Genetic and Developmental Analysis of X-Inactivation in Interspecific Hybrid Mice Suggests a Role for the Y Chromosome in Placental Dysplasia

Myriam Hemberger,*,† Haymo Kurz,‡ Annie Orth,§ Sabine Otto,* Angela Lüttges,* Rosemary Elliott, Andras Nagy,†† Seong-Seng Tan,‡‡ Patrick Tam,§§ Ulrich Zechner* and Reinald H. Fundele***

**Max-Planck-Institut für Molekulare Genetik, 14195 Berlin, Germany, †Health Sciences Centre, University of Calgary, Calgary, Alberta T2N 4N1, Canada, ‡Anatomisches Institut II, 79104 Freiburg, Germany, §Laboratoire Génome et Populations, Université de Montpellier, 34095 Montpellier, France, **Roswell Park Cancer Institute, Buffalo, New York 14263, ††Samuel Lunenfeld Research Institute, Toronto M5G 1X5, Canada, ‡‡Howard Florey Institute, University of Melbourne, Melbourne, Victoria 3010, Australia and §§Children's Medical Research Institute, University of Sydney, Wentworthville, New South Wales 2145, Australia*

Manuscript received May 15, 2000
Accepted for publication October 2, 2000

ABSTRACT

It has been shown previously that abnormal placental growth, *i.e.*, hyper- and hypoplasia, occurs in crosses and backcrosses between different mouse (Mus) species. A locus that contributes to this abnormal development has been mapped to the X chromosome. Unexpectedly, an influence of fetal sex on placental development has been observed, in that placentas attached to male fetuses tended to exhibit a more pronounced phenotype than placentas attached to females. Here, we have analyzed this sex dependence in more detail. Our results show that differences between male and female placental weights are characteristic of interspecific matings and are not observed in intraspecific *Mus musculus* matings. The effect is retained in congenic lines that contain differing lengths of *M. spretus*-derived X chromosome. Expression of the X-linked gene *Pgk1* from the maternal allele only and lack of overall activity of two paternally inherited X-linked transgenes indicate that reactivation or lack of inactivation of the paternal X chromosome in trophoblasts of interspecific hybrids is not a frequent occurrence. Thus, the difference between male and female placentas seems not to be caused by faulty preferential X-inactivation. Therefore, these data suggest that the sex difference of placental weights in interspecific hybrids is caused by interactions with the Y chromosome.

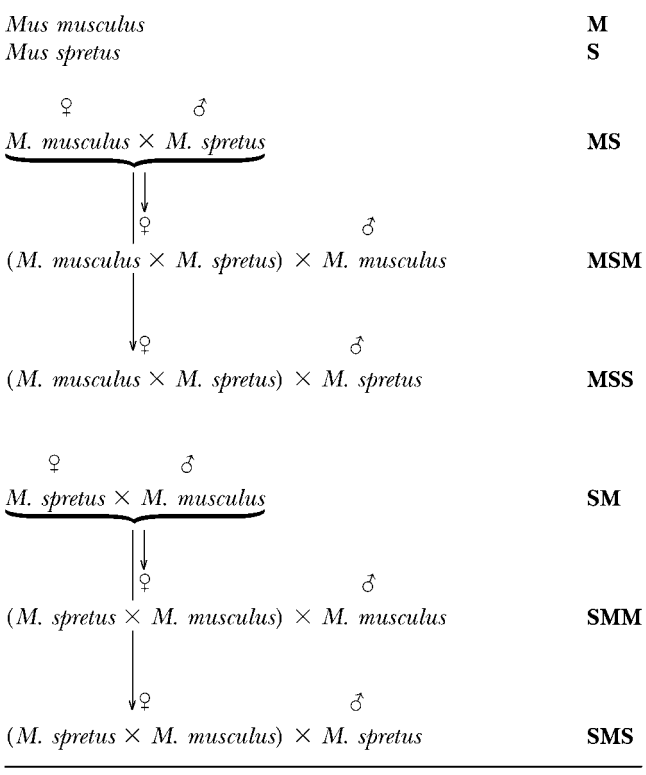
ABNORMAL placental development in interspecific crosses has been described for the genera Peromyscus (Coyne 1992), Mus (Zechner *et al.* 1996), and Equus (Allen 1975; Allen *et al.* 1993). The effects observed in the genera Peromyscus and Mus, which have evolved independently for ~16.6–20.1 × 10^6 years (Catzeflis *et al.* 1993), are very similar in that they are both associated with abnormal placental growth (Rogers and Dawson 1970; Zechner *et al.* 1996). In the mouse, opposite effects on placental growth, *i.e.*, placental hyperplasia and hypoplasia [interspecific hybrid placental dysplasia (IHPD)], are observed in reciprocal crosses and backcrosses. Hyperplastic placentas are seen in matings of *M. spretus* (**S**) female × *M. musculus* (**M**) male (**SM**) and in the (**SM**) × **M** and (**MS**) × **M** backcrosses (BCs; for crosses, see Table 1). Hypoplastic placentas appear in the **MS** cross and in the **MSS** and **SMS** BCs. Identical results are obtained when the **S** mice are substituted by mice of the two other European species *M. macedonicus* and *M. spicilegus*. In all cases the placental tissue that is mainly affected is the spongiotrophoblast. This cell layer is enlarged in the **SM**, **SMM**, and **MSM** placentas. In contrast, it is reduced in the reciprocal cross and BCs and in some cases it is missing completely. This phenotype results in fetal growth retardation or death (Zechner *et al.* 1996), which in turn may be the explanation for the transmission ratio distortion observed in several **MSS** BCs (Biddle 1987; Eicher *et al.* 1992; Rowe *et al.* 1994; Montagutelli *et al.* 1996). It has been suggested that these phenotypes are caused by the aberrant interactions between loci derived from the genomes of the different species, whereas in the usual, intraspecific context these loci interact normally (Zechner *et al.* 1996, 1997). It has been shown that a region on the X chromosome (*Ihpd*) is involved in the production of aberrant placental phenotypes (Zechner *et al.* 1996).

