Diabetic Embryopathy in C57BL/6J Mice

Altered Fetal Sex Ratio and Impact of the Splotch Allele

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Maternal diabetes (types 1 and 2) induces a broad array of congenital malformations, including neural tube defects (NTDs), in humans. One of the difficulties associated with studying diabetic embryopathy is the rarity of individual malformations. In an attempt to develop a sensitive animal model for maternal diabetes–induced NTDs, the present study uses chemically induced diabetes in an inbred mouse model with or without the splotch (*Sp*) mutation, a putatively nonfunctional allele of *Pax3*. *Pax3* deficiency has been associated with an increase in NTDs. Female C57BL/6J mice, either with or without the *Sp* allele, were injected intravenously with alloxan (100 mg/kg), and plasma glucose was measured 3 days later. A wide range of hyperglycemia was induced, and these diabetic mice were bred to C57BL/6J males, some carrying the *Sp* allele. Gestational-day-18 fetuses were examined for developmental malformations. Fetuses from matings in which either parent carried the *Sp* allele were genotyped by polymerase chain reaction. Maternal diabetes significantly decreased fetal weight and increased the number of resorptions and malformations, including NTDs. A significant correlation was found between the level of maternal hyperglycemia and the malformation rate. The sex ratio for live fetuses in diabetic litters was significantly skewed toward male fetuses. Matings involving the *Sp* allele yielded litters with significantly higher percentages of maternal diabetes–induced spina bifida aperta but not exencephaly, and this increase was shown to be associated with the presence of a single copy of the *Sp* allele in affected fetuses. Thus, *Pax3* haploinsufficiency in this murine model of diabetic embryopathy is associated with caudal but not cranial NTDs. *Diabetes* 50:1193–1199, 2001

Embryopathy resulting from maternal type 1 or type 2 diabetes is a well-established phenomenon, with the risk of a birth defect in a diabetic pregnancy being at least two and as much as six times higher than normal (1–3). Maternal diabetes has the potential to adversely affect the development of multiple organ systems, resulting in a wide range of congenital malformations. Thus, it is unlikely that a specific pattern of malformations can be attributed to embryonic effects of the maternal diabetic state (4,5). Generally, however, the birth defects most commonly associated with maternal diabetes are caudal regression, situs inversus, kidney malformations, cardiac anomalies, and neural tube defects (NTDs) (6,7).

Although the etiology of diabetic embryopathy has yet to be elucidated, several animal experiments have revealed the importance of genetic background in susceptibility of the conceptus. Eriksson et al. (8) showed that two substrains of Sprague-Dawley rats differed substantially in their teratogenic response to maternal diabetes induced by streptozotocin. A subsequent study with the susceptible and resistant substrains demonstrated that it is necessary for the mother to be both manifestly diabetic and genotypically susceptible in order to exhibit a significantly increased rate of resorption (9). Significant increases in fetal malformation rates were observed only when the mother was manifestly diabetic and both parents had the susceptible genotype. The impact of strain-specific genetics on teratogenicity is well established and has been demonstrated for a wide variety of physical and chemical teratogens in several rodent species (10).

Although it is unlikely that diabetic embryopathy can be described as a specific set of malformations, congenital defects resulting from maternal diabetes in both humans and animal models typically include a large proportion of NTDs (2,11). Thus, it was considered that added sensitivity may be conferred by a mutation in *Pax3*, a gene shown to be involved in a variety of developmental processes including neural tube closure (12). *Pax3* is one of a highly conserved family of paired-box genes encoding transcription factors and expressed primarily during development; four of the nine murine *Pax* genes have been associated with classical mouse mutants (13). The *Pax3* mouse mutant, splotch (*Sp*) (14), is semidominant (15), with heterozygotes (*Sp*/+) exhibiting a white spot on the belly and, sometimes, white paws caused by defective migration of neural crest cells. Homozygotes (*Sp*/*Sp*) die between days

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13 and 14 of gestation (16), possibly due to cardiac failure (17,18). The penetrance of NTDs is highly variable, depending on genetic background (19,20). C57BL/6J *Sp/Sp* mice reportedly exhibit 100% spina bifida aperta and 56% exencephaly (16), while outcrossing to In(1)1Rk mice, in one laboratory, resulted in only 25% spina bifida aperta but 100% exencephaly in *Sp/Sp* progeny (19,21). *Sp* has been associated with gene-teratogen interactions, resulting in increased sensitivity to NTDs caused by retinoic acid (22–24) and arsenite (25).

