DNA Methylation in Placentas of Interspecies Mouse Hybrids

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ABSTRACT

Interspecific hybridization in the genus Mus results in several hybrid dysgenesis effects, such as male sterility and X-linked placental dysplasia (IHPD). The genetic or molecular basis for the placental phenotypes is at present not clear. However, an extremely complex genetic system that has been hypothesized to be caused by major epigenetic changes on the X chromosome has been shown to be active. We have investigated DNA methylation of several single genes, *Atrx*, *Esx1*, *Mecp2*, *Pem*, *Psx1*, *Vbp1*, *Pou3f4*, and *Cdx2*, and, in addition, of LINE-1 and IAP repeat sequences, in placentas and tissues of fetal day 18 mouse interspecific hybrids. Our results show some tendency toward hypomethylation in the late gestation mouse placenta. However, no differential methylation was observed in hyper- and hypoplastic hybrid placentas when compared with normal-sized littermate placentas or intraspecific *Mus musculus* placentas of the same developmental stage. Thus, our results strongly suggest that generalized changes in methylation patterns do not occur in trophoblast cells of such hybrids.

IN mammals, interspecific hybridization leads to characteristic and consistent developmental defects (Gray 1971). The most frequent and visible defects are sterility of male hybrids and abnormal growth. These defects are obvious. It is likely that other, less obvious, hybrid dysgenesis effects occur but are not normally observed. Notably, in the three mammalian groups, equids, murids, and peromyscids, in which placental development of interspecific hybrids was analyzed to date, placentation defects were observed (Rogers and Dawson 1970; Allen *et al.* 1993; Zechner *et al.* 1996).

Initially, the findings obtained in Mus hybrids (*Mus musculus*, *M. spretus*, and *M. macedonicus*) suggested the presence of an X-linked locus that, in synergy with several autosomal loci, causes interspecific hybrid placental dysplasia (IHPD; Zechner *et al.* 1996). However, a detailed study using congenic and subcongenic mouse lines that contained varying X chromosomal regions derived from *M. spretus* (MSP) did not lend strong support to this model. Instead, these results indicated that multiple loci on the X chromosome could act synergistically to generate IHPD or that the genetic complexity of IHPD could be caused by epigenetic modifications of chromosomal regions on the MSP X chromosome in the presence of *M. musculus* (MMU)-derived chromosomes (Hemberger *et al.* 1999b). Profound epigenetic alterations had indeed been demonstrated for interspecific hybridization in marsupials. These comprised genome-wide demethylation of one parental genome, followed by retroelement amplification and chromosome remodeling (O'Neill *et al.* 1998). Justifiably, it was argued that such epigenetic changes could contribute to hybrid dysgenesis effects and thus promote speciation. Indeed, changes in epigenetic mechanisms of gene control are thought to be important during evolution as they may cause widespread secondary effects (O'Neill *et al.* 1998) and also because of the rapid evolutionary changes they may induce (Sollars *et al.* 2003). However, further studies that investigated genome-wide DNA methylation in adult interspecific hybrids between placental mammals from four different orders provided no evidence for changes on a level seen in marsupials (Roemer *et al.* 1999; Robinson *et al.* 2000). Still, this finding does not exclude the possibility that methylation changes on a smaller scale, that is, specific for gene loci, chromosomes, tissues, and/or developmental stages, are involved in hybrid dysgenesis effects, including IHPD. In this context it is noteworthy that loss-of-imprinting (LOI) has been shown to occur in hybrids between Peromyscus species and that LOI of *Peg3* is causally involved in placental dysplasia in these hybrids (Vrana *et al.* 1998, 2000). It has also been shown for the maternally expressed gene *H19* that biallelic expression in *Peromyscus polionotus* × *P. maniculatus* hybrids is correlated and probably caused by reduced methylation of the paternal *P. maniculatus* allele (Vrana *et al.* 1998).