Intriguingly, an influence of fetal (and placental) sex on placental growth became obvious. In the **MSM** BC, XY placentas with a **S**-derived X chromosome are larger than XX placentas that have inherited a **S**-derived X chromosome from their mother. In contrast to this, in the **MS** cross and in the **MSS** BC, XY placentas with a **M**-derived X are smaller than heterozygous female placentas with a maternal **M**-derived X chromosome

Corresponding author: Myriam Hemberger, Genes and Development Research Group, University of Calgary, Health Sciences Ctr. Rm. 2153, 3330 Hospital Dr., N.W., Calgary, Alberta T2N 4N1, Canada. E-mail: mhemberg@ucalgary.ca

TABLE 1

Mouse crosses



(Zechner *et al.* 1996). This is against expectations, as in the mouse the X chromosome is imprinted in the extraembryonic tissues such that the paternally derived X chromosome (X^p) is preferentially inactivated (Takagi and Sasaki 1975; West *et al.* 1977). Furthermore, there is evidence that X^p remains inactivated in the derivatives of the trophoblast (Sobis *et al.* 1991), which include the spongiotrophoblast (Rossant and Croy 1985). Thus, where X chromosome activity in the trophoblast is concerned, **MSM** $\mathbf{X^SY}$ males and $\mathbf{X^SX^M}$ females should behave identically, since the maternally inherited $\mathbf{X^S}$ should be exclusively active in trophoblast derived cells. Three possible explanations have been suggested to account for the sex-specific effect (Zechner *et al.* 1996). First, it has been argued that in trophoblasts of interspecific hybrids, X chromosome imprinting and preferential inactivation of X^p could be disturbed, leading to lack of inactivation or reactivation of X^p and consequently to quantitative differences in expression levels of all X-linked genes between males and females. The second explanation is that only the specific gene(s) causing the placental phenotypes may remain active on X^p or are reactivated. The third hypothesis is that interactions between the region of the X chromosome bearing the *Ihpd* locus and the Y chromosome cause, or contribute to, the differences between male and female placental growth phenotypes. The first possibility seemed most likely and it also had the advantage that it could easily be tested through the use of mice carrying variant alleles of X-linked genes (Nielsen and Chapman 1977) or X-linked transgenes (Tan *et al.* 1993; Hadjantonakis *et al.* 1998).

In an attempt to investigate the causes for the sex-specific effect in more detail, we have applied the approaches outlined above, making use of transgenic and variant mouse lines (Nielsen and Chapman 1977; Tan *et al.* 1993; Hadjantonakis *et al.* 1998). Mouse lines carrying X-linked transgenes can be used in this context by investigating transgene activity after paternal inheritence. Here, we used the enhanced green fluorescent protein (EGFP) and β-galactosidase (LacZ) transgenes located on the proximal and central X chromosome, respectively. These chromosomal regions have been implicated in causing IHPD previously (Zechner *et al.* 1996; Hemberger *et al.* 1999). In addition, we have performed a genetic analysis of the sex-specific effect in several congenic mouse lines and strains carrying different lengths of **S**-derived X-chromosomal regions in a **M** background (Hemberger *et al.* 1999).

MATERIALS AND METHODS

Mice: Mouse stocks used in the present analysis were *M. spretus* strain SFM, and C57BL/6 (B6), B6 × C3H ($B6C3F_1$), and CD1. **M** females were mated with SFM males to produce F_1 interspecific hybrids. In addition, mice of the transgenic lines H253 and D4XEGFP as well as mouse strain C3H/Aa were used. H253 mice carry 14 tandem copies of a ubiquitously expressed LacZ transgene that maps to the X chromosome and is subject to X-inactivation in hemizygous females (Tan *et al.* 1993). The D4XEGFP strain contains an ubiquituously expressed green fluorescent protein marker gene on the X chromosome (Hadjantonakis *et al.* 1998) that maps to band A3.1-3.3, ~12.6 cM proximal to the LacZ transgene in band A6 (P. Tam and S.-S. Tan, unpublished data). C3H/Aa mice carry a rare electrophoretic variant of the X-linked enzyme phosphoglycerate kinase (PGK1; Nielsen and Chapman 1977). The congenic strain AT24 was constructed by selecting for the **S** allele for *Hprt* onto a background of B6 using 12 BC generations followed by intercrossing (Elliott *et al.* 2001). It contains ~17 map units of chromosome X derived from **S.** Congenic lines MH1.1, MH1.3, MH1.4, and MH2.1 were generated by repeated backcrossings and have been described previously (Hemberger *et al.* 1999).

