Splotch locus mouse mutants: models for neural tube defects and Waardenburg syndrome type I in humans

Connie E Moase, Daphne G Trasler

The majority of data that contribute to our understanding of mammalian embryonic development are obtained from the study of animal models. An increasing availability of molecular tools to dissect these model systems has enabled us to establish parallels with human development that range from morphogenetic similarities to homologous DNA sequences. The mouse mutant splotch (Sp) has long been recognised as a model for human neural tube defects (NTDs) and, more recently, has become a candidate model for Waardenburg syndrome type I (WSI) in humans.12 This latter circumstance is based on similarities in some of the defects in neural crest cell (NCC) derivatives that are shared by Sp mutants and Waardenburg patients, as well as the possibility that WSI may be localised to human chromosome 2q37, a region known to share homologies with mouse chromosome 1 where the Sp locus is found.

Neurulation occurs between gestation days 8-5 and late day 9 in the mouse (plug day = day 0) and this is comparable to days 20 to 26 of human embryonic development.3 Neural crest cells originate from the neural tube and begin to emigrate just before cephalic neural tube closure, or shortly after trunk neurulation in mammalian embryos. Thus, the two events occur almost simultaneously in development. NCCs then migrate to various regions of the body and differentiate into a variety of structures including neuronal cells of the sensory and autonomic ganglia, nerve supporting structures, mesoectodermal structures, certain cells of the endocrine system, and melanocytes.43 The fact that both neurulation and NCC emigration are disrupted in the Sp mutant makes this a useful model for understanding the basis of two fundamental developmental processes.

This article reviews the results of investigations that have examined splotch locus mutants. These studies include histological analyses at the structural and ultrastructural levels, gene-teratogen interactions, as well as outcomes from immunohistochemical, biochemical, cellular, and molecular approaches. Information gathered from the analyses of these mutants has contributed to our understanding of neurulation and neural crest cell emigration, and has provided clues as to how these two fundamental processes may be developmentally related.

Splotch locus alleles

ORIGIN OF SPLOTCH LOCUS MUTANTS

Splotch locus mutants include several allelic variants that serve as mouse models for both NTDs and deficiencies in NCC derivatives. The original splotch mutation arose spontaneously on a C57BL inbred background and was first described by Russell.3 Since then, Dickie7 reported other de novo splotch mutations (Sp1, Sp2, and Sp3), as well as the appearance of an allele splotch delayed (Spδ). More recently, Beechey and Searle6 described three radiation induced mutations at this locus, two of which appeared to be the same as the original Sp mutation (Sp1H and Sp2H), and a third was referred to as splotch retarded (SpR), an allele of Sp and Spδ.

Early linkage analysis showed Sp belonged to linkage group XIII and was positioned between the coat colour mutation leaden (In) and the hair follicle mutation fuzzy (Fz).9,10 Recombination frequencies between Sp and either In or Fz were found to be similar to those of Sp1, supporting the idea that Spδ was indeed allelic to Sp. Since then, the splotch locus has been mapped more specifically to band C4 of chromosome 111 and investigations to define this locus further using DNA probes are currently in progress.

HOMOZYGOUS FEATURES

Mutations at the Sp locus are classified as semidominant lethal,7 with differences in homozygous phenotype being the factor that distinguishes between splotch and splotch delayed. Most Sp homozygotes develop spina bifida (lumbosacral rachischisis) and over half exhibit exencephaly (craniocchisis) as well, owing to lack of closure in the hindbrain region.11 On occasion, these mutants may develop only a tail flexion defect that results in a curly tail.13 However, all mutants die in utero at approximately 13 or 14 days of gestation.12 This is in contrast to the SpR mutant which develops only spina bifida and survives until birth, hence undergoing delayed death.7

Unlike Sp and Spδ, splotch retarded homozygotes are presumed to die before implantation.8 This mutation is more severe than Sp or Spδ as it involves a cytogenetically detectable deletion of the 1C4 band, which constitutes approximately 2% of the total physical length of chromosome 1.11
HETEROZYGOUS PHENOTYPE

Sp, Sp', and Sp' heterozygotes display a similar phenotype that is characterised by a white belly patch, feet, and tail tip, although Sp' heterozygotes occasionally lack the ventral spotting. This pigmentation defect results from the failure of neural crest cells to populate these regions sufficiently during development.12 Other NCC derived structures have been shown to be deficient or absent in both Sp and Sp' mutants as well. These include spinal ganglia,13 Schwann cells,14 and NCC derived structures of the heart.15,16 What distinguishes the Sp' heterozygote from Sp and Sp' gene carriers is the fact that it experiences an overall growth retardation which persists throughout adulthood.17 However, because this particular mutant has only recently become available, it has not been characterised as extensively as Sp or Sp'.