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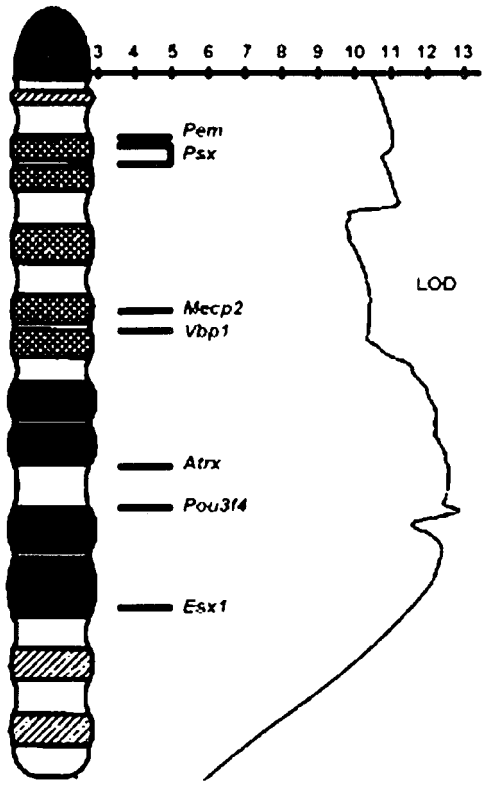

FIGURE 1.—Localization of X chromosomal genes analyzed in this study and linkage of interspecific placental hyperplasia in the MSM backcross to the whole length of the X chromosome (HEMBERGER *et al.* 1999b). All of these genes, including *Esx1*, map to chromosomal regions that exhibit significant linkage to IHPD.

Therefore, it is easily conceivable that aberrant methylation of loci on X chromosomes could be causally involved in IHPD.

To test this hypothesis, we have carried out a methylation study using *Hpa*II and *Msp*I restriction digests of DNA from tissues, including the placenta, of interspecific hybrid fetuses, followed by Southern blotting and hybridization with probes specific for X-linked genes and/or genes expressed in the placenta. These genes were *Atrx*, *Esx1*, *Mecp2*, *Pem*, *Psx1*, *Vbp1*, *Pou3f4*, and *Cdx2*. Of these, *Atrx* (GIBBONS *et al.* 1995, 2000), *Esx1* (LI *et al.* 1997; LI and BEHRINGER 1998), *Mecp2* (ADLER *et al.* 1995), *Pem* (LIN *et al.* 1994), *Psx1* (CHUN *et al.* 1999), and *Vbp1* (HEMBERGER *et al.* 1999a) are expressed in the placenta and located on the X chromosome in regions exhibiting strong linkage to the IHPD phenotype (Figure 1). *Pou3f4* maps to the X chromosome; however, it is expressed exclusively in the ear anlage and the pancreas (DE KOK *et al.* 1995). Although *Cdx2* maps to chromosome 5, it is expressed in the placenta in the tissue layer, the spongiotrophoblast, that is most severely affected in the IHPD phenotype (BECK *et al.* 1995; CHAWENGSAKSOPHAK and BECK 1996). In addition, methylation status of LINE-1 and IAP repeats was assessed. LINE-1 repeats exhibit increased density on the X chromosome and a putative role for them in the X inactivation process has been discussed (BAILEY *et al.* 2000). The long terminal repeats of retrovirally derived IAP elements have been demonstrated to act as promoter sequences driving placenta-specific expression of genes in proximity to the insertion site (CHANG-YEH *et al.* 1991, 1993). X chromosomal localization combined with the function in placental development of the majority of these genes should allow us to detect whether placental dysplasia in IHPD was accompanied by widespread methylation changes on the X chromosome. In addition, targeted mutation of *Esx1* causes a placental phenotype that is very similar to hyperplasia in IHPD (LI and BEHRINGER 1998). Specifically, overgrowth of the spongiotrophoblast and increased differentiation of spongiotrophoblast into glycogen cells are common features of both IHPD and *Esx1* mutant placentas. Finally, with *Mecp2* and *Atrx*, two genes that are involved in epigenetic control of gene expression were included in the analysis. MECP2 silences transcription by binding to symmetrically methylated DNA and then recruiting both the histone deacetylase and histone H3 methylation repressive machineries (JONES *et al.* 1998; NAN *et al.* 1998; FUKS *et al.* 2003). ATRX is a member of the SWI/SNF family of chromatin-remodeling proteins and has been shown to be involved in chromatin-mediated transcriptional control (MCDOWELL *et al.* 1999; GIBBONS *et al.* 2000).