Isolation of tissues, histology, and morphometry: After determination of fetal and placental wet weights, placentas were fixed in Serra's fixative (60% ethanol, 30% formalin, 10% acetic acid) for 24 hr at 0°. After dehydration, placentas were embedded in Paraplast X-tra (Sigma, Taufkirchen, Germany) and sectioned at 7 μm. Sections were stained with hematoxylin-eosin (HE). For morphometric analysis, the volume fraction, V_V (percentage of total placental volume), of the following compartments was determined by point counting of three HE-stained sections per placenta: chorionic plate, labyrinthine and spongious trophoblast, and decidua. Within the spongiotrophoblast, V_V (percentage of spongiotrophoblast) of glycogen cells and nonglycogen cells was determined (Kurz *et al.* 1999).

Analysis of X-inactivation: MS F_1 females were mated with H253 males and killed at various gestational stages [counting the day of the vaginal plug as day 1 (e1)]. Fetuses were frozen

for genotype analysis, and placentas were weighed and then perfused first with ice-cold PBS and then with 3.7% formaldehyde in PBS. Fixation on ice lasted for 30 min; then the placentas were again perfused with PBS and transferred into 30% sucrose in PBS overnight. Placentas were embedded in tissue freezing medium (Leica, Nussloch, Germany) and sectioned in a cryostat microtome at 10–50 μm. Sections were stained for β-galactosidase activity overnight at 37° as described (Sanes *et al.* 1986). In the H253 line, the LacZ activity is restricted to the nucleus, due to a nuclear localization signal in the transgene (Tan *et al.* 1993). Nuclear counterstaining was performed with the Feulgen reaction. Adjacent sections were incubated with peroxidase-conjugated lectin BS-IB_4 from *Bandeiraea simplicifolia* (Sigma). Staining was performed with diaminobenzidine (DAB) and H_2O_2. In the late gestation mouse placenta, this lectin specifically marks labyrinthine trophoblast and decidual cells (Hemberger *et al.* 1999; Kurz *et al.* 1999). As a control for β-galactosidase staining and LacZ expression, H253 females were mated to H253 males and placentas were processed as described above. Sections of these placentas showed overall blue staining of trophoblast nuclei.

For detection of EGFP fluorescence in the **MS** × D4XEGFP matings, placentas were fixed in 4% paraformaldehyde (PFA) in PBS. Sections of 50- and 100-μm thicknesses were cut on a series S1000 vibratome and mounted under coverslips with Mowiol (Hoechst). Nuclear staining was performed using propidium iodide or bis-benzimide (Sigma). For epifluorescence microscopy, standard FITC (EGFP), TRITC (propidium iodide), and 4′,6-diamidino-2-phenylindole (bis-benzimide) filter sets were used on a Zeiss Axiophot with digital imaging systems. Alternatively, **MS** F_1 females were mated with C3H/Aa males. Pregnant females were killed on e18. Fetuses were frozen for genotype analysis, and placentas were weighed and then frozen without prior fixation in tissue freezing medium at −70°. Placentas were sectioned in a cryostat microtome at 200 μm. Tissue samples from spongiotrophoblast and labyrinth were punched out using 0.55-mm diameter needles. Samples were homogenized and used for electrophoresis on Cellogel membranes for separation of PGK allozymes. Electrophoresis and enzyme-specific staining were performed exactly as described (Bücher *et al.* 1980). After sample isolation, 200-μm sections were fixed in 7.4% formaldehyde, washed in PBS containing 1% Triton X-100 and 0.1% Nonidet NP40, and then incubated free-floating for 60 min at room temperature with BS-IB_4 followed by peroxidase-specific staining.

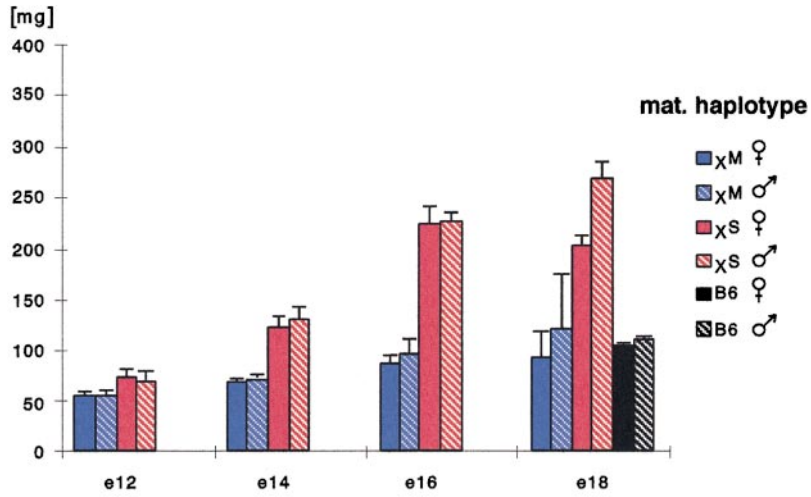
Genotyping: To determine sex chromosome and *Ihpd* constitution of conceptuses, genomic DNA was prepared from fetuses using the Kristal mammalian genomic DNA extraction kit (Cambridge Molecular Technologies, Watertown, MA). For genotyping of BC1 fetuses, the following microsatellite markers from the Massachusetts Institute of Technology were used: *DXMit86*, *DXMit143*, *DXMit8*, and *DXMit65* (Research Genetics, Huntsville, AL). Only fetuses and their placentas with a nonrecombinant X chromosome between flanking markers *DXMit86* (17.4 cM) and *DXMit65* (48.5 cM) were used in this analysis. For congenic strains and lines, additional microsatellite markers were used as described previously (Hemberger *et al.* 1999).