PHENOTYPIC VARIATIONS

Variations in expressivity have been obtained by outcrossing Sp or Sp' gene carriers to mice with different genetic backgrounds. Using Sp' on a heterogeneous BALB/c stock, Kalter18 showed that each of three teratogens known to cause exencephaly could do so at a higher frequency in Sp' homozygotes than in non-mutant litter mates. Thus, even though Sp' homozygotes do not normally express exencephaly, they appear to be liable to such an occurrence. This was further supported by the observation that exencephaly occurred at a frequency of 6-1% in day 16 Sp' homozygous embryos from F1 mice that had originated by outcrossing Sp' inbreds to an In(1)1Rk stock.19 Outcrossing Sp inbreds to In(1)1Rk mice resulted in an altered distribution frequency of NTDs in Sp homozygous embryos as well. The proportions of NTDs changed from 56% of mutants having exencephaly in addition to spina bifida12 to 100% of mutant embryos having exencephaly, with spina bifida occurring only about 25% of the time.19 In addition, Sp mutants on this background can survive longer than the usual 13 to 14 days of gestation in an inbred line, with viable embryos being present up to day 18 of gestation.19

Splotch as a model for human NTDs

In humans, NTDs are primarily the result of interactions between genetic and environmental factors. The cellular and molecular mechanisms of neuralisation have yet to be fully understood. However, one approach towards this end is to examine abnormal neural tube closure in genetic models where environmental factors can be manipulated. Various NTD mouse mutants such as loop-tail,20,21 crooked,22 rib-fusion,23 and extra-toes,24,25 express additional gross anomalies in unrelated organ systems, thus complicating the analysis of NTD pathogenesis. Another model, the curly-tail mutant,26 is a recessive mutation with incomplete penetrance that has yet to be assigned to a particular chromosome. This, in addition to the fact that only about 60% of all curly-tail embryos develop an NTD, makes it difficult to carry out investigations on abnormal neural tube closure until the development of an NTD is well under way.

In the case of Sp, however, only the skull and vertebral malformations that are associated with an open neural tube are present in conjunction with the defects in certain NCC derivatives, which also originate from the neural tube. Therefore, it is possible that a mechanism directly involved in neuralisation is the primary target of the Sp mutation. This, combined with the fact that Sp mutants can be identified before the manifestation of an NTD,19,20 make it a good model for understanding abnormal neural tube closure.

PATHOGENESIS OF SPLOTCH MUTANT DEFECTS

Some of the first studies involving the splotch mutant examined the pathology of the abnormally developed neural epithelium. Hsu and Van Dyke,29 as well as Auerbach,30 reported an extensive overgrowth of neural tissue in open regions of the neural tube. Hsu and Van Dyke29 attributed this to an increase in mitotic activity between 13-5 and 14-5 days of gestation. Using tritiated thymidine incorporation, Wilson31 showed that mesencephalic cells of day 10 and 11 Sp embryos undergo a longer cell cycle than that found in heterozygous or wild type embryos, and that proliferation is actually decreased in these mutants. However, through analysis of younger embryos, Kapron-Bras and Trasler32 found that the mitotic index was similar between all regions of day 9 Sp and control embryos. Thus, any difference in the mitotic index between mutants and non-mutants probably reflects secondary changes following the initial disruption of neural tube closure.

Other histological features of abnormally developed Sp or Sp' mutants include neural tube irregularities throughout the embryo, abnormal tail morphology, distortions of the brain lumen11,12 with significantly reduced lumen size,12 abnormal otic vesicle differentiation in those with exencephaly,13 disorganised neuro-epithelial tissue with more intercellular space,14 and significant reductions in the area of the neuro-epithelium as well as the forebrain,32 and reduced or absent neural crest cell derivatives.12,13 However, because these observations were from embryos that already exhibited the defect, it was impossible to determine whether they were primary or secondary effects of the mutation. In order to understand the aetiology of Sp locus mutations, it is necessary to examine embryos before the appearance of the defect.