Pax3 is a transcription factor that contains both a paired-box and a homeobox DNA-binding domain and is expressed primarily during embryogenesis (12). Expressed dorsally in the developing neural tube, as well as in neural crest cells and developing limb buds, Pax3 is believed to function in neural tube closure (26) and as a regulator of muscle development (27–29) and melanogenesis (30). The mutated sequence in *Sp* consists of a 5-bp change near the 3′ extremity of intron 3 of the murine *Pax3*. This mutation leads to aberrantly spliced mRNA, which has been reported to result in nonfunctional Pax3 polypeptides (15). In addition to *Sp*, there are eight known mutations at the murine *Pax3* locus (26). These include the point mutation, *Sp*-delayed (Sp^{d}), as well as Sp^{J}, Sp^{2J}, Sp^{3J}, and the X irradiation–induced deletion mutations, Sp^{1H}, Sp^{2H}, Sp^{4H}, and *Sp*-retarded (Sp^{r}). Mutations in *PAX3*, the human homologue of *Pax3*, result in the autosomal-dominant diseases known as Waardenburg syndrome type I (31) and type III (32). Waardenburg syndrome consists of developmental anomalies affecting hearing, pigmentation, cardiac morphology, and the skeletal system (33,34), and homozygotes for these *PAX3* mutations are postulated to have a higher incidence of NTDs (35).

The present study was designed to determine the effects of the *Sp* allele, introduced via the male or female, on malformations—particularly NTDs—secondary to chemically induced maternal diabetes. Experimental models of type 1 diabetes have relied on chemical induction by selective destruction of pancreatic β-islet cells or on animal models that are genetically predisposed to spontaneous development of type 1 diabetes. Typically, diabetes is chemically induced via the administration of alloxan (ALX) or streptozotocin (STZ). Both of these diabetogenic agents effectively target the β-islet cells, but they differ in their side effects. STZ is more destructive to elements of the immune system, and ALX is associated with a higher mortality rate in rats (36). Alternatively, there are several animal models that spontaneously develop type 1 diabetes, of which the most frequently studied are the NOD mouse and the BB rat (37). In the present study, chemical induction of type 1 diabetes was preferred over a genetic model, since this permitted investigation of teratogenesis secondary to the maternal diabetic milieu without the possible contribution to teratogenesis of genetic factors that predispose the genetic models to type 1 diabetes. Preliminary experiments with ALX and STZ indicated adequate survivability in the C57BL/6J strain and that most of the treated animals could be maintained in a diabetic state for several weeks and still maintain reproductive capacity (unpublished observation).

RESEARCH DESIGN AND METHODS

Animals. C57BL/6J mice, with or without the *Sp* mutation, were purchased from Jackson Laboratories (Bar Harbor, ME) or bred in our animal facility. The majority of experiments were carried out at the University of California–Los Angeles (UCLA), but the animal protocol was approved at both UCLA and the University of Cincinnati. All animals were housed and treated in accordance with the respective institutional policy. The mice were maintained on a 12-h alternating light/dark cycle and supplied with food (Purina Formulab 5,008) and tap water ad libitum. For the experimental induction of diabetes, females at least 10 weeks old were injected intravenously (into the tail vein) with 100 mg/kg ALX monohydrate (ICN Biomedicals, Aurora, OH). The mice were briefly warmed with a heating lamp to increase blood flow and facilitate injection. Three days after ALX administration, with the mouse under ether anesthesia, a blood sample was obtained from the orbital sinus, and plasma glucose levels were determined using a glucose kit (Trinder; Sigma, St. Louis, MO). Animals with values >300 mg/dl were considered manifestly diabetic and included in the study. Diabetic females were housed individually with males overnight, and the appearance of a vaginal plug in the morning indicated that copulation had occurred. That day was designated gestational day 0. The mating protocol was repeated for each female every night until the appearance of a vaginal plug, or until the diabetic female was clearly too sick to mate, in which case the animal was killed. Nondiabetic females were mated and observed for a vaginal plug in the same way.