RESULTS

Differential growth of XX and XY placentas occurs late in gestation: In our original study (Zechner *et al.* 1996), the growth difference between XX and XY placentas was analyzed on e18 only. To determine whether this difference manifests itself at earlier stages, placentas were dissected on e12, e14, e16, and e18 from **MS** females that had been backcrossed with **M** males. The associated fetuses were sexed and analyzed for the presence of **S** alleles on the X chromosome. In addition, e18 placentas were isolated from B6 × B6 crosses. This analysis showed that a significant difference between male and female placentas in the **MSM** BC1 was evident only on e18 (Figure 1). Whereas between e12 and e16, XX and XY placentas showed a proportional weight difference of a factor between 1.01 and 1.06, XY placentas were 1.32 times larger than XX placentas at e18. Intriguingly, this sex difference was observed not only in placentas that carried **S** alleles on the X chromosome but also in placentas that were **M** on the X (Figure 1). A similar proportional difference was observed with XY placentas exhibiting a 1.31 times increase in placental weight over XX placentas. In the B6 × B6 cross, no significant difference between XX and XY placentas was observed (Figure 1). Morphometric analysis of three XY and three XX placentas carrying **S** alleles at *DXMit143* (26.0 cM), *DXMit8* (32 cM), and *DXMit65* (48.5 cM) showed no differential growth of placental compartments even though the sex difference in placental weights was clearly evident in these samples (data not shown).

Differential growth of XX and XY placentas is observed in congenic lines that carry S-derived regions on the X chromosome: Several congenic lines have been established by repeated backcrossing of **MS** females to **M** males. These congenic lines carry different **S**-derived regions on the maternal X chromosome and still exhibit enlarged placentas (Hemberger *et al.* 1999). Weights of XX and XY placentas on e18 were analyzed in four of these congenic lines: MH1.4, MH2.1, MH1.3, and MH1.1. In two of these lines, MH1.4 and MH1.1, the weight differences between XX and XY placentas reached statistical significance (Figure 2). Since comparable numbers of fetuses were analyzed in these lines, the statistical values are unlikely to be significantly influenced by differences in sample sizes. XY placentas of line MH1.4, which carries an **S**-derived region between 3.8 and 26.0 cM, were 1.26 times larger than XX placentas of the same line. This value is comparable to that observed in BC1 placentas at e18. The analysis was also carried out with congenic strain AT24 that is homozygous for a **S** interval between 14.2 and 30.8 cM. This strain produces clearly enlarged placentas; however, the sex difference was statistically not significant.

The paternally derived X chromosome (X^p) is preferentially inactivated in trophectoderm-derived tissues of MS hybrids: An explanation for sex differences of placental weights could be a defect in normal X-inactivation in extraembryonic tissues of interspecific hybrids. To test this hypothesis, first, the H253 mouse line carrying an X-linked LacZ transgene was used. This transgene has been shown to be subject to normal inactivation when inherited paternally and to be active upon



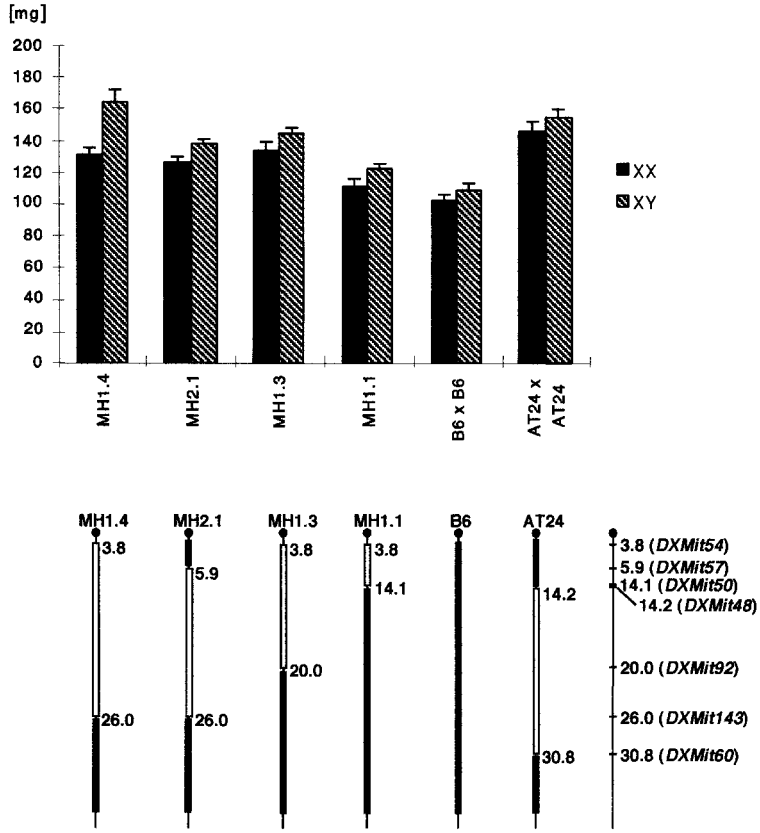
	e12 ♀	e12 ♂	e14 ♀	e14 ♂	e16 ♀	e16 ♂	e18 ♀	e18 ♂
X^M [N]	9	5	13	11	5	5	33	29
t-test	0.997		0.437		0.592		0.011	
X^S [N]	9	10	12	13	5	13	33	30
t-test	0.738		0.713		0.939		0.001	
B6 [N]							18	11
t-test							0.141	

