Genetic Dissection of X-Linked Interspecific Hybrid Placental Dysplasia in Congenic Mouse Strains

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ABSTRACT

Interspecific hybridization in the genus Mus results in male sterility and X-linked placental dysplasia. We have generated several congenic laboratory mouse lines (*Mus musculus*) in which different parts of the maternal X chromosome were derived from *M. spretus*. A strict positive correlation between placental weight and length of the *M. spretus*-derived part of the X chromosome was shown. Detailed analysis was carried out with one congenic strain that retained a *M. spretus* interval between 12.0 and 30.74 cM. This strain consistently produced hyperplastic placentas that exhibited an average weight increase of 180% over the weight of control placentas. In derived subcongenic strains, however, increased placental weight could no longer be observed. Morphometric analysis of these placentas revealed persistence of abnormal morphology. Fully developed placental hyperplasia could be reconstituted by recombination of proximal and central *M. spretus* intervals with an intervening *M. musculus* region. These results may suggest that placental dysplasia of interspecific mouse hybrids is caused by multiple loci clustered on the X chromosome that act synergistically. Alternatively, it is possible that changes in chromatin structure in interspecific hybrids that influence gene expression are dependent on the length of the alien chromosome.

ABNORMAL placental development in interspecific hybrids has been described in different mammalian groups, in equids (Allen 1975; Allen *et al.* 1993), murids (Zechner *et al.* 1996, 1997), and peromyscids (Rogers and Dawson 1970). Thus, genes active in placentation (and spermiogenesis) can be expected to evolve very rapidly (Haig 1993). To date, interspecific hybrid placental dysplasia has been described in most detail in the genus Mus (Zechner *et al.* 1996, 1997). Very similar effects on placental growth were obtained when inbred *M. musculus* (*mus*) mice were crossed and backcrossed with mice of three other closely related species (Bonhomme *et al.* 1983; Boursot *et al.* 1993), *M. spretus* (*spr*), *M. macedonicus*, and *M. spicilegus*. However, only matings between *mus* and *spr* will be discussed here. The occurrence of abnormal placentas exhibited an imprinting effect, in that increase or decrease of placental growth was dependent on the sex of the parental species (Zechner *et al.* 1996). Mainly hyperplastic placentas were found in (*spr* × *mus*)F_1 (SM) matings, in the SM × *mus* (SMM), and MSM backcrosses (BCs), and in the further BCs to *mus* males (in all matings, the female is indicated first). The opposite phenotype, placental hypoplasia, was consistently observed in the reciprocal MS, MSS, and SMS crosses and BCs. Abnormal fetal growth was not a consistent finding in the Mus crosses, where abnormal fetal weights could be explained by changes in placental efficiency (Kurz *et al.* 1999).

As in the Peromyscus matings, the main tissue affected in Mus dysplastic placentas was the spongiotrophoblast (Rogers and Dawson 1970; Zechner *et al.* 1996). This cell layer was strikingly enlarged in hyperplastic placentas, but was either reduced or sometimes completely absent in hypoplastic placentas. In addition, differentiation of glycogen cells, a cell type presumably derived from the spongiotrophoblast (Redline *et al.* 1993), was also strikingly affected. Together with the finding that at least one locus on the X chromosome, designated *Ihpd* (for interspecific hybrid placental dysgenesis), is tightly linked to placental dysplasia (Zechner *et al.* 1996), this provided the likely explanation for the imprinting effect observed in Mus interspecies matings. The spongiotrophoblast is a derivative of the trophoblast (Rossant and Croy 1985), in which the paternal X chromosome is preferentially inactivated (Takagi and

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Sasaki 1975; West *et al.* 1977). Thus, only loci on the maternal X chromosome are presumably active in spongiotrophoblast cells of placentas associated with both male and female fetuses (Sobis *et al.* 1991).

As control of placental growth is of fundamental importance in mammalian development, the identification of the genes that in combination cause abnormal growth in interspecific hybrids is of considerable interest. Here it is assumed that these genes are also involved in the regulation of spongiotrophoblast and placental growth in the normal intraspecific context. In addition, faulty interactions between genes descended from closely related species indicate that these genes evolve rapidly and, therefore, may be regarded as speciation genes.

In this article, we describe the generation of several congenic strains in which different regions of the X chromosome were descended from *spr*. This strategy was used in an attempt to reveal X-chromosomal regions critical for expression of IHPD, and it was designated as a first step towards a positional cloning approach of at least one of the X-chromosomal loci.

