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All-or-none craniorachischisis in Loop-tail mutant mouse chimeras

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Summary

Mouse embryos homozygous for the mutant gene Loop-tail (Lp) are characterized by craniorachischisis, an open neural tube extending from the midbrain to the tail. In the present study, experimental chimeric mice containing mixtures of genetically mutant (from *Lp*/+×*Lp*/+ matings) and genetically normal cells were produced. Our aim was to determine whether a 'rescue,' phenotypic gradient, or intermediate expression (i.e. alternating areas of open and closed neural tube) would be observed in these chimeras. We report our analyses of Loop-tail mutant chimeras (n=82) by gross examination, progeny testing and quantitative analysis of glucose phosphate isomerase (GPI) isozyme levels. An all-or-none craniorachischisis in Loop-tail mutant chimeras was observed. Two multicolored adult chimeras, without any gross evidence of a neural tube defect, were shown to be homozygous Loop-tail chimeras (*Lp*/*Lp*↔+/+) by progeny testing. These results indicate that the normal phenotype can be expressed in the presence of mutant cells. Conversely, six neonates with craniorachischisis were shown to be chimeras by GPI analyses. These results show that the full mutant phenotype can be expressed even when one-third to one-half of the cells are genotypically wild-type. This study did not determine which tissue is primarily responsible for the defective neurulation in this mutant, but suggests that a 'threshold' mechanism underlies the Loop-tail mutant phenotype. In some chimeras that threshold is not reached and the neural tube remains open, whereas in other chimeras the threshold is reached and the neural tube closes completely.

Key words: mouse, Loop-tail mutant, neural tube defects, craniorachischisis, spina bifida.

Introduction

Neurulation occurs with elevation of the lateral margins of the neural plate, which approach each other and fuse to form a neural tube. Both intrinsic properties of the neural plate and extrinsic tissues could be involved in neurulation (Karfunkel, 1974; Schoenwolf, 1982; Gordon, 1985). Intrinsic properties of the neural plate, such as differential proliferation, cell shape and midline or lateral regional variations, may contribute to normal neurulation (Schoenwolf, 1982; Gordon, 1985; Smith and Schoenwolf, 1987, 1988). Extrinsic tissues that could elevate the neural folds and displace them medially include: (1) surface ectoderm, (2) somites and (3) notochord. The possibility of surface ectoderm pushing the neural folds together has been suggested. In fact, neural tube defects are present in embryos with epidermal defects and embryos treated with antibodies to epidermal growth factors (O'Shea, 1986). Somites or mesenchyme may push the neural folds together and/or support the rising neural folds (Morriss and Solursh, 1978*a*,*b*; Schoenwolf, 1982). The possibility of notochord elongation affecting neural tube development has been suggested (Karfunkel, 1974; Jacobson, 1978). Neural tube mutants of the mouse such as Loop-tail and curly tail have demonstrated defects in axial elongation and notochord proliferation (Smith and Stein, 1962; Copp *et al.* 1988). However, elucidating the mechanisms involved in mammalian neurulation has been difficult due to the inaccessibility of the mammalian embryo for experimental manipulation. The analysis of genetic mutants is a means of exploring these development mechanisms.

The heterozygous and homozygous expression of the semi-dominant mutant gene Loop-tail (gene: *Lp*, Chromosome 1) in mice has been described on a gross, microscopic and ultrastructural level (Strong and Hollander, 1949; Stein and Rudin, 1953; Stein and Mackensen, 1957; Smith and Stein, 1962; Van Abeelen and Raven, 1968; Wilson and Michael, 1975; Wilson and Finta, 1980 *a*,*b*; Wilson, 1982, 1985*a*,*b*). Heterozygotes have loop-tails, enlarged ventricles of the brain, and, in one-third of the females, imperforated vaginas (Strong and Hollander, 1949; Van Abeelen and Raven, 1968). Homozygous embryos, which die shortly after birth, have been characterized as expressing the following major morphological defects: (1) craniorachischisis, an open neural tube extending from the midbrain to the tail, (2) twisted loop-tails, (3) axial skeletal abnormalities, (4) concavity of the back, and (5) dorsal skin

excision (Strong and Hollander, 1949; Stein and Mackensen, 1957; Smith and Stein, 1962). The primary tissue or cells affected by this mutant gene is unknown.