However, methylation analysis of these genes provided no indication for demethylation in dysplastic IHPD placentas. Therefore, no support is provided for the hypothesis that overall loss of methylation of X chromosomal genes contributes to the placental phenotypes observed in interspecies hybrids in the genus Mus.

MATERIALS AND METHODS

Mice: For the generation of interspecific hybrids, laboratory (MMU) F_1 hybrid mice (C57BL/6 × C3H)F_1 (B6C3) and *MSP* strain SMZ mice were used. SMZ mice were originally caught in Azzemour, Morocco, and bred in captivity in Montpellier. Both (B6C3 × SMZ)F_1 (MS) and (MS × B6C3)BC1 (MSM) mice were used in this study. Pregnant females were killed on day 18 (e18) of gestation. Plug day was counted as e1. From the e18 conceptuses, brain, liver, and the placenta were dissected for methylation analysis. Placentas contained the maternal decidua but the yolk sac was largely removed. The maternal decidua contributes only a small proportion of the mature e18 placenta. Remaining fetal tissue was used for genotyping.

DNA isolation and Southern blot hybridization: DNA was isolated according to standard phenol-chloroform extraction procedures. Phase-Lock-Gel tubes (Eppendorf, Madison, WI) were used to minimize loss of DNA. Isolated DNAs were restriction-digested with *Bam*HI, *Eco*RI, or *Hin*dIII and, subsequently, with either *Msp*I or *Hpa*II. For electrophoretic separation and Southern hybridization, again standard techniques were applied. Briefly, digested DNAs were electrophoresed through 1.5% agarose gels and transferred to Hybond-N membranes. Probes (see below) were radioactively labeled with [α-^{32}P]

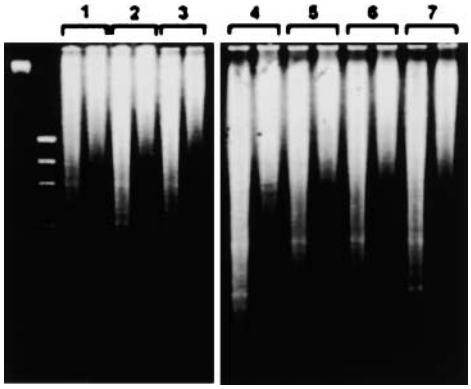

FIGURE 2.—DNA methylation in tissues of adult and fetal interspecific hybrids. Left lane, *Msp*I; right lane, *Hpa*II. Lanes 1–3 show DNAs isolated from liver, brain, and placenta of one e18 MS conceptus. Placental weight was 79.2 mg. Lanes 4–7 show DNAs isolated from brain of adult mice. Lane 4, MMU; lane 5, SM; lane 6, MSP; lane 7, MS. No significant differences can be detected in DNA methylation between adult and fetal samples and between placenta and other fetal tissues.

dCTP and hybridized to the membranes at 65° overnight followed by washes under stringent conditions.