MATERIALS AND METHODS

Mice: The following mice were used: *Mus musculus* (*mus*) C57BL/6 (BL6) and C57BL/6×C3H (B6C3), as well as *Mus spretus* (*spr*) SFM and SEG (Bonhomme and Guénet 1996). The *spr* mouse strains were established at the Laboratoire Génome et Populations, Université de Montpellier. For the production of repeated backcross lines, laboratory strain *mus* females were mated with *spr* males. The resulting F_1 females were successively backcrossed to *mus* males, and their offspring were selected for *spr*-derived alleles on the X chromosome. The established lines were characterized by recombination at different positions on the X chromosome. Congenic strain AT24 was constructed by selecting for the *spr* allele of *Hprt* on a background of BL6 using 12 backcross generations followed by intercrossing (V. M. Chapman, unpublished results). It contains ~20 map units of chromosome X derived from *spr*. Subcongenic strains were obtained by crossing AT24 with BL6 and backcrossing to BL6. Animals carrying crossovers within the *spr* interval were intercrossed (R. Elliott, D. Miller, R. Pearsall, C. Hohman, D. Poslinski, D. Tabaczynski and V. Chapman, unpublished results). The SD7 strain originated from the same strategy, but by selection for *spr* alleles on distal chromosome 7. They were made homozygous after the fifth backcross generation and have since been maintained by brother × sister matings (Hemberger *et al.* 1998). Placentas were collected and weighed on gestational day 18, counting the day of the vaginal plug as day 1.

Microsatellite mapping: Genomic DNA was prepared from mouse tail tips or embryos using the Mammalian Genomic DNA Extraction Kit (Cambridge Molecular Technologies). DNAs were typed by polymerase chain reaction (PCR) with simple sequence length polymorphism (SSLP) markers purchased from Research Genetics (Huntsville, AL). PCR conditions were 2.5 mm $MgCl_2$ for 35 cycles with an annealing temperature of 55°. PCR products were analyzed on 4% agarose gels. For LOD score analysis, DNA samples from the 64 MSM BC1 fetuses described in Zechner *et al.* (1996) plus samples from additional 23 MSM fetuses were used and analyzed with the MapMaker 3.0 program (Lander *et al.* 1987). In total, 30 X chromosomal markers distributed between *DXMit57* at 5.9 cM and *DXMit12* at 72 cM were used.

Histology: Placentas processed for routine paraffine histology were fixed in Serra's fixative (60% ethanol, 30% formalin, 10% acetic acid) at 0° overnight. Sections were treated for 3 min with 3% H_2O_2 in 1× TBS, and they were incubated for at least 1 hr with peroxidase-conjugated lectin from *Bandeiraea simplicifolia* BS-I B_4 (Sigma, St. Louis, MO) at a concentration of 40 ng/μl. Washes were carried out three times with 0.3 m NaCl, 50 mm Tris-Hcl, 0.1% NP40, and staining was performed using the Liquid DAB and Substrate Chromogen System (DAKO, Hamburg, Germany). Nuclear counterstaining was carried out with hemalaun.

Morphometric analysis: For each strain analyzed morphometrically, two placentas were investigated, one derived from a male fetus and the other from a female fetus. Fetuses were sexed by their gonadal morphology. Placentas were cut in halves, processed for paraffine histology, and sectioned at 7 μm. This procedure provided a systematic random sample of vertical sections for unbiased stereological estimates (Baddeley *et al.* 1986). The volume fraction (percentage of total