AETIOLOGY OF SPLOTCH NTDs

Neuropore measurements of embryos derived from intercrosses of Sp or Sp' heterozygotes show that presumptive homozygous mutants have longer posterior neuropores than their non-mutant heterozygous and wild type litter.
in addition, presumptive Sp mutants have longer anterior neuropores.\(^{35,36}\) This delay in neuropore closure was postulated to be the underlying factor that predisposed Sp gene carriers (that is, heterozygotes), which are morphologically normal, to develop NTDs upon exposure to a teratogenic agent\(^{35,36}\) (see section on Splotch and retinoic acid, below). These results were later corroborated by using embryos that were positively identified (see section on Chromosomal markers, below) as Sp gene carriers.\(^{38}\) Similarly, this delay in posterior neuropore closure has been shown to occur in the curly-tail mutant discussed previously.\(^{39}\) However, the mechanism responsible for this delay in closure in any of these mutants has yet to be elucidated.

**Markers for splotch locus mutants**

**GRAFTED EMBRYONIC ECTODERM**

As mentioned above, differentiation between mutant and non-mutant embryos before the expression of a malformation is necessary in order to identify potential primary causal factors of abnormal development. One method of distinguishing Sp mutants from their heterozygous and wild type litter mates involves isolating dorsal ectodermal tissue together with its underlying mesoderm from day 9 embryos, and implanting it into either embryonic chick coelom, or the anterior eye chamber of adult albino mice.\(^{12}\) Grafted ectoderm from Sp homozygotes fails to generate pigment after 16 days, whereas tissues from heterozygous and wild type embryos produce pigmentation. Using this technique to identify day 9 Sp mutant embryos, Wilson and Finta\(^ {40}\) showed the frequent occurrence of gap junctional vesicles in the lumbosacral region of the neural groove that subsequently fails to close. Gap junctional vesicles, which are normally abundant in C57B1/6J embryos a day earlier in gestation,\(^ {41}\) are suggested to represent a breakdown of gap junctions, and thus may be an indicator of decreased cell–cell communication in the Sp mutant at this stage of development.

**CHROMOSOMAL MARKERS**

**Robertsonian translocation Rb(1.3)1Bnr**

Although Sp mutants can be identified by either skin grafts or the length of their neuropores relative to non-mutant embryos at the same developmental stage, it is not possible to distinguish between heterozygous and wild type embryos by these methods. Thus, it was necessary to develop a more specific marker. Kapron-Brás et al.\(^ {42}\) devised a breeding scheme that introduced a 1.3 Robertsonian translocation (a fusion of chromosomes 1 and 3) into the Sp line. This could then be used as a marker for the chromosome carrying the wild type allele. However, using this system to predict the genotype provided 80% reliability in heterozygotes, but only 60% accuracy in homozygotes owing to cross over. Therefore, a more dependable genotyping strategy was required.

**Inversion In(1)1Rk**

A more reliable marker was established using the In(1)1Rk mouse line which is homozygous for a paracentric inversion that spans the Sp locus. Using an appropriate breeding design (fig 1), Moase and Trasler\(^ {43}\) showed that the accuracy of this marker was greater than 98% owing to recombination suppression by the inversion. Because this inverted segment encompasses certain biochemical loci that differ in isotype from the splotch line,\(^ {43}\) and also includes 42% of the total length of chromosome 1,\(^ {44}\) the genotype of each individual embryo can be established by one of two methods. Individual embryos at day 11 of gestation or older can be genotyped on the basis of their isocitrate dehydrogenase profile\(^ {45}\) (fig 2), while younger embryos can be identified using cytogenetic analysis\(^ {46}\) (fig 3). Until the Sp locus or closely linked markers are identified, this marking scheme involving the In(1)1Rk inversion is the most reliable method to date for differentiating between mutant, heterozygous, and wild type embryos.

One aspect that is revealed with the use of this marker is the fact that 4% of day 16 Sp\(^ {47}\) mice, and 9% of day 9 Sp mice which exhibit an NTD are actually heterozygotes\(^ {48}\) (unpublished observations). Using this system, however, it is not possible to determine whether NTDs are found in inbred heterozygotes as well, or only when these mice are outcrossed to the In(1)1Rk stock.

