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Interaction between *splotch* (*Sp*) and *curly tail* (*ct*) mouse mutants in the embryonic development of neural tube defects

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SUMMARY

The mouse mutations *splotch* (*Sp*) and *curly tail* (*ct*) both produce spinal neural tube defects with closely similar morphology, but achieve this by different embryonic mechanisms. To determine whether the mutants may interact during development, we constructed mice carrying both mutations. Double heterozygotes exhibited tail defects in 10% of cases, although the single heterozygotes do not express this phenotype. Backcrosses of double heterozygotes to *ct/ct* produced offspring with an elevated incidence of neural tube defects, both spina bifida and tail defects, compared with a control backcross in which *Sp* was not involved. Use of the deletion allele Sp^{2H} permitted embryos carrying a *splotch* mutation to be recognised by polymerase chain reaction assay. This experiment showed that only embryos carrying Sp^{2H} develop spina bifida in the backcross with *ct/ct*, suggesting that the genotype Sp^{2H}/+, *ct/ct* is usually lethal around the time of birth as a result of severe disturbance of neurulation. The interaction between *Sp* and *ct* was investigated further by examining embryos in the backcross for developmental markers of the *Sp/Sp* and *ct/ct* genotypes. *Sp/Sp* embryos characteristically lack neural crest derivatives, such as dorsal root ganglia, and die on day 13 of gestation. Double mutant embryos from the backcross did not exhibit either of these characteristics suggesting that homozygosity for *ct* does not cause *Sp*/+ embryos to develop as if they were of genotype *Sp/Sp*. The angle of ventral curvature of the posterior neuropore region is enhanced in affected *ct/ct* embryos whereas it was found to be reduced in *Sp/Sp* embryos compared with their normal littermates. Double mutant embryos from the backcross had an angle of curvature that resembled the *ct/ct* pattern but was less exaggerated. We conclude that the non-allelic mutations *Sp* and *ct* interact to promote the development of neural tube defects in a manner that does not involve exacerbation of the specific developmental effects of either gene. The presence of enhanced curvature of the caudal region, which is responsible for neurulation disturbance in *ct/ct* embryos, summates with the reduced neurulation potential of the neuroepithelium in the *Sp*/+ genotype leading to the development of severe spina bifida. This study demonstrates that the finding of a significant interaction between genes in double mutant mice cannot be assumed to indicate that the genes operate in the same genetic pathway.

Key words: mouse, embryo, neurulation, neural tube defects, neural crest, mutation, congenital malformation

INTRODUCTION

The construction of double mutant individuals is a powerful technique for analysing complex developmental pathways that involve the coordinated expression of several genes. In *Drosophila*, double mutant flies have provided information on gene interactions during segmentation of the early embryo (Ingham, 1983; Coulter and Wieschaus, 1988; Ingham et al., 1991) and during nervous system formation (Elkins et al., 1990). In *Caenorhabditis*, double mutants have been instrumental in elucidating the genetic pathways that control vulval development (Sternberg and Horvitz, 1991) and programmed cell death (Ellis and Horvitz, 1986). The double mutant approach has also been used in the mouse for identifying interactions between genes that control the development of the pigmentary system (Rittenhouse, 1968; Wolff et al., 1978; Lamoreux and Russell, 1979; Hirobe, 1991), the eye (Konyukhov and Sazhina, 1983, 1985; Sanyal, 1987), myelination in the central nervous system (Billings-Gagliardi et al., 1990; Sinclair et al., 1991) and the immune system (Scribner et al., 1987). Mice carrying two distinct transgenes have been constructed in a study of the synergistic effects of oncogenes (Sinn et al., 1987). To date, however, early embryogenesis in mammals has rarely been subjected to double mutant analysis.

Neurulation, the process by which the neural plate folds to

form the neural tube in the early embryo, would appear to be an ideal system in which to perform double mutant studies in the mouse. A number of mutations have been described that disrupt the process of neurulation, leading to neural tube defect malformations, including spina bifida and exencephaly (Copp et al., 1990). The presence of so many mutations with similar phenotypes suggests that the coordinated expression of a number of genes may be required for neural tube closure to be completed at all levels of the body axis.

Here, we describe the construction of double mutant mice that carry the mutations *splotch* (*Sp*) and *curly tail* (*ct*), both of which cause neural tube defects. Homozygous *Sp/Sp* embryos develop lumbosacral spina bifida and 50% also have mid/hindbrain exencephaly (Auerbach, 1954). They die on day 13 of gestation, probably as a result of heart malformations that include persistent truncus arteriosus (Franz, 1989). *Splotch* heterozygotes are viable and undergo normal neural tube closure; they usually develop a white belly spot as a result of defective neural crest cell migration. Homozygosity for *ct* produces exencephaly in 2-5% of offspring and spinal neural tube defects in 50-60% of cases; the spinal defects comprise lumbosacral spina bifida (5-20% of offspring) and tail flexion defects (40% of offspring; Gruneberg, 1954; Embury et al., 1979; Copp et al., 1982). Curly tail heterozygotes are morphologically normal.