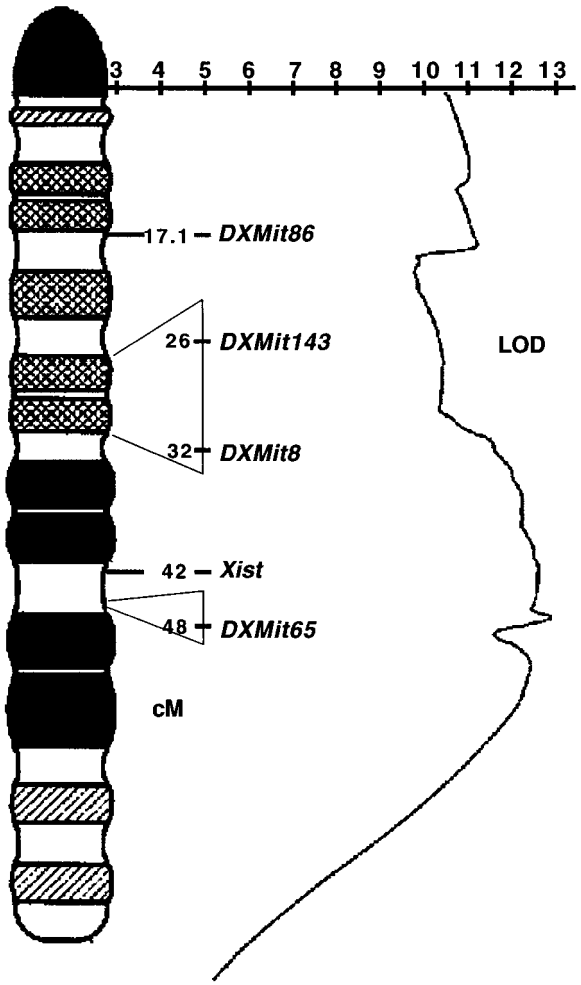


Figure 1.—Association between chromosome X markers and placental weight. LOD score analysis was carried out with 87 MSM BC1 fetuses. Linkage was found for the whole proximal and central part of the X chromosome between the centromere and 50 cM.