**Gene-teratogen interactions**

Numerous studies have examined the effect of teratogens on mice that are carriers of a mutant gene. Embryos heterozygous for a recessive or semidominant mutant gene often express the homozygous phenotype when exposed to a specific teratogen. This positive effect may be indicative of possible shared mechanisms.
through which mutant genes and teratogens exert their effects. Cole and Trasler showed that progeny obtained from a cross of either crooked or rib-fusion heterozygotes to non-mutant strains were more susceptible to NTDs after in utero exposure to teratogenic doses of insulin than were progeny from intercrosses between non-mutants. Other examples of positive interactions include 5-fluorouracil with the limb mutants luxoid and luxate, trypan blue and NTDs in brachyphalangy carriers, trypan blue or actinomycin D with tail malformations in brachyury gene carriers, and 6-aminonicotinamide with cleft lip in dancrer heterozygotes.

**SPLOTCH AND RETINOIC ACID (RA)**

**RA induction of NTDs**

Retinoic acid (RA), a vitamin A analogue, has been extensively studied with respect to its interaction with splotch and splotch-delayed. A positive gene-teratogen interaction has been shown for carriers of the Sp gene in vivo as well as in vitro using the whole embryo culture system. However, no positive interaction with retinoic acid was observed for carriers of Sp. In an extensive histological analysis to determine whether Sp and retinoic acid were acting through the same mechanism to produce NTDs, Kapron-Brais and Trasler showed that the Sp gene is associated with reducing both the number of NCCs released from the neural tube, and the amount of extracellular space surrounding the neural tube, including the area between the neural tube and surface ectoderm. On the other hand, retinoic acid caused disruptions in the spatial relationship between the notochord and neural tube, as well as in the shape of the neural tube. Thus, retinoic acid and the Sp gene affect different aspects of neurulation, but combine to induce NTDs in a greater degree than either factor by itself.

![Figure 2](image-url)  
*Figure 2* Isoeitrate dehydrogenase profiles from individual day 11 Sp/Sp, Sp+ and +/+ embryos. The slow migrating band (aa) in lane 1 is from a Sp homozygote, lane 2 is heterozygous for Idh-1* and Idh-1* (ab) isotypes and is from a Sp heterozygote, lane 3 contains the fast migrating variant (bb) and is from a +/+ embryo. (0 = origin, from Moase and Trasler.)

**RA reduction of NTDs**

Although treatment of non-mutant embryos with 30 to 60 mg/kg of retinoic acid on day 8 of gestation causes NTDs, it was also noted that, as with the curly-tail mutant, low retinoic acid doses of 5 mg/kg administered one day later in gestation (day 9) significantly reduces the incidence of NTDs in litters from Sp heterozygote intercrosses, without increasing the resorption frequency. When this study was repeated using genotypically identifiable Sp embryos, it was found that the reduction in NTD frequency was actually attributable to retinoic acid induced selective mortality of mutant embryos, and that this induced mortality was not enough to increase the resorption frequency significantly. In genotypically identifiable Sp embryos, the effect was somewhat different. Rather than reducing the NTD frequency, these low RA doses appeared to induce NTDs in day 11 heterozygotes. However, there was a higher incidence of developmentally retarded, malformed embryos in the treated group, suggesting that even a low dose of RA was indeed detrimental to embryonic development and viability.

Studies involving the curly-tail mutant have shown that administration of the DNA inhibitor mitomycin C or maternal food deprivation during embryonic neurulation, significantly reduces the NTD frequency without increasing the resorption frequency. Although the effect of DNA inhibitors has not been tested in Sp locus mutants, a pilot study by Mehin and Trasler indicates that food deprivation does not reduce the overall NTD frequency in litters obtained from Sp/+ intercrosses. This, in conjunction with other differences, indicates that Sp and curly-tail mutants do not share a common aetiological basis for NTDs other than the fact that both experience delays in neural tube closure.

![Figure 3](image-url)  
*Figure 3* A chromosomally spread from a cytogenetically marked day 9 Sp heterozygous embryo. G banding shows the pattern associated with the chromosome 1 that carries the Sp'/or Sp allele (a), and the chromosome 1 derived from the In(1)IRk line which carries the inversion and, hence, the told type allele (b). (From Moase and Trasler.)