These mutations were chosen because, although they cause superficially similar neural tube defects, they probably act by quite different embryonic mechanisms. The defect in *Sp* appears to affect the neuroepithelium primarily: neural crest cell migration is defective (Auerbach, 1954) and expression of Pax-3 mRNA, which has been shown to be encoded by the *Sp* locus (Epstein et al., 1991), is first detected during development in the neuroepithelium (Goulding et al., 1991). The mechanism by which neurulation fails in *Sp/Sp* embryos has not yet been determined. The basic defect in the *ct* mutant, in contrast to *Sp*, affects non-neural tissues with the neuroepithelium appearing unaffected (van Straaten et al., 1993). Neural tube defects in *ct/ct* embryos result from an abnormally slow rate of proliferation of the notochord and hindgut endoderm (Copp et al., 1988a), which, due to the tethering of these tissues to the overlying neuroepithelium, produces curvature of the caudal region (Brook et al., 1991). Curvature inhibits closure of the posterior neuropore and produces neural tube defect phenotypes ranging from spina bifida to tail defects, depending on the severity of the delay in neuropore closure (Copp, 1985).

In this paper, we show that the *Sp* and *ct* mutations interact in double heterozygotes and that mice with genotype *Sp/+, ct/ct* develop severe neural tube defects which are often lethal around the time of birth. Examination of the double mutant embryos suggests that the interaction represents a summation of the developmental effects of both mutants without exacerbation of the specific action of either mutant.

MATERIALS AND METHODS

Mice

Sp and Sp^{2H} mutant mice were obtained from the MRC Radiobiology Unit, Harwell, UK, and were maintained as heterozygotes in closed, random-bred colonies. The *ct* mutation has been maintained for a number of years in our laboratory as a random-bred colony of homozygous individuals (see Brook et al., 1991, for details).

Production of double mutant mice

To generate doubly heterozygous mice, four randomly selected *ct/ct* males were each mated with two *Sp/+* females and, conversely, four *Sp/+* males were each mated with two *ct/ct* females. Each of the 16 females was allowed to have two to four litters and the F_1 offspring were examined at birth for spina bifida and tail defects and at weaning for a white belly spot, as an indication of a mutant *splotch* allele. Analogous matings were performed using the Sp^{2H} mutant. Backcrosses were performed between F_1 (*Sp/+, ct/+*) mice and *ct/ct* mice (experimental backcross) and between F_1 (*+/+, ct/+*) mice and *ct/ct* mice (control backcross). The backcross series were divided equally between matings in which the F_1 mouse was the male parent and matings in which the *ct/ct* mouse was the male parent. Backcross matings each yielded six litters on average, and the offspring were examined as described above for the initial cross.

Analysis of backcross embryos

F_1 (*Sp/+, ct/+*) and F_1 (*+/+, ct/+*) males were each mated overnight with *ct/ct* females, which were considered 0.5 days pregnant if a copulation plug was found the following morning. Pregnant females were killed by cervical dislocation at 11.5, 14.5 and 18.5 days gestation and their embryos were explanted into PB1 medium (Cockroft, 1990) containing 10% fetal calf serum. Embryos were dissected free from their extraembryonic membranes and were examined for gross morphological defects. An analogous experiment was performed using the Sp^{2H} allele. In this case, pregnant females were killed at 11.5 days gestation and the yolk sacs were collected for polymerase chain reaction (PCR) analysis (see below). The embryos were inspected for defects, fixed in Bouin's fluid for at least 24 hours and embedded in paraffin wax. Serial sections, thickness 6 μm, were cut transversely at the level of the hindlimb bud and stained with haematoxylin and eosin, for examination of the morphology of the dorsal root ganglia.

Determination of Sp^{2H} genotype by PCR

Yolk sacs, collected from 11.5 day Sp^{2H} backcross embryos, were washed thoroughly in phosphate-buffered saline, pH 7.4 and DNA was isolated by the method of Estibeiro et al. (1990). PCR was performed in a total volume of 25 μl using primers b and c as described by Epstein et al. (1991). Conditions for the PCR reaction were as described (Epstein et al., 1991). PCR products were separated on 4% agarose gels in TBE buffer (0.045 M Tris, 0.045 M boric acid, 0.001 M EDTA, pH 8.3) by electrophoresis at 100 V for 1.5-2.5 hours. Bands were visualised by ethidium bromide staining. Wild-type alleles at the *splotch* locus yielded a single band of 127 bp whereas the Sp^{2H} deletion allele yielded a single 95 bp band.

Measurement of ventral curvature of the caudal embryonic region

Embryos were removed from the uterus at 10.5 days gestation, somites were counted and embryos with 27-29 somites were selected for analysis. These were examined for size of posterior neuropore using criteria described previously (Copp, 1985). Embryos with normal sized or severely enlarged neuropores were analysed further whereas those with intermediate sized neuropores were discarded. Angle of curvature was measured from camera lucida drawings of profile views of the caudal embryonic region, as described previously (Brook et al., 1991).

Statistical analysis

The incidence of embryonic defects and resorptions was compared between the different mating types using -squared tests, with

Yates correction where appropriate. Angles of curvature were compared between normal and abnormal embryos, separately for 27, 28 and 29 somite groups, by analysis of variance (general linear model) using the Minitab statistical computer package.