Probes: cDNA probes for *Mecp2* (IMAGp998F081640, 523 bp), *Pem* (IMAGp998L233263, 620 bp), and *Psx1* (IMAGp998D214447, 505 bp) were obtained from the German Resource Centre, Berlin (RZPD). cDNA probes for *Atrx* (1200 bp), *Pou3f4* (1500 bp), and *Cdx2* (3500 bp) were kindly provided by D. Picketts, Ottawa, F. Cremers, Nijmegen, The Netherlands, and V. Subramanian, Bath, United Kingdom, respectively; the cDNA probe for *Esx1* (1000 bp) was provided by R. Behringer, Houston; the probe for *POU3F4* was from F. Cremers, Nijmegen; and the *Vbp1* (374 bp) was made in our lab (HEMBERGER *et al.* 1999a). All probes were murine with the exception of the *POU3F4* probe, which was human derived. Probes were amplified by PCR using M13 primers; for *Atrx* specific primers were used: 5′-3′ CACCATCTTCTTGCCATC TCTGTAG and 3′-5′ GATGTATTCTTGCCCACTGCATG. The probes used for analysis of LINE-1 and IAP (kind gift from B. Mueller, Heidelberg, Germany) methylation were described before (TEUBNER and SCHULZ 1994; FEHRMANN *et al.* 1997).

RESULTS

Global methylation in placenta and fetal tissues: A previous analysis had demonstrated absence of global demethylation in tissues of hybrids from several eutherian orders (ROEMER *et al.* 1999). However, this negative finding did not exclude the possibility that such global demethylation occurs in fetal stages and specifically in the placenta, which exhibits hybrid dysgenesis effects. To investigate this possibility, DNA was isolated from interspecific MS and MSM tissues and analyzed. This analysis showed no difference in methylation levels between fetal and adult tissues, between placenta and fetal brain and liver, and between hyperplastic MSM, hypoplastic MS and normal placentas either from the MSM BC or from B6C3 × B6C3 matings (Figure 2).

Methylation of specific genes and LINE-1 and IAP repeats in placenta and fetal tissues: Altogether 14 placentas from MS and MSM matings with weights ranging between 56.3 and 297 mg were assessed. In all cases, brains and livers from the same conceptuses were used for methylation analysis. Of the single-copy genes that were assessed, *Cdx2* and *Esx1* exhibited no differential restriction pattern after *Hpa*II/*Msp*I digestion and were therefore not analyzed further. The other genes exhibited methylated sites detectable by *Hpa*II/*Msp*I digestion and could be used for further analysis. *Atrx*, *Mecp2*, *Vbp1*, and *Pou3f4* showed comparable *Hpa*II/*Msp*I restriction patterns in all samples analyzed, that is, fetal liver and brain, placenta, and liver from adult MS, SM, SMZ, and B6C3 mice. Thus, these genes exhibited no tissue-specific methylation as shown for *Mecp2* in Figure 3A and, specifically, no hypomethylation in the placenta compared to the other tissues. In contrast to this, the *Hpa*II/*Msp*I restriction patterns of *Pem* (Figure 3B) and *Psx1* (Figure 3C) showed that these two genes were hypomethylated in placenta compared to fetal brain and liver and adult liver (not shown). However, hypomethylation was detected in all placental samples analyzed and no quantitative differences could be detected between normal and hyperplastic placentas in the MSM BC, and MS and B6C3 × B6C3 matings. The observed methylation patterns of X-linked genes that were assessed is summarized in Table 1. A methylation pattern similar to that of *Pem* and *Psx1* was observed for the repetitive LINE-1 and IAP elements: these showed increased sensitivity to *Hpa*II digestion in placenta compared with the other fetal and adult tissues; however, no difference could be observed between normal and dysplastic placentas (Figure 4, A and B).