Teratology. On day 18 of gestation, blood samples for plasma glucose determination were obtained from both diabetic and nondiabetic females, after which the mouse was killed by cervical dislocation under ether anesthesia. Fetuses were extracted from the uterus, weighed, examined for sex determination, observed for external morphological defects and then killed by ether overdose. Approximately half the fetuses were preserved in Bouin's solution and analyzed for visceral malformations using Wilson's sectioning technique (38). The other half were double stained with alizarin red and alcian blue using the techniques of Inouye (39) and Kimmel and Trammel (40) as modified by Kuczuk and Scott (41) and examined for skeletal anomalies. All malformations were named according to the standardized nomenclature of Wise et al. (42). For matings involving *Sp* animals, individual visceral yolk sacs and amnions were collected. Genomic DNA was extracted from the yolk sacs by the method of Thomas et al. (43) as modified by Couse et al. (44) and genotyped for the wild-type and *Sp* alleles of *Pax3* by the polymerase chain reaction (PCR).

Genotyping of fetuses. So that *Sp* heterozygote (*Sp*/+) fetuses could be distinguished from their wild-type (+/+) littermates, PCR oligonucleotide primers were designed to bind specific sequences within the *Pax3* gene. An upstream (forward) 20-bp primer was synthesized to correspond to nucleotide positions 625–644 in exon 3 of the *Pax3* cDNA. The upstream primer sequence was 5′ ACA ACG CCT GAC GTG GAG AA 3′. Two oligonucleotide downstream (reverse) primers, also 20 bp in length, were synthesized to complement a segment of the *Pax3* gene that initiates at nucleotide 763 in the cDNA and spans the intron 3/exon 4 boundary. The difference between the wild-type and mutant (*Sp*) alleles is a four-nucleotide sequence and a single nucleotide deletion near the 3′ end of intron 3. Thus, the downstream primer sequences were 5′ GGC TGA TAG AAC TCA CTG GA 3′ for the wild-type allele and 5′ GGC TGA TAG AAC TCA CAC AC 3′ for the *Sp* allele. The sequence divergence between the two downstream primers is underlined. Genomic DNA was subjected to PCR in a 20-μl reaction as follows: 94°C for 2 min, 67°C for 2 min, and 72°C for 5 min (one cycle); 94°C for 30 s, 67°C for 30 s, and 72°C for 1 min (35 cycles); and 94°C for 1 min, 67°C for 1 min, and 72°C for 10 min (one cycle). The resultant PCR product was 1.24 kb in length and could be readily observed on a 0.8% agarose gel stained with ethidium bromide. PCRs for the wild-type and mutant alleles were carried out separately for each fetus. As expected, based on the mating schemes, all fetuses were positive for the wild-type (+) allele. The presence of a product in the reaction containing the primer for the *Sp* allele was considered diagnostic for the heterozygote genotype.

Statistical analysis. A one- or two-tailed *t* test was used to determine statistically significant differences in glucose levels, implantation sites per litter, fetal weight, and proportions of resorptions and malformations among groups. Since the maternal diabetic insult affects the entire litter, the fetuses are not statistically independent. Thus, the litter was used as the statistical unit for comparison, and mean litter percentages of malformations were calculated for all litters in each group. Proportions for all litters were compared by *t* test for significant differences. Since maternal diabetes is an established teratogen, a one-tailed *t* test was used for comparison of diabetic litters with controls. For all other comparisons, a two-tailed *t* test was used. Comparisons of malformation frequencies between groups were undertaken in all cases in which a malformation was observed in more than one instance. The Bonferroni adjustment was then used to compensate for multiple

TABLE 1
Reproductive effects of ALX-induced (100 mg/kg, intravenous) maternal diabetes in C57BL/6J mice with and without the *Sp* allele