Experimental mouse chimeras have been used to study mutants, and 'rescue' of the mutant phenotype, expression of phenotypic gradients as well as cell autonomous gene action has been reported. Mintz (1964) reported that the lethal mutant t^{12}/t^{12} mouse, which usually arrests its development at the morula stage, in the presence of normal cells continues to survive to the blastocyst stage. Muggleton-Harris *et al.* (1987) have demonstrated that the congenital cataractous lens mouse mutant can be rescued by forming chimeras between a noncataractous strain and the mutant. Studies with chimeras have also shown a gradient phenotypic expression depending on the quantitative proportion of normal and mutant cells present. Forsthoefel *et al.* (1983) has shown a gradient expression of the Strong's luxoid gene, *lst*, whereby the presence of more mutant *lst* cells correlates with more skeletal deformities. In another chimeric study of cleft palates, positive correlation between cleft palate and the percentage of cells from a strain of mice susceptible for cleft palate was found (Sulik and Atnip, 1978). Chimeras have also been used to determine the defective cell types in neurological mutants (Mullen and LaVail, 1976; Mullen, 1977; Herrup and Mullen, 1979; Mullen and Herrup, 1979; Goldowitz and Mullen, 1982).

The use of chimeras to study the morphogenetic process of neurulation has never been done to our knowledge. In the present study, experimental chimeric mice containing mixtures of genetically mutant (from $Lp/+ \times Lp/+$ matings) and genetically normal cells were produced. Our aim was to determine whether in these chimeras a 'rescue,' phenotypic gradient, or intermediate expression (i.e. alternating areas of open and closed neural tube) would be observed. If the latter, it might be possible to correlate open and closed regions of the neural tube with mutant and normal cell genotypes, respectively, to shed light on the defective cell type of the Loop-tail mutant.

In this paper we report our analyses of Loop-tail mutant chimeras by gross examination, progeny-testing and quantitative analysis of glucose phosphate isomerase (GPI) isozyme levels. Newborn chimeras were visually examined for complete or intermediate expression of craniorachischisis. Glucose phosphate isomerase isozyme levels were measured to determine whether the abnormal neonates were chimeras and to estimate the proportion of normal and mutant cells. Normal neonates were studied as adults by progeny tests to determine their genotypes ($Lp/Lp \leftrightarrow +/+$, $Lp/+ \leftrightarrow +/+$, or $+/+ \leftrightarrow +/+$). In this study, no intermediate expression of neural tube closure was observed. An all-or-none craniorachischisis in Loop-tail mutant mouse chimeras was observed. The full mutant phenotype could be expressed even in the presence of normal cells. Conversely, the normal phenotype can be expressed in the presence of mutant cells. Part of this work has been previously reported in abstract form (Cichocki (Musci) and Mullen, 1988).

Materials and methods

Mice and chimera production

The mice were bred and maintained in our laboratory for use in these experiments. The Loop-tail strain (LPT/Le) was established from stocks obtained from The Jackson Laboratory, Bar Harbor, Maine. The inbred strains and crosses used to produce the embryos are given in Table 1, together with their designations, relevant genotypes and coat colors. Different strains of mice were used to form the chimeras in this study to test the possibility of strain differences (Wilson and Center, 1977; Moore and Mintz, 1972). However, no strain differences were noted, at least not in the coat colors of the multicolored chimeras.

Chimeras were made by a standard embryo aggregation method (Mullen and Whitten, 1971). Superovulation of female mice was induced by the use of 2 to 3 i.u. of pregnant mare serum (Sigma) followed in 44 h by the same dose of human chorionic gonadotropin (Sigma). Two days after the detection of vaginal plug (E0), 4- to 8-cell embryos derived from matings of heterozygous mutants ($Lp/+ \times Lp/+$) and normal BALB, C3H and B6 matings were obtained by flushing oviducts of the superovulated females. The zona pellucida of these embryos was lysed by pronase (Calbiochem). Embryos were aggregated and cultured overnight

Table 1. *Embryos used to produce chimeric mice*

Strains	Designations of embryos	Coat Color	Genotype at *Gpi-1* locus
Inbred strains			
LPT/Le-*Lp*/+	LP	agouti	B/B
BALB/c	BALB	albino	A/A
C3H/HeN	C3H	agouti	B/B
C57BL/6	B6	black	B/B
*Hybrids**			
BALBLPT-*c*/*c*	CLP	albino	A/A, A/B, or B/B
BALBLPT-+/−×LPT	HLP†	agouti	A/B or B/B
BALBLPT-+/−×BALPLPT-+/−	HLP†	agouti or albino	A/A, A/B or B/B

* All hybrids were $Lp/+$. The +/− refers to the genotype at the albino locus.
† In this paper, these two hybrids will be grouped together unless otherwise indicated.

until the late morula or blastocyst stage. These chimeric embryos were then transferred surgically into the uteri of pseudopregnant hosts (Swiss) that had mated with vasectomized males the day after the mating of donor females.

