stage (collected at 9.5 dpc) showed a marked difference in expression pattern between the two genes (Fig. 5). The distribution of *Igf2* expression was identical with that in the control embryos, whereas *H19* expression was detected only in the trophoblast and virtually no expression was seen in the embryo and the yolk sac. Since both the maternal *H19* copies were expressed in parthenogenetic embryos (not shown), the expression pattern of *H19* in the embryo proper of androgenetic and parthenogenetic embryos is therefore in complete agreement with its established imprinting pattern. The expression of the paternal *H19* copies in the androgenetic trophoblast was inconsistent with what we would expect from the direction of imprinting, but agreed with the activity from the paternal copy seen with some cultured 8.5 dpc normal embryos (Fig. 3C, right). This could be attributed to the extensive in vitro manipulation of these embryos but also to the absence of the maternal genome.

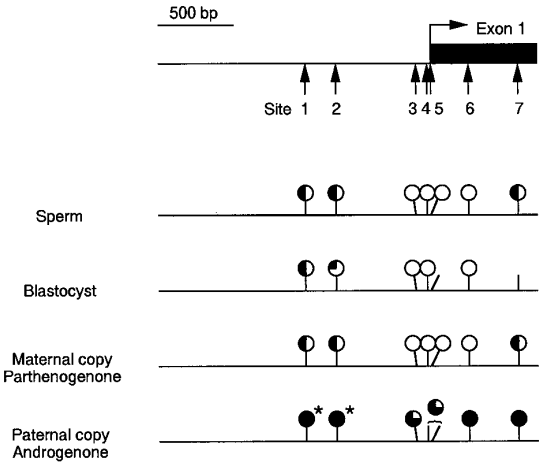

Fig. 6. Summary of the methylation studies. The data for sperm is from Ferguson-Smith et al. (1993). For site 1 and 6, the data by Brandeis et al. (1993) and Tremblay et al. (1995) are also incorporated. Open circles indicate no methylation and filled circles full methylation. Asterisks show the sites on the paternal chromosomes that become methylated more slowly in the trophoblast than in the embryo proper. These sites are less methylated in the androgenetic trophoblast as well. Although not specified, some other sites also appear to be less methylated in the trophoblast cells.

DISCUSSION

DNA methylation is an important component regulating the parental-origin-specific activity of imprinted genes as embryos mutated in the DNA methyltransferase gene show deregulation of these genes (Li et al., 1993). In the present study, we demonstrated that the CpG-rich promoter of the paternally derived *H19* gene is initially undermethylated in blastocysts and then becomes hypermethylated upon implantation, but with delayed progression of methylation in the extraembryonic lineages, especially in the trophoblast (summarized in Fig. 6). The initiation of methylation at around the time of implantation coincides with the onset of de novo methylation of the bulk genome (Monk, 1990). CpG islands of X-linked genes also become methylated at similar developmental stages (Lock et al., 1987; Singer-Sam et al., 1990; Grant et al., 1992). Furthermore, the relative undermethylation of the paternal *H19* copy in the extraembryonic lineages compared with the embryonic lineages agrees well with the previous observations on non-imprinted sequences and X-linked genes (for review see Sanford et al., 1985; Monk, 1990). Thus the occurrence of the overall hypermethylation of the paternal *H19* copy follows

the general rules of DNA methylation in mouse development. The acquisition of hypermethylation by the paternal *H19* promoter, however, clearly contrasts with the behavior of another imprinted gene, *Igf2*, in which allele inactivation occurs without detectable methylation at the CpG-island promoter (Sasaki et al., 1992).

We also wished to determine whether both parental genomes are essential in establishing the appropriate allelic methylation pattern. This is because it has been shown that parental-origin-specific *trans*-acting factors or modifiers can affect methylation imprinting of transgenes (Allen et al., 1990). However, in the case of *H19*, appropriate methylation does occur in postimplantation androgenetic embryos in the absence of a maternal genome. Conversely, neither of the maternal copies in postimplantation parthenogenetic embryos is methylated. (See Fig. 6 for summary). This suggests that they do not require the activity of parental-origin-dependent modifiers at least for *H19*. However, we cannot formally dismiss the involvement of such an effect for other imprinted genes, which will need to be examined.