FIGURE 1.—Analysis of placental weights in interspecific backcross 1 mice at various developmental stages. Placental weights of backcross 1 **MSM** fetuses at different stages of development are shown. Placental weights of male and female fetuses that inherited *Mus musculus* (**M**)-derived alleles as tested between markers *DXMit86* (17.4 cM) and *DXMit65* (48.5 cM) on the maternal X chromosome are shown in blue. Red bars indicate placental weights of fetuses with a *M. spretus* (**S**)-derived maternal X chromosome. Error bars indicate the standard error for all groups. Significant weight differences are evident for both groups at e18, but are not present in **M** (B6) control placentas. Numbers of fetuses investigated (N) and *t*-test values of all stages are given.

maternal inheritence (TAN *et al.* 1993 and data not shown). To determine whether preferential inactivation of X^p in trophoblast-derived tissues proceeds normally in mouse interspecific hybrids, F_1 females were mated with H253 males. Nine females were sacrificed at different gestational stages: three females at e12, four at e14, and two at e18. A total of 23, 23, and 11 fetuses were recovered from these females at e12, e14, and e18, respectively, and of these, 4, 6, and 5 were females that were heterozygous for **M** and **S** alleles on the X chromosome. LacZ staining provided no evidence for consistent abnormal X-inactivation in trophoblast-derived tissues (Figure 3, A–E). With two exceptions, only a small proportion of spongiotrophoblast cells had not inactivated X^p, as shown by blue staining of the nuclei. The proportion of blue nuclei within the spongiotrophoblast did not change significantly between e12 and e18 (~0.5%), thus indicating that neither delayed inactivation of X^p early in placental development nor reactivation of X^p in late placental development occurs. Similar proportions of blue nuclei were observed in female littermates that were homozygous **M** or heterozygous **SM** on the X chromosome. However, stained nuclei were consistently observed in the chorionic plate and the labyrinth (Figure 3B). From their spatial orientation it can be concluded that all or most of these LacZ-positive nuclei belong to the endothelial cells of fetal blood vessels that are mesoderm derived and thus subject to random X-inactivation.

Large numbers of cells with active X^p were observed in the placentas of an e12 female that was homozygous **M** on the X (Figure 3F) and in an e14 female that was heterozygous **MS** at *DXMit8* (Figure 3G). In the e12 placenta, the majority of nuclei in all placental compartments apart from decidua exhibited staining. This placenta did not show the expected mosaic staining pattern in extraembryonic mesoderm that is normally caused by random X-inactivation. Moreover, this placenta exhibited transgene activity from the X^p in the trophoblast. It is probable that in this placenta the transgene was aberrantly activated in both extraembryonic mesoderm and trophoblast, although it normally follows the endogenous inactivation pattern (TAN *et al.* 1993). In the e14 placenta, cells with active X^p were restricted to the spongiotrophoblast. Labyrinthine trophoblast cells stained by LacZ were not detected. This placenta gave no indication for preferential inactivation of the nontransgenic X^m in mesoderm-derived cells.

Ten F_1 females that had been mated with D4XEGFP males were killed between e10 and e19 of gestation,



	MH1.4		MH2.1		MH1.3		MH1.1		B6		AT24	
	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂	♀	♂
[N]	22	18	14	31	31	33	18	16	18	11	9	25
t-test	0.0003		0.056		0.073		0.032		0.141		0.137	

FIGURE 2.—Placental weights in congenic lines and strains. Placental weights of congenic lines (MH1.4, MH2.1, MH1.3, and MH1.1) and congenic strain (AT24) are shown. Haplotypes of the maternally inherited X chromosome are shown with the **S**-derived intervals indicated by open boxes. Sex difference of placental weight is statistically significant in lines MH1.4 (*t*-test, 0.0003) and MH1.1 (*t*-test, 0.032). DXMit marker positions delimiting the congenic intervals are given in centimorgans.

resulting in 34 XX placentas that were analyzed. As seen in the $F_1 \times$ H253 matings, fluorescent cells containing an active X^p were observed mainly in the chorionic plate and in the labyrinth (data not shown). Again, the positive cells in the labyrinth are likely to be endothelial cells lining the fetal blood vessels. In the panel of placentas assessed by us, very few positive cells were seen in the spongiotrophoblast. In this, no difference was observed between placentas that carried **M** or **S** alleles on the proximal to central region of X^m (data not shown). Thus, the overall distribution of fluorescent cells was very similar to that observed for the LacZ-positive cells (Figure 3). This indicated again that reactivation of X^p is not a frequent occurrence in *Mus* interspecific hybrids.

Seven e18 placentas weighing between 125 and 293 mg derived from five **MS** $F_1 \times$ C3H/Aa litters were investigated for reactivation of X^p by PGK isozyme analysis. These conceptuses were all heterozygous for markers on the X chromosome. A total of 46 spongiotrophoblast and 15 labyrinth-derived samples were assessed for the presence of paternally inherited PGK-1A. Only a sample isolated from the chorionic plate of a 252-mg placenta exhibited a strong PGK-1A band, probably derived from extraembryonic mesoderm-derived cells. No other samples from the chorionic plate were dissected. However, the presence of both PGK-1A and PGK-1B was seen in samples isolated from livers of the fetuses associated with these placentas (Figure 3, H and I).