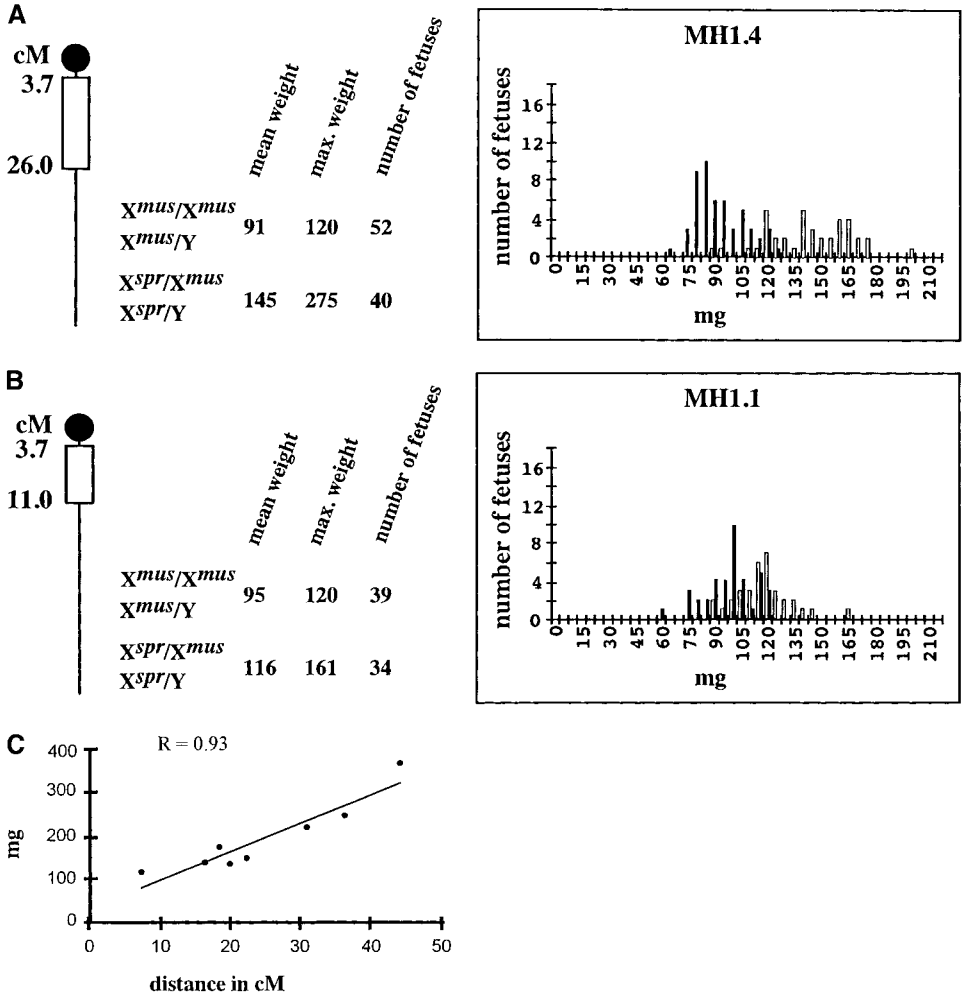


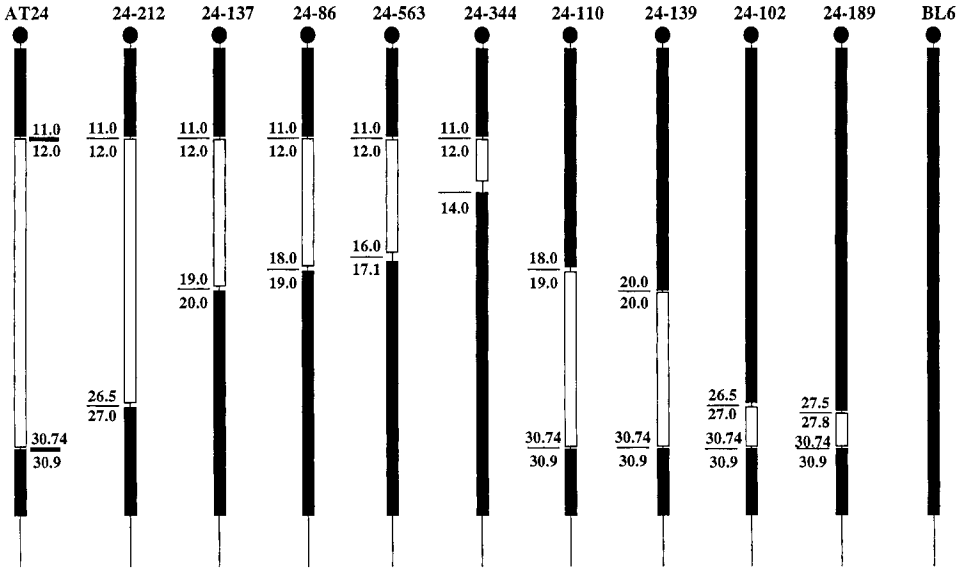
Figure 2.—Heterozygous congenic lines. Placental weight distribution and correlation with the length of the *spr*-derived region on the X chromosome. (A and B) Placental weight distribution of offspring obtained from heterozygous X^{spr}/X^{mus} females and *mus* males. The X chromosomal haplotype is shown on the left, with the *spr*-derived interval indicated by the white box. Black bars represent placental weights of fetuses that inherited the *mus* X chromosome; open bars indicate placental weights of fetuses that inherited the *spr*-derived X chromosome. Mean and maximum placental weights (in milligrams) and the number of fetuses investigated are given. (C) Mean placental weights from eight independent lines are depicted against the length of the *spr*-derived interval on the X chromosome. The regression slope is indicated. The correlation coefficient between mean placental weight and the length of the *spr*-derived interval on the X chromosome is 0.93 ($P < 0.001$).

placental volume) of the following compartments was determined by point counting in three fields of each of the three lectin-hemalaun-stained sections per placenta: labyrinthine trophoblast, spongiotrophoblast, and decidua. The volume fraction of the glycogen cells was determined separately.

RESULTS

Linkage analysis of placental dysplasia: To reveal associations between X chromosomal loci and placental hyperplasia, 87 MSM BC1 fetuses were analyzed. The obtained LOD score ranged between 10 and 13 for the whole proximal and central X chromosome, from the centromere to 48 cM (Figure 1), suggesting that placental dysplasia in interspecific crosses could be under polygenic control.

Occurrence of hyperplastic placentas in BC mice with defined *spr*-derived regions on the X chromosome: MSM females were further backcrossed with *mus* males to generate lines with defined *spr*-derived regions on the X chromosome. Because of male sterility resulting from the incompatibility at the pseudoautosomal region (Matsuda *et al.* 1991), these lines could not be made homozygous and, thus, the offspring of genotyped heterozygous females were analyzed for placental weight distribution. Altogether, 12 different lines were established (not shown). Eight lines, MH1.4, MH2.1, MH1.3, MH1.1, MH1.5, MH4.2, MH6.1, and MH7.1, were analyzed in more detail in that both placental weights and genotypes of associated fetuses were determined for at least 22 conceptuses from each line. In that analysis, values derived from BC4 to BC8 fetuses were pooled as no significant weight differences were detected between the BC generations. Detailed presentations of placental weight distribution are shown for two representative lines (Figure 2, A and B). The overall results for all lines show a positive correlation between the length of the *spr*-derived X chromosome and placental weight (Figure 2C) in that lines with shorter stretches of *spr*-derived X chromosomes generated smaller mean placental weights ($r = 0.93$; $P < 0.001$). In contrast to this, conceptuses from these lines that had inherited the *mus* X chromosome from their heterozygous mother exhibited normal placental weights (Figure 2, A and B). In the following, only conceptuses with *spr*-derived X chromosome regions will be referred to (Table 1). Line MH1.1, which was *spr* between 3.7 and 11.0 cM (*DXMit54* and *DXMit50*), produced placentas with a mean weight of



mean weight (mg)	167	119	113	114	92	97	111	104	103	106	92
SD (mg)	33	19	17	20	10	16	17	17	14	20	13
number of fetuses	56	50	83	101	25	13	81	59	93	87	36
maximum weight (mg)	251	166	167	164	108	115	158	156	140	179	123

Figure 3.—Placental weight analysis of congenic and subcongenic strains with the *spr*-derived interval indicated by the white box (only haplotypes are shown). Centimorgan positions of microsatellite markers representing the borders of *spr*- and *mus*-derived regions are given on the left of each chromosome and are divided by the horizontal line. Placental mean weights, standard deviation, number of fetuses investigated, and maximum placental weights are depicted for each strain. Although strain AT24 exhibits placental hyperplasia, none of the subcongenic strains clearly retains this placental enlargement.