RESULTS

Matings between *splotch* and *curly-tail* mice

(1) Production of double heterozygotes

In an initial cross, *splotch* heterozygotes (*Sp/+*) were mated with homozygous *curly-tail* (*ct/ct*) mice, as shown in Fig. 1. Two sets of reciprocal crosses were performed to test for possible effects of genetic imprinting: there were no significant differences in the results obtained from the two types of intercross, so the data were combined for subsequent analysis (Table 1). As expected, approximately half of the F_1 offspring carried *splotch* (i.e. *Sp/+, ct/+*). Of these doubly heterozygous mice, 10% had tail flexion defects similar to those seen in *ct/ct* homozygotes (Table 1). By contrast, the F_1 offspring that did not carry *splotch* (i.e. *+/+, ct/+*) were always straight-tailed. This result is not due to allelism of the two mutations: *Sp* is known to map to the proximal part of chromosome 1 (Seldin et al., 1991) whereas a recent study has located *ct* on the distal part of chromosome 4, with linkage to *b*, *Pmv-19*, *D4Nds2* and *D4Mit13* (Neumann, Copp, Frankel, Coffin and Bernfield, unpublished data). Thus, there appears to be an interaction between *Sp* and *ct*.

(2) Backcross of double heterozygotes to *curly tail*

In order to investigate further the interaction between the mutations, F_1 (*Sp/+, ct/+*) animals were backcrossed to *ct/ct* (experimental backcross; Fig. 1). F_1 (*+/+, ct/+*) animals were backcrossed to give a measure of the penetrance of the curly-tail phenotype on the same background, but in the absence of *splotch* (control backcross; Fig. 1). Penetrance of *ct* is known to vary with genetic background (Embury et al., 1979). The backcross offspring were scored at weaning and, as before, reciprocal crosses were examined for imprinting effects. No evidence of imprinting was found, so the data from the two types of experimental backcross were pooled (Table 2).

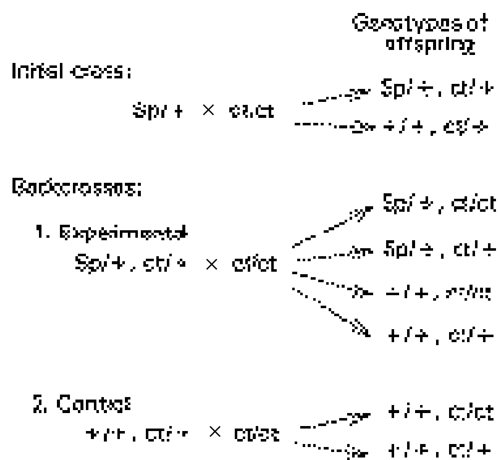

Fig. 1. Diagram showing the three types of genetic cross performed in the study. The expected genotypes of the offspring of each cross are shown on the right. The initial cross provided double heterozygote mice that served as parents in the backcrosses. The three genetic crosses, shown here for the *Sp* mutant, were also performed using the Sp^{2H} allele.

Table 1. Offspring of matings between *Sp*/+ and *ct/ct* mice

	Non-*splotch* offspring		*splotch* offspring	
	Tail defect	Normal tail	Tail defect	Normal tail
Sp/+ male parent	0	48	5	51
Sp/+ female parent	0	54	6	48
Total offspring (%*)	0 (0)	102	11 (10.0)	99

*Percentage with tail defects differs significantly between non-*splotch* and *splotch* offspring, ^{2}=8.8, P<0.005.

In the experimental backcross, there was an apparent deficit of animals carrying *splotch*: only 93 offspring were scored as *splotch* compared with 131 non-*splotch* animals. Of the *splotch* offspring in this backcross, 39.8% had tail flexion defects. By contrast, mice not expressing *splotch* had a much lower incidence of tail defects: only 13.7% of the non-*splotch* offspring in the experimental backcross and 12.7% of offspring in the control backcross were affected. These findings provide further evidence for an interaction between *Sp* and *ct*, and raise the possibility that a proportion of the double mutant mice die before the age of weaning. For instance, the genotype *Sp/+, ct/ct* could be lethal before or around the time of birth in a proportion of individuals. An alternative explanation could be that homozygosity for *ct* could suppress the expression of *Sp* in mice of the genotype *Sp/+, ct/ct*, thereby accounting for the apparent deficit of *splotch* mice in the experimental backcross. As a test of the first model, based on lethality of *Sp/+, ct/ct* mice, we inspected embryos from the two backcrosses to determine whether they had developmental defects compatible with perinatal lethality.

Examination of double mutant embryos

Experimental and control backcross litters were examined at various stages of gestation. The resorption frequency did not differ significantly between the two types of backcross (Table 3) and, among the embryos and fetuses obtained from these litters, neural tube defects were the only gross abnormalities observed. The overall incidence of exencephaly did not differ significantly between experimental and control embryos, whereas there was a significantly higher incidence of spinal defects in the experimental embryos compared with the controls (Table 3). Lumbosacral spina bifida showed a more than 13-fold excess (16.6% in experimentals compared with 1.2% in controls) and tail flexion defects were elevated nearly three-fold (29.1% in experimentals compared with 10.7% in controls). This proportionally greater increase in the frequency of spina bifida, compared with tail defects, suggests that the mutant interaction causes delay in closure of the posterior neuropore. It has been demonstrated previously that, in the *ct* mutant, severely