Some of the chimeras were delivered naturally by the female host at 19 days of pregnancy. Unfortunately, neonates born with abnormalities such as craniorachischisis are usually destroyed by the mother. To circumvent this problem, pups were taken from the host by Caesarian section at 18 days of pregnancy. Normal pups of these litters were fostered on other lactating females.

Progeny testing of normal chimeras

Chimeras were weaned at approximately three weeks of age, at which time, the percentage of agouti to albino or black fur in the chimeras was visually estimated and recorded. All of these normal chimeras were then mated to BALB or B6 (+/+) mice and the resultant progeny were classified by coat color as being derived from the Loop-tail component or the normal component of the chimera. Agouti progeny from LP↔BALB, LP↔B6, HLP↔BALB, or HLP↔B6 chimeras would be derived from the mutant component, while albino or black progeny would be of the BALB or B6 component, respectively. In the case of CLP↔C3H, albino mice would be of the loop-tail component, while agouti mice would be of the normal C3H component. Progeny from the normal component were excluded from the data.

Since heterozygous matings were used to produce the mutant embryos, a 1:2:1 ratio of +/+:*Lp*/+:*Lp*/*Lp* embryos was expected. The ratio of phenotypes (loop-tail compared to straight-tail) among the progeny from the mutant component of each chimera allowed us to deduce the genotype (+/+, *Lp*/+, or *Lp*/*Lp*) of the chimera (Mullen and Herrup, 1979). If the chimera produced progeny that were 100% straight-tailed, it was deduced that the genotype of the chimera was +/+↔+/+. Production of 50% loop-tail and 50% straight-tail progeny from the mutant component indicated that the chimera was of the genotype *Lp*/+↔+/+. If the phenotypes of the progeny were 70–100% loop-tails, the deduced genotype of the chimera was *Lp*/*Lp*↔+/+. The phenotypes do not have to be completely 100% loop-tail because there is only 70% penetrance of the loop-tail phenotype in heterozygotes (Stein and Rudin, 1953). Chi-square analysis was used to determine whether the progeny test of the deduced homozygous chimeras, *Lp*/*Lp*↔+/+, was statistically significant.

Glucose phosphate isomerase analysis

The inbred strains LP, C3H and B6 are homozygous for the GPI-1B (fast variant) allele which expresses a GPI that migrates more cathodally than the GPI of BALB mice which are homozygous for the GPI-1A (slow variant) allele. Genetic hybrids between LP and BALB strains may also express both parental forms of the enzyme and a heterozygous band with intermediate mobility (i.e. GPI-1AB) (Peterson *et al.* 1978).

Abnormal pups with craniorachischisis were prepared for glucose phosphate isomerase analysis. The following tissues of the abnormal pups were dissected and stored at −70°C: (1) exencephalic brain, (2) skin, (3) liver, (4) eyes, (5) intestines and (6) neural axis, i.e. spinal cord, vertebral column and adjacent tissue. The tissues were disrupted by homogenization in 50 mM Tris buffer (pH 7.5) in micro glass homogenizers at 4°C. Adult liver of LP and BALB mice were similarly prepared to serve as controls for each gel. Adult Chimera 811 was anesthetized with Avertin, (0.02 ml g^{-1}), and the left eye was surgically removed. The neural retina was dissected out in physiological saline at 4°C, and homogenized in 50 mM Tris buffer, pH 7.5. The proportions of GPI isozymes was determined by running cellulose acetate gels using modified methods previously described (Eicher and Washburn, 1978). 5 μl samples were applied to 94×76 mm Helena, Titan III electrophoresis plates. Using a Tris–glycine buffer (5.16 g Tris–base plus 3.48 g glycine per liter) pH 8.8, electrophoresis was conducted for 2.5 h at 200 V. The plates were stained with an agar overlay consisting of a mixture of 160 μl phenyl methosulfate, 5 μl glucose-6-phosphate, 2.24 ml distilled water, and 400 μl each of 50 mM $MgCl_2$, 50 mM fructose-6-phosphate, 10 mM β-nicotinamide adenine dinucleotide phosphate, and nitro blue tetrazolium (10 mg ml^{-1}) added to 4 ml of 2% agar in 100 mM Tris–base, pH 8.0, at 50–55°C. The plates were incubated for approximately 15 to 30 min with the stain agar overlay. The proportions of the different GPI allozymes for each chimera were then quantified by a scanning densitometer (Gilford 2600 Instruments) to determine the genotypic composition of the chimeric tissue. The accuracy of the isozyme analysis was determined by serial dilutions of mixtures containing known proportions of both GPI isozymes. For the ratio of 4:1, i.e. BALB:LP, the proportion of both GPI isozymes yielded results within ±5% of those expected. The accuracy of the proportions decreased when one of the genotype measurements was below 20%. This may be due to systematic decrease in the relative staining intensity of GPI-1B with a decreasing enzyme concentration (Peterson *et al.* 1978).