116 ± 16 mg ($\bar{X}$ ± SD), and the maximum placental weight was 161 mg (N = 34). Lines MH1.4, MH2.1, and MH1.3, which were *spr* between 3.7 and 26.0 cM (*DXMit143*), 5.9 cM (*DXMit57*) and 26.0 cM, and 3.7 cM and 20.0 cM (*DXMit92*), respectively, had mean placental weights of 145 ± 31 mg (N = 40), 133 ± 17 mg (N = 45), and 138 ± 23 mg (N = 64). The maximum placental weights observed in these lines were 275, 183, and 186 mg. The other four strains were *spr* between 3.7 cM (*DXMit54*) and 48 cM (*DXMit65*) (MH1.5), 17.1 cM (*DXMit86*) and 48 cM (*DXMit65*) (MH4.2), 29.75 cM (*DXMit146*) and 66 cM (*DXMit28*) (MH6.1), and 43.6 cM (*DXMit158*) and 62 cM (*DXMit35*) (MH7.1). The mean placental weights observed in these strains were 365 ± 87 mg (N = 9), 217 ± 70 mg (N = 9), 245 ± 54 mg (N = 9), and 171 ± 24 mg (N = 7). Maximum placental weights were 494, 384, 331, and 217 mg, respectively.

These results further strengthened the hypothesis that several loci on the X chromosome have to act synergistically to produce hyperplastic placentas.

Analysis of congenic strains homozygous for *spr*-derived regions and subregions on the X chromosome: Independently from the lines described above, a congenic strain, AT24, was established that contains ~20 map units of *spr*-derived X chromosome between 12.0 cM (*DXMit48*) and 30.74 cM (*DXMit60*). The males of this strain were fertile and, thus, the strain could be made homozygous for the *spr* region. As expected from the results obtained with the heterozygous lines, females of strain AT24 contained hyperplastic placentas. Mean placental weight was 167 ± 33 mg (N = 56), and maximum placental weight was 251 mg (Figure 3). Thus, it seemed possible that further reduction of the *spr* portion would lead to the identification of a region containing one of the loci involved in the production of hyperplastic placentas. Consequently, nine subcongenic strains were produced with shorter intervals from *spr* that together covered the complete AT24 region (Figure 3). Analysis of placental weights showed that none of these subcongenic strains produced a measurable placental hyperplasia (Figure 3).

To determine whether pronounced placental hyperplasia can be reconstituted by recombining proximal and central *spr*-derived regions, AT24 males were mated with females of line MH7.1. Double heterozygous females were then mated with BL6 males, and the female offspring were assessed for recombination. To date, two recombinant females were dissected on day 18 of gestation. The mean weight of placentas from the litters that

TABLE 1

Placental weights of congenic lines

Line	Proximal marker	Position (cM)	Dist. marker	Position (cM)	Mean (mg)	SD (mg)	Max (mg)	*N*
MH1.1	*DXMit54*	3.7	*DXMit50*	11.0	116	16	161	34
MH1.4	*DXMit54*	3.7	*DXMit143*	26.0	145	31	275	40
MH1.3	*DXMit54*	3.7	*DXMit92*	20.0	138	23	186	64
MH2.1	*DXMit57*	5.9	*DXMit143*	26.0	133	17	183	45
MH1.5	*DXMit54*	3.7	*DXMit65*	48.0	365	87	494	9
MH4.2	*DXMit86*	17.1	*DXMit65*	48.0	217	70	384	9
MH6.1	*DXMit146*	29.75	*DXMit28*	66.0	245	54	331	9
MH7.1	*DXMit158*	43.6	*DXMit35*	62.0	171	24	217	7

N, number of fetuses.

carried both *spr*-derived regions on the X chromosome was 245 ± 36 mg ($N = 10$). This contrasts with the mean weights of the parental strains AT24 and MH7.1, which had mean placental weights of 167 ± 33 mg ($N = 56$) and 171 ± 24 mg ($N = 7$). Thus, an increase in placental weight of recombinants over the parental strains AT24 and MH7.1 was evident.