DISCUSSION

Several analyses of DNA methylation in specific tissues and stages of mouse development have shown that embryonic and extraembryonic lineages are differentially methylated and that extraembryonic DNA exhibits overall hypomethylation (CHAPMAN *et al.* 1984; RAZIN *et al.* 1984; ROSSANT *et al.* 1986; MONK *et al.* 1987, 1991). As in the present study, *Hpa*II/*Msp*I restriction was used in these investigations to assess methylation of repetitive elements and specific genes. Therefore, our findings that both repetitive elements and some specific genes tend to be hypomethylated in e18 mouse placenta are not surprising and fit well into accepted knowledge. The only discrepancy between our and the previous results is that the majority of specific genes assessed by us, that is, *Atrx*, *Mecp2*, *Vbp1*, and *Pou3f4*, exhibited no differential methylation between placenta and fetal liver and brain. This is in contrast to the studies by RAZIN *et al.* (1984) and ROSSANT *et al.* (1986). Razin and colleagues analyzed three specific genes, dihydrofolate reductase, the major histocompatibility gene *H-2K*^{b}, and the β-major globin gene. All of these genes exhibited hypomethylation in extraembryonic tissues. Rossant *et al.* analyzed

TABLE 1

Map localization and methylation status of X-linked genes

Locus	Map position (cM)	Methylation status[a]
Pem	12.7	Decreased
Psx1	A2-A4	Decreased
Mecp2	29.6	No change
Vbp1	30.8	No change
Atrx	43.8	No change
Pou3f4	48.4	No change
Esx1	57.0	Unmethylated

[a] Methylation level of placenta compared to brain and/or liver is shown.

α-fetoprotein, albumin, and the major urinary protein gene family and showed hypomethylation for these genes in extraembryonic tissues. In addition, hypomethylation of actin and metallothionein is mentioned but not shown. As these authors also used cDNA probes, with the exception of the α-fetoprotein probe that was a 4.5-kb genomic probe, technical problems associated with the use of cDNA probes in our study cannot be responsible. A possible explanation is that the extraembryonic tissues analyzed in the previous studies were isolated at early stages of development. Thus, in the study by Rossant *et al.* (1986), e7.5 (corresponds to our e8) extraembryonic tissues, including ectoplacental cone, a derivative of the trophectoderm, were analyzed. This stage is prior to the development of a placenta proper. Although in the study by Razin *et al.* (1984) e9–e10 placenta were assessed, this is well before the e18 stage that we have studied. The dramatic changes in morphology and gene expression (Hemberger *et al.* 2001) observed between early and late placental stages make it possible that these are accompanied by changes in DNA methylation.

In contrast to the previous results cited above, in a more recent study in which the restriction landmark genomic scanning (RLGS) technique was applied, CpG islands in placenta and trophoblast were not found to be hypomethylated in comparison to other tissues such as kidney or brain (Shiota *et al.* 2002). Thus, it is also possible that the overall hypomethylation of trophoblast DNA described in the previous reports was in part due to analysis of repetitive elements and to gene sampling bias.

In any case, the main aim of this study was to determine whether placental dysplasia, a specific hybrid dysgenesis effect in the genus Mus that cosegregates with the X chromosome, is caused by deregulated DNA methylation, specifically in placental tissues and X-linked loci. This possibility had been suggested by findings that in the marsupial group Macropodidae interspecific hybridization results in genome-wide demethylation associated