A recent study showed that the *H19* promoter is equally undermethylated in androgenetic and parthenogenetic embryonic stem (ES) cells (Feil et al., 1994), which is consistent with our blastocyst data. Paradoxically, however, it was also reported that the *H19* promoter is more methylated in androgenetic ES cells than in parthenogenetic ES cells (Szabó and Mann, 1994). Other papers have described that the parental *H19* copies are differentially methylated in normal ES cells (Bartolomei et al., 1993; T. Tada and N. Takagi, personal communication). The reasons for these discrepancies are unknown but it could be that the regulation of methylation is relaxed in culture or that some ES cells proceed to a slightly advanced developmental stage even though they still preserve the potential to form chimeras.

By contrast to the lineage- and stage-dependent methylation, the expression of *H19* was monoallelic from the earliest stages of mouse development with some exceptions (see below). This data is consistent with that from other laboratories (Tremblay et al., 1995). The finding that the repression of the paternally derived copy occurs without detectable methylation at the CpG-rich promoter indicates that hypermethylation of the promoter is not the primary inactivation mechanism.

The above findings notwithstanding, it is important to note that in two groups of embryos produced by in vitro fertilization and in vitro culture until the blastocyst stage, we observed biallelic expression of *H19*. The paternal copy was initially expressed in blastocysts and became inactivated progressively as its methylation level increased after implantation. The repression occurred more slowly in the extraembryonic tissues than in the embryo proper, especially in the trophoblast. Since in two other experiments with similarly prepared embryos there was monoallelic expression, it is not possible to attribute the activation of the paternal copy solely to the in vitro culture. Also, the biallelic expression did not result from 'imprinting polymorphisms' since the results were obtained with pooled materials. Although the precise cause of the phenomenon is unknown, it is possible that the paternal-specific repression of *H19* is unstable/variable under some specific conditions in the tissues where this copy is not fully methylated.

We also observed by in situ hybridization that the paternal copies of *H19* were expressed in the androgenetic trophoblast. This is consistent with the previous observation that *H19* is expressed following differentiation of androgenetic ES cells (Allen et al., 1994) and may be due to the extensive in vitro manipulation of these embryos or to the absence of a maternal genome. Studies in the human have indicated that *H19* is biallelically expressed not only in complete hydatidiform moles (Mutter et al., 1993) but also in the normal fetal placenta (Zhang and Tycko, 1992). A more recent study has further shown that the activity of the paternally derived *H19* copy in the human placenta declines gradually during fetal development (Jinno et al., 1995). Thus it appears that biallelic expression of *H19* is a normal phenomenon in the human placenta. In any case, it is likely that the undermethylated state of the paternal promoter allows this copy to be activated in androgenetic trophoblast.

It is assumed that the primary imprint originates from the germline of parents and persists through fertilization. Stöger et al. (1993) have argued that differential methylation which they found in an intron of *Igf2r* constitutes such an imprint because this modification was inherited from the egg, thus marking the maternal copy for activity. As for *H19*, the region upstream of the promoter contains several CpG sites which are methylated in sperm but not in oocytes and this difference in methylation is maintained through the preimplantation period at least at some sites (Tremblay et al., 1995). Thus it is proposed that differential methylation of the far 5′ region is the gametic signal for *H19* imprinting (Tremblay et al., 1995). A recent study by targeted deletion of the *H19* locus showed that a 13 kb region containing this differentially methylated 5′-flanking region and the coding gene itself can cause loss of imprinting of at least three genes, namely *H19*, *Igf2* and *Ins2* (Leighton et al., 1995). However, the minimum sequence element that is essential and sufficient for imprinting of the *H19* genes is unknown at present.

In conclusion, it appears that there is a strong tendency for the *H19* gene to be expressed monoallelically in the early embryos even when the promoter methylation has not been fully established. However, there are some exceptional cases that we and others have noticed when the paternal allele is expressed such as in vitro and in the androgenetic material. Although the precise cause of this apparent instability is unknown at present, it seems likely that such instability occurs prior to promoter methylation. The imprint is probably more stable after the promoter is fully methylated.

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