Genotype mated*	Treatment	Number of litters	Plasma glucose†	Implants per litter ± SD	Mean fetal weight ± SD	% Resorbed
+/+ × +/+	Control‡	12	173 ± 12	8.3 ± 2.05	1.05 ± 0.10	11.1
+/+ × *Sp*/+	Control	10	179 ± 5	8.3 ± 1.57	1.09 ± 0.12	13.3
Sp/+ × +/+	Control	17	173 ± 8§	8.4 ± 1.00	1.13 ± 0.07	12.7
+/+ × +/+	ALX	17	677 ± 139‖	6.8 ± 2.22	0.69 ± 0.18‖	35.7
+/+ × *Sp*/+	ALX	38	704 ± 177‖	7.3 ± 2.36	0.76 ± 0.23‖¶	38.4‖
Sp/+ × +/+	ALX	19	678 ± 141‖	7.0 ± 2.09‖	0.73 ± 0.18‖	49.3‖

*Female genotype listed first; †plasma glucose levels (mg/dl) obtained on day 18 of gestation; ‡+/+ × +/+ control animals treated with water (10 μl/g) on day 8 of gestation; §determined in four dams; ‖significantly different than the same-genotype control ($P \leq 0.05$); ¶fetal weight determined in 33 +/+ × *Sp*/+ litters.

comparisons between groups. For correlation of malformation data with fetal genotype, percentages of total fetuses are presented, and comparisons were made using the χ^2 statistic for significance. For all statistical procedures, $P \leq 0.05$ was considered significant.

For correlation of resorption and malformation data with maternal plasma glucose levels, a random-effects logistic regression model (45) was used with random-litter effect to produce parameter estimates. The random-litter effect is included to account for the correlation among littermates. Computations were performed using the most recent version of the software, Bayesian inference Using Gibbs Sampling (BUGS) (46). All maternal plasma glucose values were from gestational-day-18 animals.

RESULTS

Plasma glucose levels in untreated C57BL/6J mice in our colony are typically 170–180 mg/dl (unpublished observation). As indicated in Table 1, glucose levels are essentially unchanged when measured on gestational day 18 in control females. Three days after intravenous injection of nulliparous wild-type (+/+) or heterozygote (*Sp*/+) females with ALX (100 mg/kg), the mean values among the three ALX-treated groups (+/+ × +/+, +/+ × *Sp*/+, and *Sp*/+ × +/+) ranged from 526 ± 122 to 560 ± 130 mg/dl (data not shown) and were not significantly different from each other. On day 18 of gestation, immediately before sacrifice, plasma glucose levels were obtained again, and the values for individual treatment groups are shown in Table 1. This parameter was not significantly different among the three ALX-treated groups.

ALX-induced maternal diabetes substantially reduced the rate of pregnancy in females observed to have a vaginal plug. This rate in our C57BL/6J colony is typically 70–80% (unpublished observation). In the segment of this study for which pregnancy-rate data were obtained, 121 vaginal plugs were observed, of which 53 females (43.8%) exhibited indications of being or having been pregnant (i.e., fetuses and/or resorption sites or metrial glands) on day 18 of gestation. Fetuses obtained from diabetic dams were of significantly lower weight than control fetuses, and more resorption sites were observed in diabetic litters than controls, although the increase in the number of resorption sites was not significant in the +/+ × +/+ group (Table 1). This lack of significance was due to a high standard deviation in +/+ × +/+ control group resorption rate, which resulted from a single fully resorbed litter in this group.

Mean litter percentages of the most common externally observed malformations seen in fetuses of diabetic dams are presented in Table 2. The anterior NTD, exencephaly, was the most commonly observed external malformation, and the incidence of this defect was significantly increased from the control values of zero (detected for all three crosses) in all ALX-diabetic groups. In contrast, low levels of spina bifida aperta were seen in the +/+ × *Sp*/+ and *Sp*/+ × +/+ control groups, and the incidence of this defect was significantly increased only in the +/+ × *Sp*/+ diabetic group (Table 2). The lack of statistical signifi-

TABLE 2
Mean litter percentages of selected externally observed malformations* secondary to ALX-induced (100 mg/kg, intravenous) maternal diabetes in C57BL/6J mice with and without the *Sp* allele