DISCUSSION

Three tentative explanations have been offered to explain the weight differences between XX and XY placentas in interspecific hybrids (ZECHNER *et al.* 1996): (1) lack of preferential inactivation or reactivation of the paternally inherited X chromosome leading to quantitative differences in expression levels of several or all X-linked genes; (2) lack of inactivation or reactivation of one or a few specific genes on X^p, which also results in quantitative differences; and (3) aberrant interactions between X and Y chromosomes that are derived from different species. We used several experimental approaches to test two of these hypotheses.

The parental allele-specific expression analysis of X-linked *Pgk1* and the X-linked LacZ and EGFP trans-

genes that map within the IHPD-causing interval argue against an overall inactivation defect or reactivation of X^P. Thus, these results clearly exclude the first hypothesis to explain the sex difference in placental weights of interspecific hybrids.

Although the second hypothesis cannot be ruled out, our data suggest a genetic mechanism other than an inactivation defect of one or a few X-linked gene(s). It has been shown previously that the severity of IHPD is dependent on length of the **S**-derived X-chromosomal interval (see also Figure 2) whereas the precise location of the **S**-derived interval on the X chromosome seems to be less important (Hemberger *et al.* 1999). Thus, placental hyperplasia was observed in different congenic lines and strains with nonoverlapping **S**-derived regions, and no single genetic region cosegregating with placental dysplasia could be identified. These findings can be explained by additive or epistatic interactions of multiple genes on the X that are involved in the development of IHPD. Thus, these genetic dissection experiments argue against the presence of only one or a few genes causing IHPD. If the second of our initial hypotheses to explain sex-specific weight differences holds true, all of these genes involved in IHPD not only have to cause phenotypically identical alterations in placental morphology, but, in addition, they have to be subject to leaky X-inactivation. Presumably, lack of inactivation of a large number of genes on X^P would have been detected by our approaches to test the X^P inactivation state.

Thus, the behavior of the imprinted X^P seems to be in contrast to that of autosomal imprinted genes where biallelic expression in interspecific hybrids has been described. In hybrids between *Peromyscus maniculatus* × *P. polionotus*, three out of eight imprinted genes exhibited biallelic expression in either fetus or placenta or both. In the reciprocal cross, six out of eight imprinted genes were expressed biallelically (Vrana *et al.* 1998). In one of the parental species, *P. polionotus*, where imprinting could be tested, monoallelic expression was seen just as in the house mouse. The findings by Vrana *et al.* (1998) of altered imprinting status of autosomal genes may point to a major difference between interspecific hybridization in the genera Mus and Peromyscus. To date no evidence for loss of imprinting of autosomal loci has been demonstrated in hybrid fetuses between **M** and **S.** Most recently, imprinted X-inactivation has also been investigated in Peromyscus hybrids (Vrana *et al.* 2000). Consistent with our findings in Mus, no indication for relaxation of imprinted X-inactivation was evident in extraembryonic tissues in Peromyscus.