Morphometric analysis of placentas from congenic and subcongenic strains: To determine whether placental morphology was normal in the subcongenic strains with normal placental weights, histological sections of strains 24-86 and 24-189 were analyzed morphometrically. For controls, placental sections derived from MSM BC1, AT24, BL6 × BL6, MH7.1 × AT24 recombinants, and SD7 matings were also measured. This analysis showed that in BL6 matings, the spongiotrophoblast contributed 25.7% of total placental volume. The mean weights of the three sections analyzed for each placenta were 26.8 ± 2.11 and 24.6 ± 1.46%. As expected, this value was increased to 43% in MSM BC1 placentas. In placentas from strain AT24, the spongiotrophoblast layer contributed 46%. In subcongenic strains 24-86 and 24-189, the relative volume of spongiotrophoblast was still increased, with values of 40.9 and 38.5%, respectively. Of all strains analyzed, the most pronounced difference between the two placentas was observed for subcongenic strain 24-86, with 36.4 ± 1.86% for the placenta associated with the female fetus and 45.33 ± 1.77% for the placenta associated with the male fetus. In the congenic strain SD7, in which the distal part of chromosome 7 is derived from *spr*, the relative volume of spongiotrophoblast was normal, with a value of 22% (Figure 4a). In addition to placental weights and spongiotrophoblast volume fraction, the degree of glycogen cell differentiation was determined as a third criterion for assessing placental dysplasia (Figure 4b). Again, subcongenic placentas exhibited intermediate degrees of glycogen cell differentiation, whereas MSM, AT24, AT24 × MH7.1, and BL6 placentas represented the most extreme differences (Figures 4b and 5).

DISCUSSION

In the current analysis, a large region of the X chromosome was investigated for linkage with placental dys-

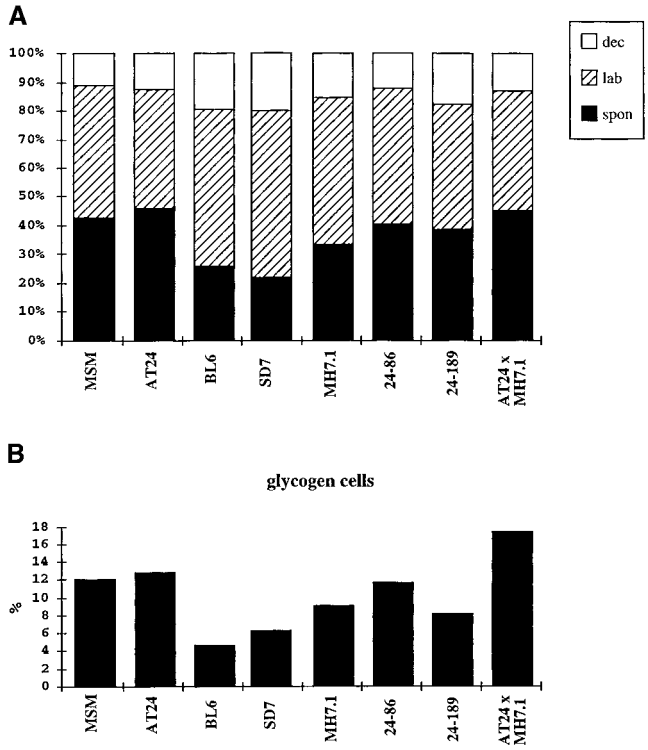


Figure 4.—Morphometric analysis of placentas. (A) Volume fractions of the spongiotrophoblast, labyrinthine trophoblast, and decidua are shown. The spongiotrophoblast volume fraction is most pronounced in MSM, AT24, and AT24 × MH7.1 recombinant placentas. In BL6 and SD7 controls, the spongiotrophoblast accounts for only 26 and 22% of the total placenta, respectively. In the subcongenic strains 24–86 and 24–189, the spongiotrophoblast volume is still increased, although placental weights are normal. (B) Similar results are obtained when the degree of glycogen cell differentiation is determined. Again, subcongenic strains 24–86 and 24–189 exhibit intermediate values between the extremes represented by MSM, AT24, and AT24 × MH7.1 placentas and by BL6 and SD7 placentas.