Table 2. Offspring of the experimental (*Sp*/+, *ct*/+ × *ct*/*ct*) and control (+/+, *ct*/+ × *ct*/*ct*) backcrosses

	Non-*splotch* offspring		*splotch* offspring	
	Tail defect	Normal tail	Tail defect	Normal tail
1. Experimental backcross:				
Double heterozygote male parent	14	65	19	32
Double heterozygote female parent	4	48	18	24
Total offspring	18	113	37	56
(%*)	(13.7)		(39.8)	
Grand totals	131		93	
2. Control backcross:				
Total offspring	23	158		
(%*)	(12.7)			
Grand total	181			

*Percentage with tail defects differs significantly between non-*splotch* and *splotch* offspring in the experimental backcross (χ^2=19.9, P<0.001) but not between non-*splotch* offspring in the experimental and control backcrosses (χ^2=0.07, P>0.05).

delayed neuropore closure results in spina bifida whereas less severe delay produces tail flexion defects (Copp, 1985).

Approximately 50% of *ct/ct* individuals with spina bifida in our breeding colony die around the time of birth (A. J. C. unpublished data). Newborn pups with spina bifida are often anaemic due to bleeding from the spinal defect, and in some cases have hind-limb paralysis; many of these pups die either as a direct result of the anaemia or due to cannibalism by the mother. In the present study, the spina bifida lesions observed in the backcross embryos and fetuses were of a similar or greater severity than those usually seen in *ct/ct* embryos, suggesting that a high proportion of these affected individuals would not have survived beyond birth. Thus, the deficit of *splotch* offspring in the experimental backcross can be explained by loss of *Sp/+, ct/ct* individuals around the time of birth due to severe spina bifida.

Table 3. Embryos derived from experimental (*Sp*/+, *ct*/+ × *ct*/*ct*) and control (+/+, *ct*/+ × *ct*/*ct*) backcrosses*

	Experimental backcross	Control backcross
No. implants	157	85
No. resorptions	6	1
(% implants)	(3.8)	(1.2)
No. viable embryos	151	84
Tail defects alone	44	9
(% viable embryos)	(29.1)	(10.7)
Spina bifida ±tail defects	25	1
(% viable embryos)	(16.6)	(1.2)
All spinal defects	69	10
(% viable embryos)	(45.7)	(11.9)
Exencephaly	4	1
(% viable embryos)	(2.6)	(1.2)

Statistical comparisons: tail defects (χ^2=10.5, P<0.01) and spina bifida (χ^2=11.4, P<0.001) were significantly more common among offspring of the experimental backcross compared with the control backcross, whereas resorptions (χ^2=0.6, P>0.05) and exencephaly (χ^2=0.1, P>0.05) did not differ significantly.

*Embryos were dissected from the uterus at either 11.5, 14.5 or 18.5 days gestation. Data in this table are the pooled results from all time points. Fig. 3 shows the results separately for each gestational day studied.

Direct demonstration that backcross embryos with spina bifida carry *Sp*

In order to determine whether embryos affected by spina bifida in the experimental backcross carried the *splotch* mutation (i.e. were of genotype *Sp/+, ct/ct*), we repeated the backcross using the Sp^{2H} allele. This allele involves a 32 bp deletion in the paired homeodomain of the *Pax-3* gene (Epstein et al., 1991), which can be demonstrated by PCR amplification of a DNA fragment spanning the deletion. Thus, Sp^{2H}/+ embryos can be distinguished from +/+ embryos by the presence of a 95 bp PCR product in addition to the wild-type 127 bp product.

Embryos were harvested from experimental (Sp^{2H}/+, *ct*/+ × *ct/ct*) and control (+/+, *ct*/+ × *ct/ct*) backcrosses at 11.5 days gestation, inspected for malformations and genotyped by PCR. In the experimental backcross, spina bifida was

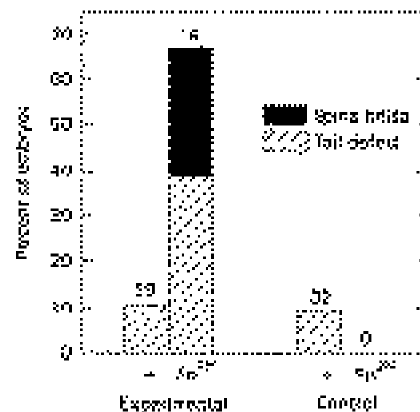

Fig. 2. Incidence of spina bifida and tail defects among 11.5 day embryos of the experimental (Sp^{2H}/+, *ct*/+ × *ct/ct*) and control (+/+, *ct*/+ × *ct/ct*) backcrosses. The *splotch* genotype of embryos was determined by PCR and data for each backcross are presented separately for Sp^{2H} and + embryos. Figures above the bars show the total number of embryos of each genotype examined. Note that the experimental backcross produced approximately equal numbers of Sp^{2H} and + embryos whereas all embryos in the control backcross carried the + allele. The data demonstrate that the high incidence of spina bifida and tail defects in the experimental backcross is due to the presence of Sp^{2H}/+ embryos.