Genotype mated	Treatment	Exencephaly ± SEM†	Spina bifida aperta ± SEM†	An/Microphthalmia ± SEM†	Cardiovascular malformations ± SEM†‡	Axial skeletal malformations ± SEM†
+/+ × +/+	Control§	0 (88‖)	0	1.1 ± 1.1	0 (45¶)	6.8 ± 4.9 (43#)
+/+ × *Sp*/+	Control	0 (72‖)	1.1 ± 1.1	1.3 ± 1.3	0 (37¶)	0 (18#)
Sp/+ × +/+	Control	0 (124‖)	0.8 ± 0.8	1.7 ± 1.2	2.0 ± 2.0 (62¶)	2.9 ± 2.9 (62#⟩
+/+ × +/+	Alloxan	14.6 ± 5.0 (74‖)**	1.3 ± 1.3	13.5 ± 4.4**	19.0 ± 8.5 (41¶)**	30.6 ± 10.2 (31#)**
+/+ × *Sp*/+	Alloxan	20.0 ± 4.4 (172‖)**	9.4 ± 3.6**‡‡	11.1 ± 4.2**	15.0 ± 4.6 (97¶)**	28.3 ± 8.7 (59#)**
Sp/+ × +/+	Alloxan	33.6 ± 6.2 (74‖)**††	4.0 ± 2.4	27.1 ± 8.8**	36.7 ± 10.7 (35¶)**	59.7 ± 11.3 (28#)**‡‡

* Infrequently occurring (i.e., not significantly different from the control value of zero) external malformations in litters from diabetic dams were aglossia, agnathia, encephalocele, micrognathia, cleft palate, cleft face, and one example each of astomia, low-set ears, left forelimb ectrodactyly, cleft lip, and omphalocele (many fetuses exhibited more than one malformation); †mean litter percentages ± SE of the mean; ‡other visceral malformations or variations, the incidence of which did not differ significantly from control values, in fetuses from diabetic dams included hydronephrosis, right-sided or midline esophagus, hydrocephaly, abnormal lung lobation, and one example each of small kidney, kidney agenesis, cryptorchidism, enlarged kidney, and double ureter; §+/+ × +/+ control animals treated with water (10 μl/g) on day 8 of gestation; ‖total number of fetuses analyzed for external malformations in this mating scheme; ¶total number of fetuses analyzed for visceral malformations in this mating scheme; #total number of fetuses analyzed for skeletal malformations in this mating scheme; **significantly different than the same-genotype control ($P \leq 0.05$); ††significantly different from ALX-diabetic +/+ × +/+ group ($P \leq 0.05$); and ‡‡significantly different than axial skeletal malformations in ALX-treated +/+ × *Sp*/+ ($P \leq 0.05$).

TABLE 3
Sex of the fetuses from diabetic and nondiabetic female C57BL/6J mice

	Live fetuses		
	Male	Female	Sex ratio
Nondiabetic female	149	135	1.10
Diabetic female	185	131	1.41*

All three mating schemes are pooled, as there were no significant differences between them with respect to fetal sex. *χ^2 indicates a significant increase ($P \leq 0.025$) in the ratio of males to females when the maternal animal is diabetic.

cance of this malformation rate in the *Sp*/+ × +/+ diabetic group may be due to the smaller number of litters produced for this cross (Table 2). The incidence of exencephaly was not higher in one fetal sex than in the other (data not shown); however, χ^2 analysis revealed that female fetuses were significantly less common and may have been preferentially resorbed in the diabetic litters versus the nondiabetic controls (Table 3). The specific eye malformations, anophthalmia and microphthalmia, occur spontaneously in our C57BL/6J colony of mice at a combined rate of ~1%. These eye defects increased significantly in all three diabetic groups (Table 2), when left, right, and bilateral anophthalmia and microphthalmia are considered as a group.