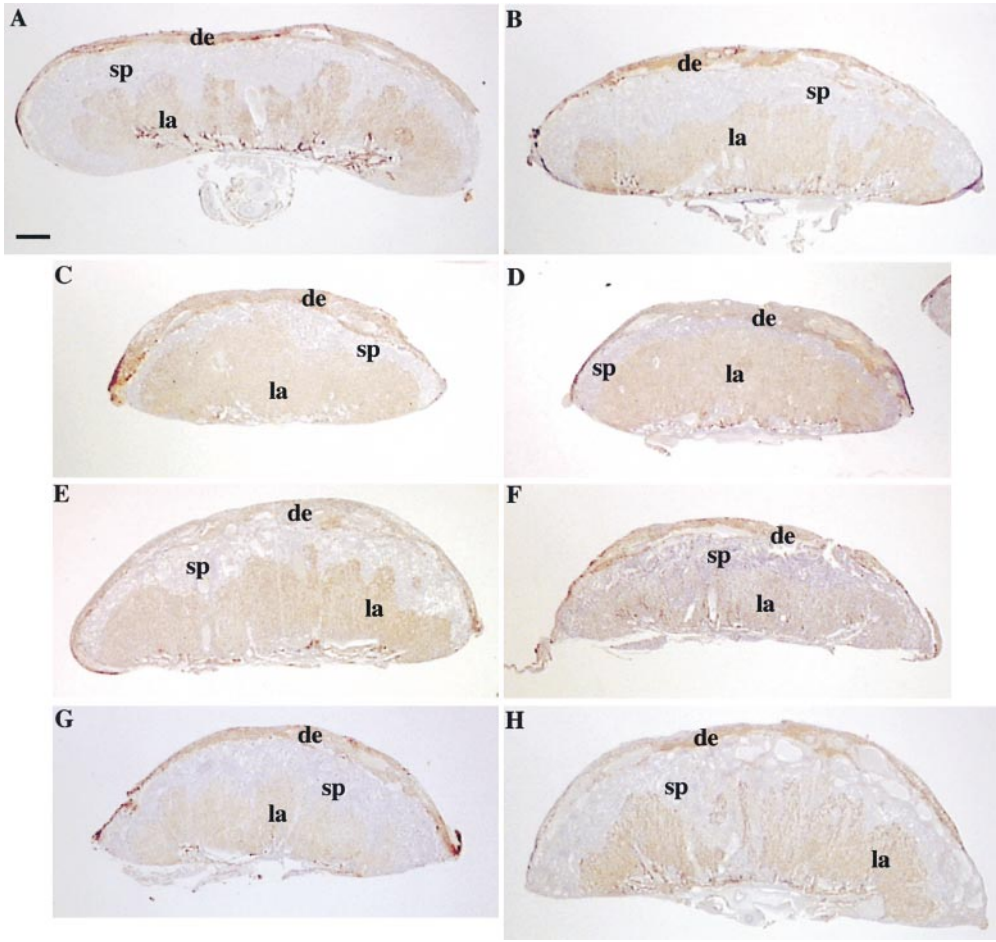


Figure 5.—Placental histology of strains analyzed morphometrically. Sections were stained with lectin BS-I B_4, which specifically marks the decidua and labyrinth. Placental sections are derived from the following strains: (A) MSM, (B) AT24, (C) BL6, (D) SD7, (E) MH7.1, (F) 24-86, (G) 24-189, and (H) AT24 × MH7.1. Bar, 600 μm. *de*, decidua; *la*, labyrinth; *sp*, spongiotrophoblast.

plasia. Contrary to our expectations, we were not able to narrow down a single genetic region segregating with placental dysplasia. The first evidence for an unusual genetic system was obtained from an LOD score analysis that showed linkage of both the proximal and central regions of the X chromosome with placental dysplasia and provided no further evidence for an especially tight linkage to *DXMit8* at 32 cM, as described previously (Zechner *et al.* 1996). Interestingly, independent support for linkage of *Ihpd* to a region in the vicinity of *DXMit8* has been provided by studies of transmission ratio distortion (TRD) in the MSS BC (Montagutelli *et al.* 1996). TRD directed against the *mus*-derived X chromosome in the MSS BC has been reported repeatedly (Biddle 1987; Eicher *et al.* 1992; Rowe *et al.* 1994; Boyd 1996), and placental insufficiency of the strongly hypoplastic placentas observed in the MSS BC provided a first explanation for fetal lethality (Zechner *et al.* 1996). Although the present analysis seems to be incompatible with a single locus around *DXMit8*, it may be misleading to compare the TRD in the MSS BC (Biddle 1987; Eicher *et al.* 1992; Rowe *et al.* 1994), which may be caused by fetal lethality, with placental weights in the MSM BC. In contrast to the MSS BC, the MSM BC does not exhibit fetal lethality (Zechner *et al.* 1996).