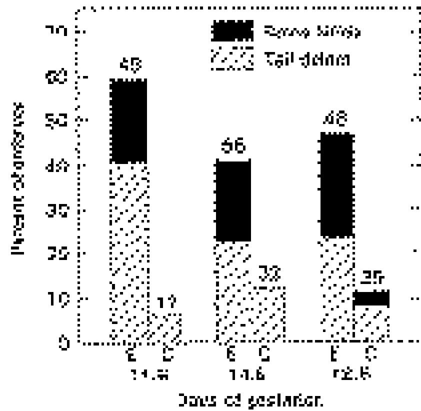

Fig. 3. Incidence of spina bifida and tail defects among embryos of the experimental (E) and control (C) backcrosses, analysed at 11.5, 14.5 and 18.5 days gestation. Figures above the bars show the numbers of embryos examined. There was no significant change in either the incidence of total spinal defects (^{2}=5.5, d.f.=2, P>0.05) or the relative frequency of spina bifida and tail defects (^{2}=6.4, d.f.=4, P>0.05) with advancing gestational age in the experimental backcross, in contrast to the situation in *Sp/Sp* embryos that die at 13 days of gestation (Auerbach, 1954).

observed in 13.5% of embryos, and was associated with the Sp^{2H} allele in every case (Fig. 2). Tail defects occurred in 24.3% of experimental backcross embryos and were associated with Sp^{2H} in approximately half of these cases. The incidence of tail defects in the control backcross was 9.4%, similar to the incidence (7.4%) of embryos with tail defects and a wild-type *splotch* allele in the experimental backcross. Spina bifida was not observed in the control backcross. We conclude that embryos with spina bifida in the backcross experiments are *splotch* heterozygotes and are likely to have the genotype *Sp/+, ct/ct*.

Developmental basis of the interaction between *Sp* and *ct*

The interaction between *Sp* and *ct* could involve an exacerbation, by *ct*, of the *splotch* mutant effect so that *Sp/+* embryos develop as if they were of genotype *Sp/Sp*. Conversely, the interaction could represent an exacerbation, by *Sp*, of the partially penetrant *ct* mutant effect, so that the incidence and severity of spina bifida is increased in *ct/ct* embryos. A third possibility is that the interaction represents a summation of the mutant effects, without either mutant's specific pattern of developmental disturbance being affected. To distinguish between these possibilities, experimental backcross embryos were examined for a series of 'developmental markers' that characterise the *Sp/Sp* and *ct/ct* phenotypes.

Splotch homozygotes die on day 13 of gestation (Auerbach, 1954), probably as a result of neural crest-related cardiac defects (Franz, 1989). Backcross litters were examined before (11.5 days) and after (14.5 days) this stage to determine whether they were dying at the same time as