**Splotch extracellular matrix components**

According to histological observations, the closed neural tube in the trunk region of Sp/Sp
embryos is in close apposition to the overlying ectoderm, and, unlike control sections, has very little extracellular space between the two tissues. Because fewer NCCs were observed in these regions compared to controls, it was suggested that the lack of extracellular space could be due to alterations in extracellular matrix components and that this, in turn, may inhibit NCC emigration from the neural tube.

Several immunohistochemical and ultrastructural analyses have focused on the temporal and spatial localisation of specific extracellular matrix constituents in Sp and Sp<sup>d</sup> mutants. Using late day 9 or day 11 Sp<sup>d</sup> embryos that exhibited the mutant phenotype, O’Shea and Liu<sup>35</sup> found that the basal lamina of the neuroepithelium was disorganised, and that there was less laminin and collagen IV, and more fibronectin and heparan sulphate proteoglycan (HSPG) in the dorsolateral region of the neural tube compared to controls. Fibronectin and HSPG were also found to be displaced in the mutants during secondary neurulation. McLone and Knepper<sup>36</sup> also observed alterations in certain glycolconjugates and glycosaminoglycans in day 10 Sp mutants. Using enzymatic digestion to quantitate particular extracellular matrix components, these authors reported that the distribution of hyaluronic acid (HA) and chondroitin sulphate (CSPG) was approximately equal in mutant neuroepithelium, whereas in normal embryos HA is predominant in the open neural tube and CSPG is predominant in the closed neural tube. McLone and Knepper<sup>36</sup> also found differences in lectin staining patterns between Sp mutants and controls, with a persistent and intense concanavalin-A staining on the abnormal luminal surface.

Other studies have examined Sp and Sp<sup>d</sup> mutant embryos at slightly earlier stages of development in order to assess extracellular matrix aspects before the abnormal phenotype is apparent. These embryos were identified as mutant by the In(1)Rk marker. Trasler and Morriss-Kay<sup>37</sup> observed greater amounts of both CSPG and HSPG antibody fluorescence in the neuroepithelial basement membrane of cranial and caudal sections from 5 to 15 somite stage Sp and Sp<sup>d</sup> mutant embryos compared to non-mutants. In an extensive ultrastructural and morphometric analysis of caudal Sp<sup>d</sup> mutant sections at 15 to 18 somite stage of development, Yang and Trasler<sup>38</sup> observed that the basal lamina of the overlying surface ectoderm was not as well formed as that of controls, that there were fewer mesodermal cells in mutant embryos, and that the neuroepithelium was disorganised and contained a greater amount of intercellular space. This latter observation corroborated similar findings in slightly older Sp mutants.<sup>34</sup> Furthermore, it showed that these morphogenetic differences were present even before the defects become apparent, suggesting that one of the primary effects of the mutant gene is to elicit these changes in the neuroepithelium. In contrast, Yang and Trasler<sup>38</sup> did not observe the significant differences in gap junctional vesicle formation between Sp<sup>d</sup> mutants and controls that were reported by Wilson and Finta<sup>40</sup> from their examination of early day 9 Sp mutants. Sp mutants have been shown to be more severely affected than Sp<sup>d</sup> mutants in other respects, such as viability, type of NTD, and reductions in spinal ganglia and emigrating NCCs. Thus, it is not surprising that differences in numbers of gap junctional vesicles are observed between Sp and Sp<sup>d</sup> as well.