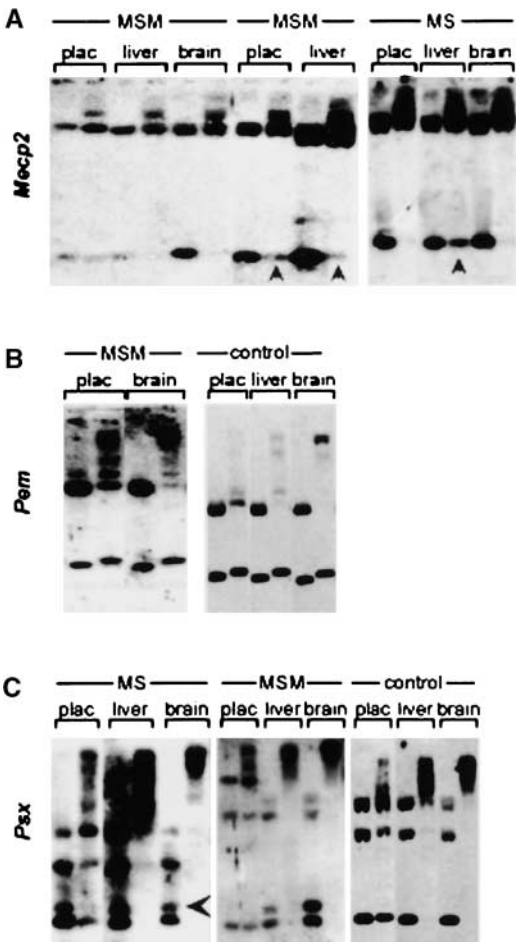

Figure 3.—Methylation analysis of specific genes in tissues from MSM, MS, and intraspecific MMU × MMU matings. Left lane, *Msp*I; right lane, *Hpa*II. (A) Southern blots of *Hin*dIII/*Msp*I- and *Hin*dIII/*Hpa*II-digested DNA hybridized with *Mecp2* probe. Left to right: e18 MSM conceptus with hyperplastic placenta of 297 mg; e18 MSM conceptus with hypoplastic placenta of 56.6 mg; e18 MS conceptus with placenta of 83.0 mg. Methylation patterns are similar for all fetal tissues and the different placental weight classes; the lower band at ~0.6 kb (arrowhead) appeared in some *Hpa*II digests of placenta and liver but not of brain DNA. A is a composite of two blots. (B) Southern blots of *Eco*RI/*Msp*I- and *Eco*RI/*Hpa*II-digested DNA hybridized with *Pem* probe. Left to right: e18 MSM conceptus with placental weight of 95.6 mg; e18 control conceptus from intraspecific MMU × MMU mating. Demethylation of *Pem* sequence in placenta compared to both liver and brain is clearly visible in both the MSM and the control conceptus placenta; however, liver exhibits intermediate methylation in relation to brain and placenta. No difference can be seen between the methylation in the e18 MMU placenta and the e18 MSM placenta. B is a composite of two blots. (C) Southern blots of *Eco*RI/*Msp*I- and *Eco*RI/*Hpa*II-digested DNA hybridized with *Psx1* probe. Left to right: e18 MS conceptus with placental weight of 93.1 mg; e18 MSM conceptus with hyperplastic placenta of 297 mg; e18 control conceptus from intraspecific MMU × MMU. Placenta exhibits clear-cut demethylation compared with liver and brain independent of genotype. The additional band at ~0.6 kb indicated by the arrowhead is due to a restriction variant in MSP. C is a composite of three blots. plac, placenta.