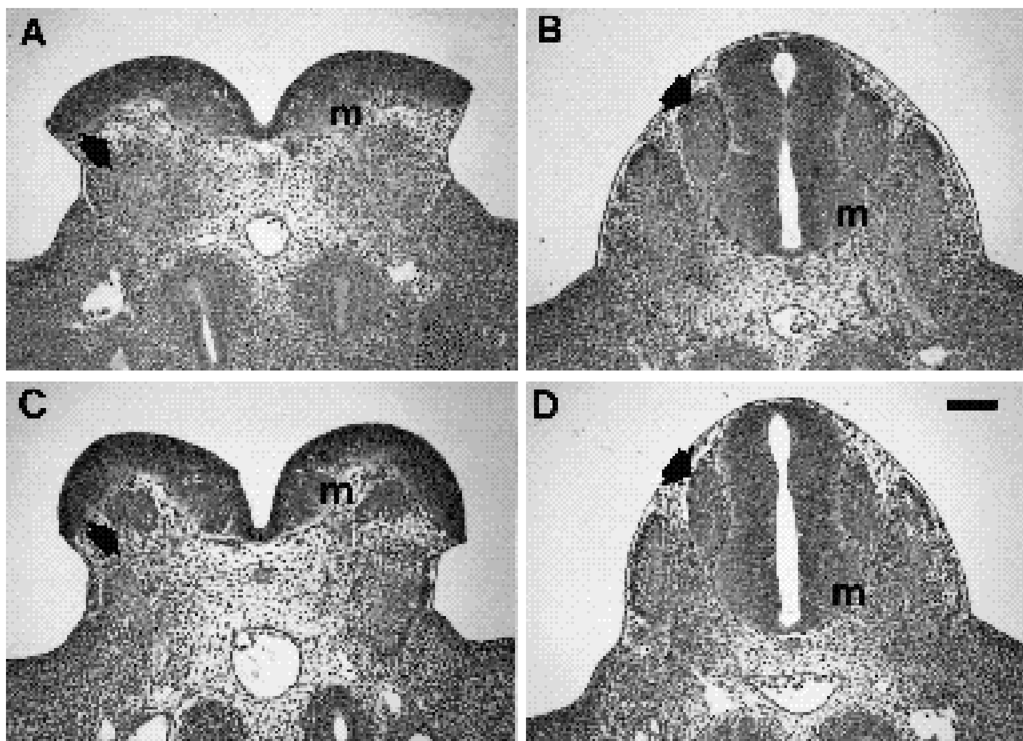

Fig. 4. Transverse histological sections through the hind limb bud region of 11.5 day embryos to illustrate the morphology of the dorsal root ganglia. (A) Sp^{2H}/Sp^{2H} embryo with spina bifida: the neuroepithelium is exposed on the dorsal surface of the embryo. There is a complete absence of dorsal root ganglia (arrow) in this and adjacent sections, indicating failure of neural crest migration. Motor neuron (m) development, by contrast, appears normal. (B) Genotypically +/+ littermate of the embryo in A showing a closed neural tube and normal sized dorsal root ganglia (arrow). (C) Double mutant embryo from the experimental backcross shown by PCR to be Sp^{2H}/+ (presumed genotype Sp^{2H}/+, *ct/ct*). Spina bifida is present with a closely similar morphology to the Sp^{2H}/Sp^{2H} embryo in A, but the dorsal root ganglia are of approximately normal size indicating relatively undisturbed migration of the neural crest at this level of the body axis. (D) Normal littermate of the double mutant embryo in C with presumed genotype +/+, *ct/ct* or +/+, *ct/+*. The neural tube and dorsal root ganglia are both normal. At least three embryos of each type were examined, with the same results in each case. Sections stained with haematoxylin and eosin. Scale bar, 0.1 mm.

Sp/Sp embryos. A third group of embryos was examined at 18.5 days gestation, to determine whether malformed embryos observed at earlier stages would have survived to birth. The incidence of tail defects and spina bifida in the experimental backcross did not differ significantly at the three developmental stages studied (Fig. 3) suggesting that the lethality of embryos with genotype *Sp*/+, *ct/ct* does not occur until after 18.5 days, unlike in *Sp/Sp* embryos.

The defective neural crest cell migration in *Sp/Sp* embryos produces, among other effects, the almost complete absence of dorsal root ganglia in the caudal region (Auerbach, 1954). We confirmed that this abnormality is also present in Sp^{2H}/Sp^{2H} embryos (Fig. 4A) compared with their normal +/+ littermates (Fig. 4B). Double mutant embryos with spina bifida (shown by PCR to be genotypically Sp^{2H}/+, and presumed to be of genotype Sp^{2H}/+, *ct/ct*) were found to have approximately normal sized dorsal root ganglia, even in the region of the spina bifida (Fig. 4C), suggesting that failure of neurulation in these embryos is not accompanied by defective neural crest migration, unlike in Sp^{2H}/Sp^{2H} embryos.

A morphological characteristic of *ct/ct* embryos that develop spina bifida is the presence of excessive ventral curvature of the caudal region at the 27-29 somite stage (Fig. 5A; Brook et al., 1991). We performed a similar analysis of curvature in a series of *splotch* embryos and found a significant *reduction* in the angle of curvature (Fig. 5B) in embryos with enlarged neuropores (presumed *Sp/Sp*; see Yang and Trasler, 1991) compared with littermates that had normal sized neuropores (presumed *Sp*/+ and +/+). This opposite effect of the *Sp* and *ct* mutations on the angle of curvature of the caudal region provides further evidence that the cellular mechanism of neurulation failure in the two mutants is quite different. Measurements on a series of embryos from the experimental backcross showed a pattern of curvature that was similar to the *ct* parent strain: the angle of curvature was increased in affected embryos, but the difference between embryos with large and small neuropores was less extreme than in the *ct* mutant, contrary to expectation if the interaction of *Sp* and *ct* leads to an exacerbation of the *ct/ct* developmental defect.

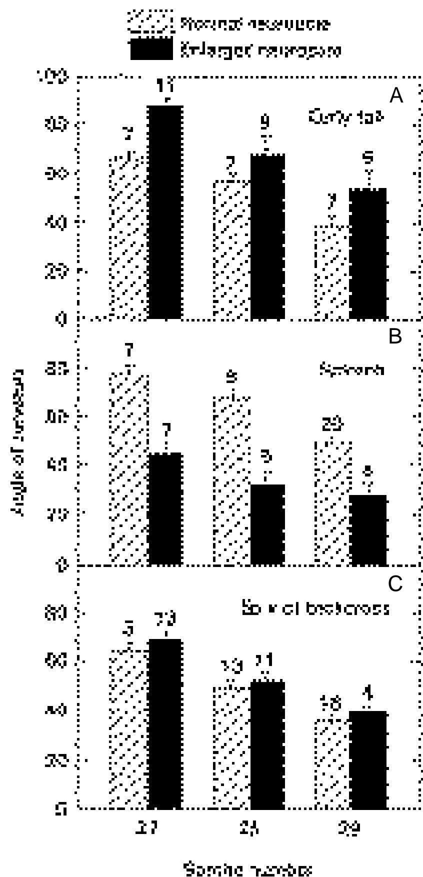

Fig. 5. Angle of ventral curvature of the caudal region (mean angle in degrees±s.e.m.) of *curly tail* (A), *splotch* (B) and experimental backcross (C) embryos at the 27-29 somite stage. In each case, data are presented for embryos with either normal (hatched bars) or severely enlarged (solid bars) posterior neuropores. Figures above the bars show the number of embryos examined. (A) *ct/ct* mutant embryos with enlarged neuropores (destined to develop neural tube defects) have a significantly increased angle of curvature compared with their normally developing littermates (F=11.9; d.f.=1, 41; P<0.005). These data are reproduced from Brook et al. (1991). (B) *splotch* embryos from matings between *Sp*/+ mice. Embryos with enlarged neuropores are assumed to be of genotype *Sp/Sp* (see Yang and Trasler, 1991), whereas embryos with normal sized neuropores are assumed to be either *Sp*/+ or +/+. In contrast to the *ct* mutant, abnormal *splotch* embryos have a significantly decreased angle of curvature compared with their normal littermates (F=67.0; d.f.=1, 46; P<0.001). (C) Embryos from the experimental backcross. The angle of curvature in embryos with enlarged neuropores is increased at all three somite stages, but the effect is less marked than in the *ct* mutant and is not statistically significant (F=1.6; d.f.=1, 54; P>0.05). Statistical analysis was by two-way analysis of variance (general linear model), performed separately for each of the three data sets. In each case there was significant variation in angle of curvature with somite number (P<0.001).