Our results show that there is a tendency for XY pla-

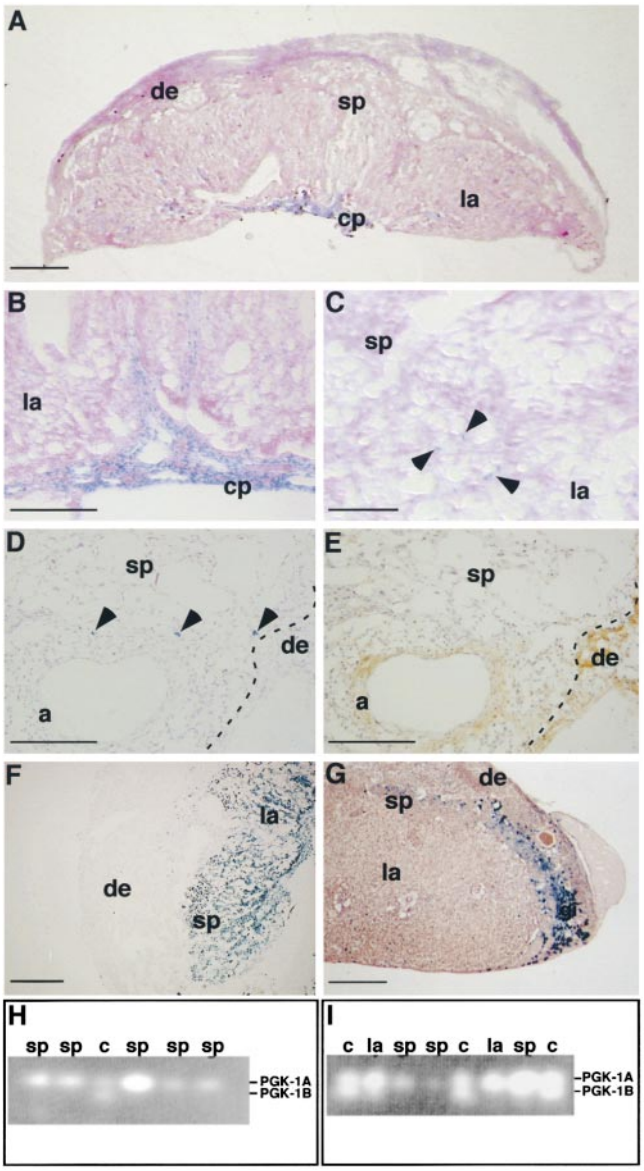


Figure 3.—Analysis of X^P inactivation in $\mathbf{X^S X^M}$ placentas of **MSM** BC1 fetuses by testing activity (blue staining) of a paternally inherited X-linked LacZ transgene. (A) Section of an e14 placenta (bar, 600 μm) with blue-stained nuclei in endothelial cells of fetal blood vessels in the chorionic plate and labyrinth (magnification shown in B; bar, 100 μm). (C) Magnification of the spongiotrophoblast and labyrinthine region of the same placenta shown in A. Again, staining is only observed in endothelia of fetal blood vessels in the labyrinth, as marked by the arrowheads (bar, 50 μm). (D) Blue staining of few nuclei (arrowheads) in the spongiotrophoblast of an e18 placenta (bar, 100 μm). (E) Lectin BS-IB_4 staining on an adjacent section to D marks decidual and labyrinthine cells (bar, 100 μm). Comparison of D and E clearly shows localization of LacZ-expressing cells (in D) in the spongiotrophoblast. (F) Biased X-inactivation in a placenta of an e12 fetus. The majority of nuclei in spongiotrophoblast and labyrinth are stained (bar, 200 μm). (G) Strong positive staining in a single e14 placenta within the spongiotrophoblast region (bar, 600 μm). (H and I) Electrophoretic separation of PGK-1 alleles of punches from spongiotrophoblast (sp), labyrinth (la), and fetal liver as control (c). Paternally inherited PGK-1B allozyme is only detected in liver control punches where random X-inactivation occurs. a, maternal artery; cp, chorionic plate; de, decidua; la, labyrinth; sp, spongiotrophoblast.

centas to be larger than XX placentas in all fetuses that were analyzed. This might reflect overexpression of X-linked genes that are not dosage-compensated at early stages of development in females (Latham 1996). However, extraembryonic tissues of female **MSM** conceptuses are not developmentally retarded between e12 and e16. Thus, early gene-dosage differences between males and females are unlikely to cause the increased growth of XY placentas that occurs only late in gestation. The increased weight of XY placentas might also be explained by a more pronounced antigenicity of male compared to female fetuses. Placental size has been shown to be affected by antigenic dissimilarity between mother and fetus (James 1965). In intraspecific matings, however, there is only a minor difference between XX and XY placental weights. Therefore, the presence of a Y chromosome alone cannot account for the pronounced differences observed at e18 in interspecific matings.

As discussed above, we cannot fully exclude the existence of multiple genes on the X that escape normal X^p inactivation in the interspecific hybrid condition. However, the abundance of these genes that is suggested by the genetic dissection analysis seems to make this situation unlikely. Consequently, we favor the third of our initial hypotheses, that the Y chromosome plays a critical role in causing the sex-specific effect. Interspecific hybrids between several Drosophila species have provided evidence for the presence of abnormal interactions between X and Y chromosomes derived from different species that are responsible for male sterility in the F_1 hybrids (Johnson *et al.* 1992, 1993; Heikkinen and Lumme 1998). Thus, in hybrids between specific Drosophila species the Y chromosome and Y-X interactions have been shown to be pivotal in contributing to Haldane's rule of interspecific sterility (Haldane 1922; Heikkinen and Lumme 1998). While, to the best of our knowledge, such effects have not been previously described in mice, our data suggest that the weight difference between placentas associated with male and female fetuses is caused by similar mechanisms.

The authors are grateful to Dr. F. Bonhomme for critically reading the manuscript. We also thank U. Schroeder for taking care of the difficult interspecific hybrids. This work was supported by grants Fu188/2-3 and Fu188/7-1 of the Deutsche Forschungsgemeinschaft to R.F., the French Centre National de la Recherche Scientifique to A.O., by National Institutes of Health grant GM33160 to R.E., and by the Max-Planck-Gesellschaft.

LITERATURE CITED

Allen, W. R., 1975 The influence of fetal genotype upon endometrial cup development and PMSG and progestagen production in equids. J. Reprod. Fertil. **23** (Suppl.): 405–413.

Allen, W. R., J. A. Skidmore, F. Stewart and D. F. Antczak, 1993 Effects of fetal genotype and uterine environment on placental development in equids. J. Reprod. Fertil. **98:** 55–60.

Biddle, F. G., 1987 Segregation distortion of X-linked marker genes in interspecific crosses between *Mus musculus* and *M. spretus*. Genome **29:** 389–392.

Bücher, T., W. Bender, R. Fundele, H. Hofner and I. Linke, 1980 Quantitative evaluation of electrophoretic Allo- and Isozym patterns. FEBS Lett. **115:** 319–324.

Catzeflis, F. M., A. W. Dickerman, J. Michaux and J. A. W. Kirsch, 1993 DNA hybridization and rodent phylogeny, pp. 159–174 in *Mammal Phylogeny*, edited by F. S. Szalay, M. J. Novacek and M. C. McKenna. Springer Verlag, New York.

Coyne, J. A., 1992 Genetics and speciation. Nature **355:** 511–515.