In cases where the chimera was A/B↔A/A a different analysis was necessary to estimate the genotype proportions. The ratio of the two isozyme types (GPI-1 AA and GPI-1 BB) and the intermediate heterozygous band (GPI-1 AB) in a hybrid would be expected to be 1:2:1. To test whether our technique upheld this ratio, the tissue of a LP×BALBF_1 mouse, heterozygous at the *Gpi*-1 locus, was analyzed for GPI. A ratio of 1:2.4:1 was observed. This ratio is not significantly different than 1:2:1. Hence, on the gel of Chimera 1, the AA band would contain enzyme from both the HLP and BALB components. To correct for this, we assumed that the HLP BB band would equal the amount of HLP AA and this was subtracted from the total AA band to estimate the BALB component.

Results

82 mice resulted from embryo aggregation, 41 were LP↔BALB chimeras, 27 were HLP↔BALB, 9 were CLP↔C3H, and 5 were LP↔B6 chimeras. 63 of the aggregated mice showed normal neural tube development and survived to adulthood, whereupon 36 expressed multicolored coats, while 27 had solid-colored coats. 19 of the neonates were abnormal, 18 had craniorachischisis and one had exencephaly.

Analysis of adult chimeras

The range of the two embryonic components contributing to each chimera's coat color was 0 to 100%. A mosaic coat color is unequivocal evidence that a mouse is a chimera although solid colored mice have also been shown to be chimeric in other tissues (Forsthoefel *et al.* 1983). The 27 solid colored mice were mated with BALB mice to determine whether they were chimeric in the germ line. Five of these mice (four loop-tails and one straight-tail) were found to be chimeras by producing progeny with coat colors indicative of mosaicism in

Table 2. *Summary of phenotypes of chimeras*

	Phenotype			
	Craniorachischisis	Loop-tail	Straight-tail	Total
Chimeras				
Multicolored adults	0	15	21	36
Solid colored adults	0	4	1	5
Mosaic neonates	7*	–	–	7
	7*	19	22	48
Nonchimeric or unknown	12	6	16	34
	19	25	38	82

* One chimera has only exencephaly, the spinal cord appeared grossly normal.

the germ line. These solid colored chimeric mice were added to the 36 multicolored chimeras for progeny testing (n=41). The remaining animals were eliminated from further study.

The phenotypes of the chimeras produced is summarized in Table 2. Nineteen of these chimeras had loop-tails, while the remaining 22 had straight-tails. The straight-tailed chimeras still could be heterozygous or homozygous mice because past evidence has shown that the loop-tail phenotype of this mutant is only 70% penetrant (Stein and Rudin, 1953). The range of coat color and tail phenotypes can be seen in Fig. 1.

Progeny testing of adult chimeras, as described in the Materials and methods, was required to determine their genotypes. The ratios of phenotypes among the progeny, allowed us to determine that 12 were +/+↔+/+, 20 were *Lp*/+↔+/+, and 2 were *Lp*/*Lp*↔+/+ (Table 3). Seven chimeras (4 with loop-tails, and 3 with straight-tails) did not produce any progeny.