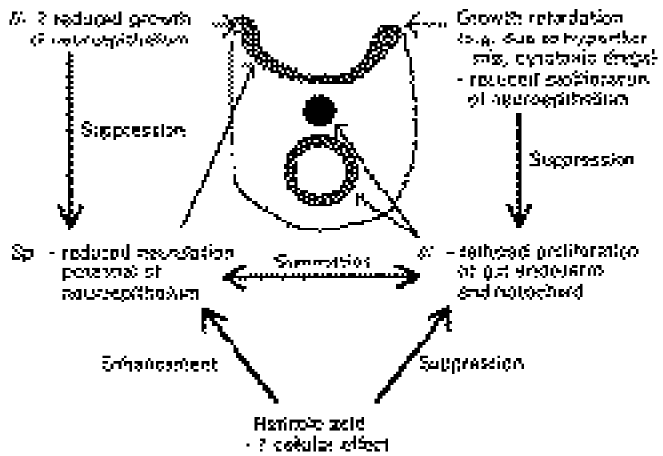

Fig. 6. Diagram showing gene-gene and gene-teratogen interactions that affect the development of spinal neural tube defects in the mouse embryo. Heavy arrows indicate the interactions and light arrows indicate the site of action of some of the factors on the tissues of the caudal embryonic region, which are shown in a diagrammatic transverse section.

Taken together, these results favour the third model, outlined above: the interaction between *Sp* and *ct* appears to represent a summation of the two mutant effects, without exacerbation of either mutant's specific pattern of developmental disturbance.

DISCUSSION

In this study, we have constructed double mutant mice as a first step towards determining the developmental relationship between mutant genes that disrupt neurulation. We found that mutations at the *splotch* and *curly tail* loci interact to promote disruption of neurulation in the developing spine. Although neither of the single heterozygotes, *Sp/+* or *ct/+*, develop neural tube defects, 10% of double heterozygotes have tail flexion defects resembling those seen in affected *ct* homozygotes. Moreover, using a backcross of double heterozygotes to *ct/ct*, we found that embryos of genotype *Sp/+*, *ct/ct* develop neural tube defects with a much greater incidence and severity than is seen in *ct/ct* embryos on this genetic background.

Embryonic basis of the interaction between *Sp* and *ct*

Examination of double mutant (backcross) embryos for several 'developmental markers' characteristic of each mutant has revealed that the interaction between *Sp* and *ct* represents a summation of the effects of the mutants, without exacerbation of either of the specific phenotypes that develop in *Sp/+* or *ct/ct* embryos. This suggests that the interaction between the mutants occurs distally in the chain of neurulation events. Previous studies of the two mutants have indicated that they act primarily on different tissues within the caudal embryonic region. The *ct* mutant affects non-neural tissues, the notochord and gut endoderm, which proliferate abnormally slowly in affected *ct/ct* embryos compared with the overlying neuroepithelium which grows at a normal rate (Copp et al., 1988a,b). Direct demonstration of the normality of the neuroepithelium in *ct/ct* embryos has come from recent experiments in which neuropore closure was shown to proceed at a similar rate in isolated neuroepithelia of *ct/ct* and non-mutant embryos, whereas neuropore closure was delayed in intact caudal regions of *ct/ct* embryos in vitro (van Straaten et al., 1993). The imbalance of tissue growth rates in *ct/ct* embryos causes the caudal region to curve ventrally (Brook et al., 1991), imposing mechanical constraints on elevation of the neural folds at the posterior neuropore and leading to delay of neuropore closure (Gruneberg, 1954; Copp, 1985). Although the finding of developmental abnormalities in the notochord and gut of *ct/ct* embryos suggests that the *curly tail* gene may be expressed primarily in these tissues, the normal pattern of *ct* gene expression has not yet been determined.

In contrast to *ct*, the *Sp* mutant is thought to disturb development through an effect on the neuroepithelium (Moase and Trasler, 1990), not on ventral tissues. Pax-3, the gene product of the *splotch* locus, is expressed prior to neural tube closure in the dorsal part of the neuroepithelium (Goulding et al., 1991). In *Sp* mutant embryos, the N-CAM polypeptide profile is altered (Moase and Trasler, 1991), cells of the neuroepithelium are less closely packed than normal (Yang and Trasler, 1991) and may adopt unusual orientations within the neural plate (Morris and O'Shea, 1983). These changes in cell-cell relationships could be responsible for hindering release of neural crest cells and for defective elevation of the neural folds in *Sp/Sp* embryos. This view is consistent with our finding that *Sp/Sp* embryos have a reduced angle of ventral curvature in the caudal region compared with normal littermates. This is the opposite situation from that seen in *ct/ct* embryos (Brook et al., 1991) and presumably indicates that, during longitudinal growth of the caudal region in *Sp/Sp* embryos, the defective neuroepithelium is underlain by normally proliferating notochord and gut, leading to a relative straightening of the caudal region.