**In vitro analysis of splotch NCCs**

Although NTDs occur in most embryos that are homozygous for Sp or Sp<sup>d</sup>, genotypic analysis has shown that 11% of mutant embryos do not exhibit such a defect. However, histological examination showed that these particular embryos did have severe reductions or absence of NCC derived spinal ganglia.<sup>14</sup> This indicated that defects observed in NCC derivatives are not the result of NTDs in these mutants. In fact, morphometric analysis showed that even some heterozygous embryos have significantly reduced volumes of spinal ganglia compared to wild type embryos, but presumably this deficiency is not detrimental to the survival of heterozygotes. Thus, since one defect was not contingent on the others’ occurrence, it was possible that neural tube closure and the release of NCCs from the neural tube may instead share a regulatory event that, if disrupted, could result in the defects seen in both developmental pathways. Kapron-Brás and Trasler<sup>39</sup> have shown that few NCCs are released from mutant neural tubes. However, it was unclear as to whether this occurred as a result of an abnormality in the neuroepithelium, the ECM into which NCCs emigrate, or whether the NCCs themselves were at fault. Examination of NCC emigration from explanted neural tubes<sup>38</sup> showed that there was approximately a 24 hour delay in the release of NCCs from mutant neural tubes compared to non-mutants, with Sp being more severely affected than Sp<sup>d</sup>. Provision of an enriched three dimensional extracellular matrix containing laminin, collagen IV, HSPG, and enactin failed to enhance NCC release significantly, and assessment of mitotic indices showed no difference in NCC proliferation between mutant and non-mutant genotypes. Therefore, these findings indicated that the neuroepithelium from which NCCs arise may be faulty in the Sp mutant with respect to the mechanism involved in the release of NCCs.<sup>38</sup>

**Cell adhesion molecules (CAMs) in splotch**

Since specific cell adhesion molecules are known to be involved in neural development, it is of interest to examine some of these with respect to Sp. Using immunofluorescent techniques, Moase and Trasler<sup>39</sup> found that neuroepithelial tissue in sections from mutant embryos fluoresced with greater intensity in response to antibodies against the neural cell adhesion molecule N-CAM than did control sections. Further analysis by immunoblotting
showed that both Sp and Sp\(^{-}\) mutants, as well as day 9 heterozygous embryos, exhibited altered N-CAM profiles compared to wild type embryos. These alterations may involve a change in the conversion of high molecular weight N-CAM to lower molecular weight forms, similar to the observation in another neurological mutant, staggerer.\(^{60}\) However, in the case of splotch, this difference is apparent early in development rather than postnatally. N-CAM is coded by a gene localised to chromosome 9 in mice. Therefore, this alteration in splotch probably relates to regulation or post-translational processing of the N-CAM protein, which may or may not be directly influenced by the Sp locus on chromosome 1. In view of the fact that N-CAM copurifies with certain ECM components, including HSPG and CSGP,\(^{61,62}\) which appear necessary for N-CAM mediated cell adhesion, it is possible that the altered N-CAM could bring about the cascade of abnormalities observed in Sp locus mutants. However, further investigation is necessary to substantiate this idea.

**Present and future directions**

In addition to investigations that analyse Sp locus mutations at the cellular level, progress is also being made towards defining this locus at the molecular level. Carriers of the Sp\(^{-}\) allele are useful in this respect owing to the large deleted segment at this locus. Four genes which fall within this deleted region have been identified;\(^{63}\) however, these markers are present in the other allelic mutants. Thus, it is necessary to find more markers and to establish a multilocus linkage map of this region in order to ascertain homologous sequences between mouse and man. Foy et al.\(^{64}\) have tentatively established linkage between Waardenburg syndrome type I and placental alkaline phosphatase, which is localised to human chromosome 2q37. These findings have been corroborated by another group that examined a single large WSI pedigree.\(^{2}\) This is interesting in view of the fact that chromosome 2q37 is known to share homologies with the region containing the splotch locus on mouse chromosome 1. The fact that phenotypic similarities occur in splotch mice and WSI patients (that is, pigmentation disturbances and occasional NTDs in WSI subjects\(^{65,66}\) suggests that Waardenburg syndrome may be the human counterpart to the mouse mutant splotch. In addition, continuing studies involving molecular analysis of the Sp locus may eventually show mouse-human homologies in DNA sequences that are associated with the development of NTDs.

**Note added in proof**

Recently Pax-3, a murine DNA binding protein expressed during early neurogenesis was mapped to mouse chromosome 1.\(^{70}\) Pax-3 was found to be deleted in Sp\(^{P}\) mice and the Sp\(^{PP}\) allele was shown to have a 32 nucleotide deletion within the paired homeo domain coding portion of Pax-3.\(^{71}\) Therefore, Pax-3 must have a key role in neurulation. Further, Pax-3 is the mouse homologue of the human HuP2 gene,\(^{72}\) which suggests the latter may map near 2q37 and may be altered in WSI.

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31. Kapron-Braïs CM, Trasilog D. Histological comparison of the effects of the splotch gene and retinoic acid on the
Splotch locus mouse mutants