In the past, quantitative trait loci (QTL) have been mapped through the use of congenic mouse strains. Examples for successful applications of this strategy were described for the mapping of QTL for epilepsy (Frankel *et al.* 1995), blood pressure (Dukhanina *et al.* 1997), diabetes (Yui *et al.* 1996), and body weight (Rance *et al.* 1997). Thus, this experimental strategy seemed feasible for the identification of the loci involved in placental dysplasia. However, with decreasing length of the *spr*-derived X chromosome, the placental phenotype became less pronounced. To complicate the story even further, the components of placental dysplasia seemed to behave independently, in that the increase in placental weight disappeared, whereas overgrowth of spongiotrophoblast and increased glycogen cell differentiation were still maintained, although to a lesser extent than in enlarged placentas. Thus, one explanation for these findings could be the presence of multiple loci on the proximal and central parts of the X chromosome that act synergistically to produce interspecific hybrid placental dysplasia (IHPD) with all its facets. However, if this multiple-locus model holds true, then in each of the subcongenic regions, one or several genes that produce more or less identical "subphenotypes" of IHPD would have to exist. An example for the presence of multiple

loci on the same chromosome involved in a single differentiation pathway has been provided by Sala *et al.* (1997). In that study, it was suggested that at least eight different genes in Xq21 may be associated with premature ovarian failure (POF), each of which is sufficient to cause POF. In contrast to this, all or most of the putative *Ihpd* loci derived from *spr* have to be present to produce extreme IHPD, as observed in the BC1 with placental weights approaching 400 or 500 mg. These findings may be reconciled with a multiple-locus model only when epistatic interactions between different loci are assumed.

Additive and epistatic interactions between several genes have been discussed extensively as the causes of speciation in the genus Drosophila (reviewed in Orr 1997). Consistent with Haldane's rule (Haldane 1922), male sterility or inviability is often observed in interspecific Drosophila hybrids. Introgression experiments in which chromosomal pieces of one species were brought into the genetic background of another species (Cabot *et al.* 1994), have proven that at least three genes sometimes have to interact to cause hybrid male sterility. None of these factors, when introgressed alone, is sufficient to cause sterility. The basis for this evolutionary preference of complex interactions is given by the Dobzhansky-Muller model (Dobzhansky 1937; Muller 1942). Herein, two allopatric populations with identical genotypes evolve independently at two or more loci. Whereas the derived alleles are functional on their own genetic background, they may be incompatible with derived alleles of another species. For combinatorial reasons, the appearance of complex incompatibilities is favored with an increasing number of interacting loci (Orr 1995). Epistatic interactions have thus been implicated in speciation in Drosophila. Here, a similar phenomenon is evident in mammalian development that is likely to form a postmeiotic barrier to interspecific hybridization in mammals.

An alternative explanation for the complications associated with IHPD may be provided by a model in which an *spr*-derived X chromosome, or parts thereof, in the presence of *mus*-derived autosomes, develops an abnormal chromatin structure. In F_1 hybrids between different wallaby species, the occurrence of genome-wide undermethylation, retroviral element amplification, and chromosome remodeling was shown to occur (Waugh O'Neill *et al.* 1998). Thus, alterations in the methylation status and/or chromosomal changes caused by the activation of mobile elements may lead to changes in the overall chromatin structure which, in turn, could facilitate rapid karyotypic evolution. It is feasible that an altered chromatin structure also appears in interspecific hybrids. This may result in an altered expression of few or perhaps even a single gene on either the distal part of the X chromosome (*cis*) or an autosome (*trans*). This model could explain the positive correlation between the length of the *spr*-derived chromosome and placental phenotype. It can be assumed that a shorter stretch of altered chromatin structure will have a weaker effect both *in cis* and *in trans*. Thus, the putative IHPD effect could be comparable to position-effect variegation (PEV), a phenomenon known from Drosophila (Singh 1994) and mouse (Cattanach 1974). While PEV is commonly observed *in cis*, effects of chromatin structure *in trans* have also been reported in Drosophila (Donaldson and Karpen 1997). However, to our knowledge, PEV *in trans* has not yet been described in Mus.

In conclusion, this study of IHPD in mouse interspecific hybrids has revealed an unexpected complexity that shares intriguing similarities with speciation mechanisms acting in insects. This complexity may also appear in further studies of aberrant phenotypes associated with interspecific hybridization. Further studies that combine molecular, cytogenetic, and classical genetic techniques, as demonstrated by Waugh O'Neill *et al.* (1998), may provide a powerful tool to solve some of the causes for the problems present in interspecific placentation.

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