We propose, therefore, that increased ventral curvature of the caudal embryonic region, due to homozygosity for the *ct* mutation, summates with reduced neurulation potential of the neuroepithelium, as a result of heterozygosity for *Sp*, to produce a severe neural tube defect phenotype in double mutant embryos. There is evidence of reduced neurulation potential in *Sp/+* embryos: in addition to their neural crest abnormalities, *splotch* heterozygotes have an increased predisposition to retinoic acid-induced neural tube defects compared with wild-type littermates (Dempsey and Trasler, 1983). Moreover, the abnormal species of N-CAM detected in *Sp/Sp* embryos are also present in heterozygotes (Moase and Trasler, 1991). Presumably, the degree of abnormality in *Sp/+* embryos falls within the range that can be tolerated by the developing embryo, without disturbing neurulation. On the other hand, if *Sp/+* embryos are made homozygous for *ct*, the combination of deficient neuroepithelium and mechanical constraints imposed by ventral curvature exceeds the limits of tolerance of the system, and severe neural tube defects result.

This study demonstrates the need for careful analysis of developmental mechanisms when evaluating interactions between mutant genes. The mere fact that double mutant mice exhibit a more severe phenotype than either of the single mutants cannot be taken as evidence that the two genes operate in the same genetic pathway. As in the case of *ct* and *Sp*, the interaction may represent a distal summation of developmental effects. This consideration could prove to be of importance for future studies of 'gene-knockout' mice, where mutations that by themselves have only mild developmental phenotypes are studied in pairwise combinations.

Relationship between neural tube closure and neural crest migration

We find that failure of neural tube closure can be associated either with concomitant failure of neural crest migration (as in Sp^{2H}/Sp^{2H} embryos) or with apparently unaffected neural crest migration (as in $Sp^{2H}/+$, *ct/ct* embryos). This suggests that closure of the neural tube and initiation of neural crest migration are independent events in the lower spinal region of the mouse embryo: neural crest migration does not appear to depend on prior fusion of the neural folds. This is consistent with the finding that the neural crest begins to emigrate before neural tube closure in the cranial region of

the rodent embryo (Morriss-Kay, 1981). Thus, neural tube closure and neural crest migration can be viewed as events that have in common certain molecular functions (e.g. correct expression of the Pax-3 gene product) but are independent with respect to others (e.g. correct expression of the ct gene product).

Gene-gene and gene-teratogen interactions during mouse neurulation

Fig. 6 summarises the gene-gene interactions, and some of the gene-teratogen interactions that have been found to affect the development of neural tube defects in the mouse. Apart from the present study of the interaction between *Sp* and *ct*, and the reported absence of an interaction between *Sp* and the neural tube defect mutant *Axd* (Essien et al., 1990), there has been only one other study of a gene-gene interaction that influences the incidence of neural tube defects in the mouse. Konyukhov and Mironova (1979) produced double mutant mice carrying both *Sp* and the mutant gene fidget (*fi*). The incidence of spina bifida was only 1.7% in an experimental intercross between mice of genotype *Sp/+*, *fi/fi*, whereas a control intercross between *Sp/+*, *+/+* mice yielded spina bifida in 14.4% of cases. Thus, *fi* suppresses the effect of *Sp* although the mechanism of this interaction is as yet unknown.

In addition to the gene-gene interactions, there is accumulating evidence that interactions between genes and teratogens may influence the development of neural tube defects (Fig. 6). A variety of factors that produce growth-retardation of the embryo have been shown to reduce the incidence and severity of *ct*-induced spinal defects; this is true for hyperthermia in vitro, maternal food deprivation in utero (Copp et al., 1988b) and the cytotoxic drugs hydroxyurea (Seller and Perkins, 1983) and mitomycin C (Seller and Perkins, 1986). These factors appear to act by re-balancing growth rates within the caudal embryonic region. The *ct* mutation reduces proliferation of the gut endoderm and notochord, and this effect is counterbalanced by generalised growth-retarding effects, which have their greatest effect on the rapidly proliferating neuroepithelium (Copp et al., 1988b). The effect of growth-retarding influences on expression of the *Sp* mutation has not yet been investigated. Retinoic acid is a well-known teratogen that interacts with both the *Sp* and *ct* mutations. The nature of these interactions are, however, opposite in type: retinoic acid increases the incidence and severity of *Sp*-induced defects (Dempsey and Trasler, 1983; Kapron-Bras and Trasler, 1984, 1985, 1988; Moase and Trasler, 1987), but decreases the incidence of *ct* spinal neural tube defects (Seller et al., 1979; Seller and Perkins, 1982). Studies are in progress to determine the cellular and molecular basis of this apparently paradoxical effect of retinoic acid. Continued in-depth analysis of gene-gene and gene-teratogen interactions, in future, may provide insight into the mechanisms controlling mouse neurulation.

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