Visceral and skeletal analyses of fetuses revealed significant increases in cardiovascular and axial skeletal malformations (Table 2). Cardiovascular defects detected in litters from wild-type diabetic females mated with wild-type males (+/+ × +/+) consisted solely of outflow-tract malformations, including transposition of the great vessels, dilated great vessels, and right-sided aortic arch and pulmonary trunk. Addition of the *Sp* allele, via the male or the female (+/+ × *Sp*/+ or *Sp*/+ × +/+), resulted in the production of ventricular septal defects and an atrial septal defect, in addition to examples of the outflow-tract malformations previously noted. These two latter groups of matings also produced a single instance each of dextrocardia and double-outlet right ventricle. Several examples each of the malformations—enlarged bladder, dilated renal pelvis, and supernumerary kidneys—were observed in fetuses from matings involving the *Sp* allele but none was seen in +/+ × +/+ litters (control or diabetic). The appearance of these malformations, though not common enough to be statistically significant, seems to be related to the *Sp* genotype, rather than to maternal diabetes. The axial skeletal malformations noted in Table 2 consisted of fusions of the vertebral bodies and/or arches, misshapen cervical arches, centrum defects, fused ribs, rudimentary ribs, and supernumerary ribs.

As shown in Table 2, the incidence of spina bifida aperta but not exencephaly is significantly higher when the paternal genotype includes the *Sp* allele (+/+ × *Sp*/+). In order to confirm that this increase is due to the presence of the *Sp* allele in affected fetuses, PCR genotyping of fetuses was conducted, and the results are presented in Table 4. While the increase in spina bifida aperta showed significant association with the *Sp* allele, the incidence of exencephaly was randomly distributed between the two genotypes. No other malformation demonstrated association with the *Sp* allele.

Figure 1 demonstrates a correlation between the level of maternal hyperglycemia on gestational day 18 and the estimated probabilities of three negative gestational outcomes. The range of hyperglycemia in the total of 70 diabetic females among the three genotype groups was 364–1,274 mg/dl, with a majority (59%) ranging from 600 to 800 mg/dl. Most malformations did not occur frequently enough in individual genotype groups to demonstrate a significant correlation with maternal plasma glucose levels. However, resorptions, exencephaly, and anophthalmia/microphthalmia did show significant correlation when the three genotype groups were considered together. The summation of these outcomes over the three different genotype groups is considered appropriate because the genetics of the groups are identical with only one exception. That exception, the mutation in a single copy of *Pax3*, was found not to be associated with any of the teratogenic outcomes, except for spina bifida aperta. The eye defects grouped together for Fig. 1 consisted of left, right, or bilateral anophthalmia and/or microphthalmia.

DISCUSSION

Past studies of diabetic embryopathy in animal models have, as a group, been plagued with difficulties concerning differentiation between true congenital malformations versus developmental delay and between teratogenicity produced by the maternal diabetic condition versus direct effects of diabetogenic chemicals (47). Additionally, many studies introduce a potentially confounding variable by administering insulin to increase maternal survival and the rate of successful pregnancy. In the present study, in order to prevent some of these difficulties, ALX was given four or more days before gestation, and diabetic females were not treated with insulin. It is presumed that the malformations presented in this work represent true congenital malfor-

TABLE 4
Fetuses from +/+ × *Sp*/+ and *Sp*/+ × +/+ maternal-diabetes matings determined to have the wild-type (+/+) or heterozygous (*Sp*/+) genotype

	+/+ × *Sp*/+ 144 fetuses (30 litters)		*Sp*/+ × +/+ 63 fetuses (16 litters)	
	Wild-type (+/+)	Heterozygote (*Sp*/+)	Wild-type (+/+)	Heterozygote (*Sp*/+)
Total fetuses per genotype	74 (51.4%)	70 (48.6%)	29 (46.0%)	34 (54.0%)
Fetuses with exencephaly	14 (18.9%)	17 (24.3%)	12 (41.4%)	11 (32.4%)
Fetuses with spina bifida	1 (1.4%)	10 (14.3%)*	0	3 (8.8%)

Percentages of NTDs secondary to ALX-induced (100 mg/kg intravenous) maternal diabetes in fetuses of each genotype are also presented. * χ^2 indicates a significant association ($P \leq 0.05$) between spina bifida and presence of the *Sp* allele.

mations, as they persisted from the time of organogenesis to immediately before birth (gestational day 18).

As has been found in past in vivo studies of diabetic embryopathy (8,9), fetal weight in the present investigation was significantly depressed in all diabetic groups. This finding is in contrast to increased fetal weight typically noted in studies of human diabetic pregnancy (48), although decreased fetal weight is seen in diabetic pregnancies complicated with vasculopathy (49). The macrosomia observed in human births and decreased birth weight seen in animal models of maternal type 1 diabetes may be reflective of the relatively large ratio of embryonic versus fetal period in rodents compared with humans (50).