Eicher, E. M., B. K. Lee, L. L. Washburn, D. W. Hale and T. R. King, 1992 Telomere-related markers for the pseudoautosomal region of the mouse genome. Proc. Natl. Acad. Sci. USA **89:** 2160–2164.

Elliott, R. W., D. R. Miller, R. S. Pearsall, C. Hohman, Y. Zhang *et al.*, 2001 Genetic analysis of testis weight and fertility in an interspecies hybrid congenic strain for Chr. X. Mamm. Genome (in press).

Hadjantonakis, A. A., M. Gertsenstein, M. Ikawa, M. Okabe and A. Nagy, 1998 Non-invasive sexing of preimplantation stage mammalian embryos. Nat. Genet. **19:** 220–222.

Haldane, J. B. S., 1922 Sex ratio and unisexual sterility in hybrid animals. J. Genet. **12:** 101–109.

Heikkinen, E., and J. Lumme, 1998 The Y chromosomes of *Drosophila lummei* and *D. novamexicana* differ in fertility factors. Heredity **81:** 505–513.

Hemberger, M. C., R. S. Pearsall, U. Zechner, A. Orth, S. Otto *et al.*, 1999 Genetic dissection of X-linked interspecific hybrid placental dysplasia in congenic mouse strains. Genetics **153:** 383–390.

James, D. A., 1965 Effects of antigenic dissimilarity between mother and foetus on placental size in mice. Nature **205:** 613–614.

Johnson, N. A., D. E. Perez, E. L. Cabot, H. Hollocher and C. I. Wu, 1992 A test of reciprocal X-Y interactions as a cause of hybrid sterility in Drosophila. Nature **358:** 751–753.

Johnson, N. A., H. Hollocher, E. Noonburg and C. I. Wu, 1993 The effects of interspecific Y chromosome replacements on hybrid sterility within the *Drosophila simulans* clade. Genetics **135:** 443–453.

Kurz, H., U. Zechner, A. Orth and R. Fundele, 1999 Lack of correlation between placenta and offspring size in mouse interspecific crosses. Anat. Embryol. **200:** 335–343.

Latham, K. E., 1996 X chromosome imprinting and inactivation in the early mammalian embryo. Trends Genet. **12:** 134–138.

Montagutelli, X., R. Turner and J. H. Nadeau, 1996 Epistatic control of non-mendelian inheritance in mouse interspecific crosses. Genetics **143:** 1739–1752.

Nielsen, J. T., and V. M. Chapman, 1977 Electrophoretic variation for X-chromosome-linked phosphoglycerate kinase (PGK-1) in the mouse. Genetics **87:** 319–325.

Rogers, J. F., and W. D. Dawson, 1970 Foetal and placental size in a *Peromyscus* species cross. J. Reprod. Fertil. **21:** 255–262.

Rossant, J., and B. A. Croy, 1985 Genetic identification of tissue of origin of cellular populations within the mouse placenta. J. Embryol. Exp. Morphol. **86:** 177–189.

Rowe, L. B., J. H. Nadeau, R. Turner, W. N. Frankel, V. A. Letts *et al.*, 1994 Maps from two interspecific backcross DNA panels available as a community genetic mapping resource. Mamm. Genome **5:** 253–274.

Sanes, J. R., J. L. Rubenstein and J. F. Nicolas, 1986 Use of a recombinant retrovirus to study post-implantation cell lineage in mouse embryos. EMBO J. **5:** 3133–4312.

Sobis, H., A. Verstuyf and M. Vandeputte, 1991 Histochemical differences in expression of X-linked glucose-6-phosphate dehydrogenase between ectoderm- and endoderm-derived embryonic and extra-embryonic tissues. J. Histochem. Cytochem. **39:** 569–574.

Takagi, N., and M. Sasaki, 1975 Preferential inactivation of the paternally derived X chromosome in the extraembryonic membranes of the mouse. Nature **256:** 640–642.

Tan, S.-S., E. A. Williams and P. P. L. Tam, 1993 X chromosome inactivation occurs at different times in different tissues of the post-implantation mouse embryo. Nat. Genet. **3:** 170–174.

Vrana, P. B., X.-J. Guan, R. S. Ingram and S. M. Tilghman, 1998 Genomic imprinting is disrupted in interspecific *Peromyscus* hybrids. Nat. Genet. **20:** 362–365.

Vrana, P. B., J. A. Fossella, P. Matteson, T. del Rio, M. J. O'Neill *et al.*, 2000 Genetic and epigenetic incompatibilities underlie hybrid dysgenesis in *Peromyscus*. Nat. Genet. **25:** 120–124.

West, J. D., W. I. Frels, V. M. Chapman and V. E. Papaioannou, 1977 Preferential expression of the maternally derived X chromosome in the mouse yolk sac. Cell **12:** 873–882.

Zechner, U., M. Reule, A. Orth, F. Bonhomme, B. Strack *et al.*, 1996 An X chromosome linked locus contributes to abnormal placental development in mouse interspecific hybrids. Nat. Genet. **12:** 398–403.

Zechner, U., M. Reule, P. S. Burgoyne, A. Schubert, A. Orth *et al.*, 1997 Paternal transmission of X-linked placental dysplasia in mouse interspecific hybrids. Genetics **146:** 1399–1405.

Communicating editor: P. D. Keightley