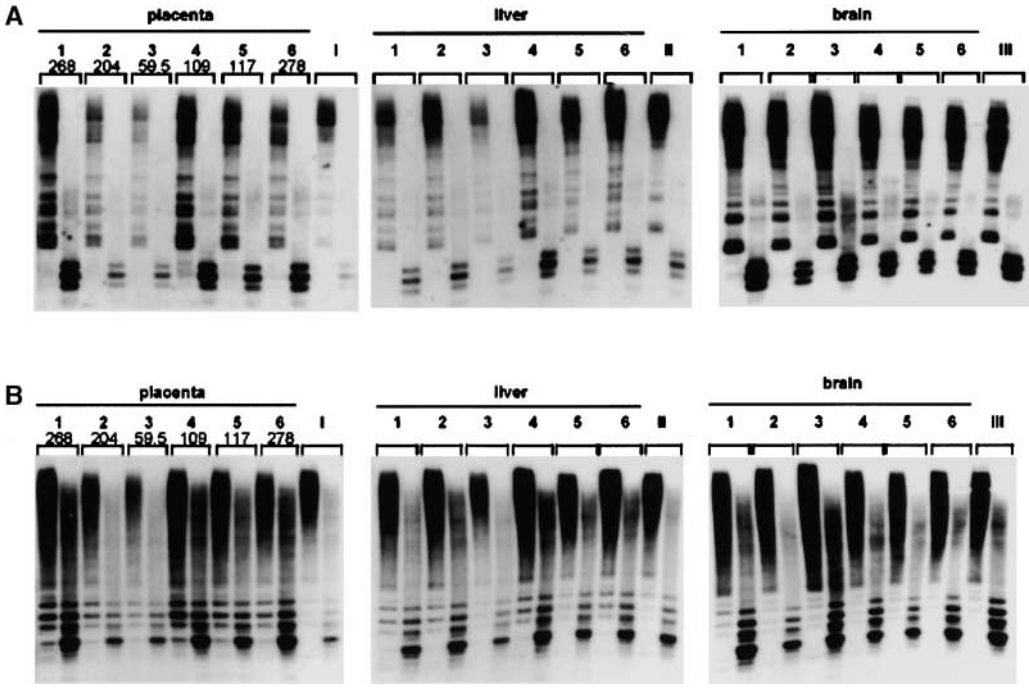

FIGURE 4.—(A) Methylation analysis of IAP repeats in MSM BC1 tissues. *Bam*HI/*Hpa*II (left lane) and *Bam*HI/*Msp*I (right lane) digests are shown for placenta, liver, and brain from six e18 conceptuses. DNA isolated from the different tissues of each conceptus was applied in the same order on each gel. Placental weights of each conceptus were: lane 1, 268 mg; lane 2, 204 mg; lane 3, 59.5 mg; lane 4, 109 mg; lane 5, 117 mg; and lane 6, 278 mg. Conceptuses 1–3 and 4–6, respectively, were littermates. Lane I shows liver DNA of the female MS F_1 hybrid from which conceptuses 1–3 had been isolated. Lanes II and III show F_9 cell control. Placental DNA shows some demethylation compared with liver and brain DNA. However, no difference in degree of demethylation between normal-sized (4 and 5), hyperplastic (1, 2, and 6), and hypoplastic (3) placentas can be seen. (B) Methylation of LINE-1 repeats in MSM BC1 tissues. Exactly the same blots as in A are shown in the same order. Demethylation of LINE-1 repeats in placentas is more pronounced than that of IAP repeats; however, again no differences between the different placental size classes are apparent. Please note that *Hpa*II/*Msp*I lanes are inverted in Figure 4 compared to Figures 2 and 3.

with retrotransposon amplification and chromosome remodeling (O'NEILL *et al.* 1998). However, the present study indicates that systemic DNA demethylation is not a feature of IHPD. Combined with previous results by us (ROEMER *et al.* 1999) and others (ROBINSON *et al.* 2000), the present results thus show conclusively that normal methylation patterns are conserved in interspecific hybridization between placental mammals.

However, our present results do not exclude the possibility that few and specific genes exhibit abnormal methylation. This is in fact suggested by analyses of placentas of cloned mice. Intriguingly, cloned mice exhibit a placentomegaly phenotype that is strikingly similar to the hyperplastic IHPD phenotype (TANAKA *et al.* 2001). Thus, cloned placentas exhibit an enlarged and disorganized spongiotrophoblast with exaggerated glycogen cell differentiation (TANAKA *et al.* 2001), just like IHPD placentas (ZECHNER *et al.* 1996). Placentomegaly was also observed in cloned animals from other species (DE SOUSA *et al.* 2001; HEYMAN *et al.* 2002). Recent DNA methylation studies of cloned placentas using the RLGS technique have shown that only very few loci are aberrantly methylated in placentas of cloned mice (OHGANE *et al.* 2001; SHIOTA and YANAGIMACHI 2002). Interestingly, it seems that these loci vary between clones (OHGANE *et al.* 2001; SHIOTA and YANAGIMACHI 2002). It will be interesting to apply the RLGS approach to IHPD placentas, which can be produced more readily than cloned placentas, to determine whether there are core loci that exhibit abnormal methylation more frequently than others. If such loci are detected, it would be feasible to determine whether these are also detected in cloned placentas and whether abnormal expression levels of these loci are associated with abnormal methylation.

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