The present study is concordant with past studies demonstrating embryopathy secondary to maternal diabetes in that a wide range of malformations was produced, and no single pattern of malformations specifically attributable to the maternal disease state was discernible (47). Among the most frequent single malformations was exencephaly, which is widely considered to be the murine equivalent of anencephaly (51), a congenital defect frequently found in human diabetic embryopathy (11). Another malformation consistently noted in studies of human diabetic embryopathy is spina bifida aperta (7,11). In animal models of maternal diabetes, however, spina bifida aperta has appeared very infrequently (47). The results of the present study demonstrate that heterozygosity for the murine *Sp* allele confers genetic susceptibility to spina bifida aperta produced by maternal ALX-induced diabetes. The *Sp* mutation on the C57BL/6J background has previously been demonstrated to increase susceptibility to NTDs induced by sodium arsenite (25). Exencephaly was affected in this arsenite study, however, as well as spina bifida aperta. In the present study of diabetic embryopathy, only spina bifida aperta increased in association with the presence of the *Sp* allele, perhaps suggesting a different underlying mechanism responsible for exencephaly secondary to maternal diabetes versus arsenite treatment.

The increased incidence of spina bifida aperta in the *Sp*/+ offspring of diabetic females (Table 4) is presumably due to haploinsufficiency of *Pax3*, since the semidominant *Sp* allele produces aberrant mRNA transcripts leading to truncated polypeptides (15) and complete fetal insufficiency of *Pax3* (*Sp/Sp*) results in virtually 100% spina bifida aperta (16). This effect may be particularly exacerbated in the embryos of diabetic mice, since maternal diabetes has been shown to result in reduced embryonic expression of *Pax3* (52,53). Reduced expression of *Pax3* was correlated, in those studies, with high concentrations of apoptotic cells in the developing neural tube, suggesting a possible pathway involved in diabetes-associated NTDs. It is also important to note that binding of two members of the *Pax* family of transcription factors to target DNA sites is reduced and can even be eliminated by oxidative stress (54,55), a hallmark of the diabetic milieu (56). Recently, the deleterious effect of oxidant stress on *Pax* DNA binding was also demonstrated for *Pax3* (56a). The involvement of reactive oxygen species in diabetic embryopathy is supported by studies demonstrating amelioration of congenital malformations with antioxidants (57–59). Additionally, a rat substrain resistant to dysmorphogenesis produced by maternal diabetes has been shown to have increased levels of both superoxide dismutase and catalase mRNA when compared with a more sensitive substrain (60).

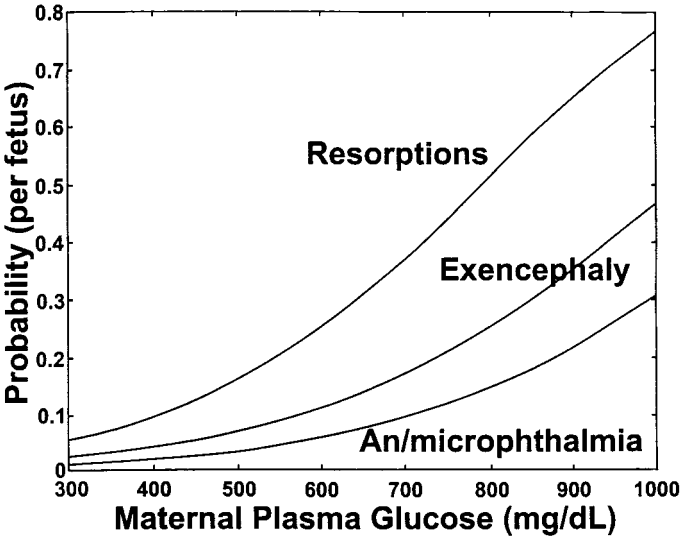

FIG. 1. Correlation between gestational-day-18 maternal plasma glucose values and the estimated probability of various adverse gestational outcomes per fetus summed across the three genetic crosses (+/+ × +/+, +/+ × *Sp*/+, and *Sp*/+ × +/+) examined in this study. "An/microphthalmia" incorporates all fetuses with left, right, and/or bilateral anophthalmia and/or microphthalmia.

With the exception of spina bifida aperta, the congenital malformations observed in this study were not increased by Pax3 haploinsufficiency indicated by the presence of a fetal *Sp* allele. However, for each of the malformations presented in Table 2, again with the exception of spina bifida aperta, there is a substantial, though not always statistically significant, increase in litters from ALX-treated *Sp*/+ dams (*Sp*/+ × +/+). Thus, there appears to be a maternal affect of the *Sp* allele on the developing conceptus. Although the initial report of murine *Pax3* expression found transcripts only in embryos (61), more recent studies in humans (62) have demonstrated *PAX3* mRNA to be present in every adult tissue tested, with particularly high expression in striated and cardiac muscle tissue.

The lack of a sex difference in diabetes-induced exencephaly is noteworthy, as uneven distribution between the sexes is a common hallmark of NTDs. Anencephaly in humans, for example, is more frequently observed in females (63). Likewise, spontaneous exencephaly in certain mouse strains and exencephaly secondary to gene inactivation both occur more frequently in females when one sex predominates (20,51). Finally, murine exencephaly produced by both hyperthermia (64) and arsenite (25) has been reported to occur more often in females. The observation in the present study that exencephaly was evenly distributed between male and female fetuses could support the concept that maternal diabetes produces exencephaly by a different mechanism than any of the above-noted physical, chemical, or genetic means. Alternatively, and perhaps more likely, if the skewing of the sex ratio of live fetuses toward the male (Table 3) was accomplished via the selective resorption of malformed female fetuses, this phenomenon may have obscured a predominance of exencephalic female fetuses. Studies of diabetic embryopathy in animal models typically do not address the sex

ratio of either normal or malformed fetuses. Likewise, epidemiological studies that address diabetes-induced birth defects do not mention sex of either normal or malformed infants (1,9). The finding that the fetal sex ratio is altered in this murine model of diabetic embryopathy bears further investigation as to causation and whether this phenomenon also occurs in humans.

Although several previous studies have demonstrated congenital malformations secondary to chemically induced maternal diabetes, in general, the correlation between the degree of maternal hyperglycemia and the rate or severity of teratogenesis has been poor (47). However, few if any studies of chemically induced diabetes have produced both the frequency of congenital defects and the extreme range of maternal glucose levels seen in the present investigation (47). Here, resorptions and the two most common single malformations, exencephaly and eye defects, occurred with sufficient frequency to demonstrate a statistically significant positive correlation with maternal hyperglycemia. It should be noted that maternal plasma glucose values were obtained 3 days after ALX administration and at gestational day 18. In almost all cases, plasma glucose increased during this time, but the precise levels during organogenesis are unknown, and it is not possible to say whether glucose levels increased linearly over time or in some other fashion. Additionally, the elapsed time between the first and second plasma glucose measurement varied depending on how long the animal took to become pregnant. Thus, the day-18 values are consistently presented and were used for all computations. Finally, the correlation noted here does not serve to argue that maternal hyperglycemia, in and of itself, is responsible for teratogenesis but merely that in this study, the level of hyperglycemia was a significantly reliable indicator of the teratogenic potential of maternal diabetes. It should be noted that variability in plasma glucose levels was possibly exacerbated by the fact that animals were not fasted before orbital-sinus bleeding for plasma glucose determination.

By producing a murine model that is genetically sensitive to diabetes-induced spina bifida aperta, the present study demonstrates a gene/teratogen interaction between *Pax3* in a haploinsufficient state and maternal type 1 diabetes. Most studies of congenital lumbosacral defects resulting from maternal diabetes have evaluated relatively minor malformations, such as spina bifida occulta and ossification defects (65), due to the rarity of open spinal NTDs in rodents, particularly spina bifida aperta. The *Pax3*-haploinsufficiency model presented here may prove valuable in the effort to elucidate molecular mechanisms underlying caudal NTDs secondary to maternal diabetes, as well as in studying the phenomenon of haploinsufficiency.

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