Two multicolored chimeras (HLP↔BALB) were shown to be homozygous Loop-tail chimeras (*Lp*/*Lp*↔+/+) by observing the simultaneous presence of both the mutant coat color (pigmented) and loop-tail in their progeny (Table 4). Chimera 811 was 60% mutant in coat color (by visual estimation) and had the characteristic loop-tail (Fig. 2). Thirty loop-tail and one straight-tail pigmented progeny were produced by mating Chimera 811 to a BALB mouse. Chimera 809 was 80% mutant in coat color and also had a loop-tail. The mating of Chimera 809 to a BALB mouse produced 40 loop-tail and 2 straight-tail pigmented progeny (Table 4). Thus, 97 and 98% of the progeny produced by the two chimeras, 811 and 809, respectively, expressed the mutant phenotype. It was shown to be

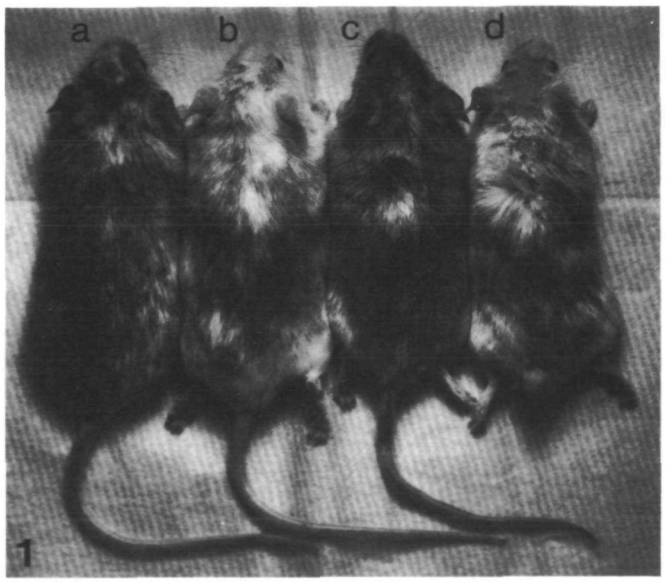

Fig. 1. Multicolored adult chimeras with various phenotypes. (a) LP↔BALB with 70% mutant (pigmented) coat color and a straight tail. (b) LP↔BALB with 50% mutant (pigmented) coat color and a straight tail. (c) HLP↔BALB with 85% mutant (pigmented) coat color and a straight tail. Although this chimera has a straight tail, progeny testing showed that it was a *Lp*/+. (d) HLP↔BALB with 50% mutant (pigmented) coat color and a loop-tail. The coat color percentages are estimated at weaning and may differ in the adult chimeras shown here.

Fig. 2. Chimera 811, determined by progeny testing to be a homozygous Loop-tail chimera (*Lp*/*Lp*↔+/+). The mouse expresses a 60% mutant (pigmented) coat color and a loop-tail.

Table 3. *Progeny test of chimeras to determine genotype of chimera*

Phenotype of progeny from mutant component of chimera	Deduced genotype of the chimeras	Observed chimeras*
100% *Lp*/+†	*Lp*/*Lp*↔+/+	2
50% *Lp*/+, 50% +/+	*Lp*/+↔+/+	20
100% +/+	+/+↔+/+	12

* Based on statistically significant number of progeny.
† Due to incomplete penetrance of the tail phenotype (70%), 70% to 100% mutant phenotype would indicate a *Lp*/*Lp*↔+/+ genotype.

Table 4. *Progeny test of the deduced homozygous Lp/Lp↔+/+*

	Chimera's phenotypes		Tail phenotype of pigmented progeny	
	coat color	tail	loop-tail	straight-tail
Chimera				
809	80% mutant	loop-tail	40	2*
811	60% mutant	loop-tail	30	1

* One of these progeny was later shown to be a *Lp*/+ by producing *Lp*/+ offspring.

statistically significant ($P<0.001$) by Chi-square analysis that these chimeras were not *Lp*/+. One of the two straight-tailed progeny of Chimera 809 was shown to be heterozygous *Lp*/+ by progeny testing. The straight tail is a result of incomplete penetrance of the gene. Unfortunately, the other straight-tailed progeny of Chimera 809 and Chimera 811 were not available for progeny testing but, in all probability, these progeny were in fact *Lp*/+ but expressed a straight-tailed phenotype. Although both of the *Lp*/*Lp* chimeras that survived to adulthood were from the hybrid strain, the numbers are too small to suggest that their survival was due to their being hybrid.

Homozygous Loop-tail Chimera 811 (Table 4) was analyzed by GPI electrophoresis to determine whether the neural tissue, specifically the neural retina, was comprised of Loop-tail and BALB cells. The densitometer readings indicated that the composition of the chimeric neural retina was 49% mutant and 51% normal (Fig. 3). Although this chimera did not express any gross evidence of a neural tube defect, mutant (*Lp*/*Lp*) cells contribute to its neural tissue, at least the neural retina.

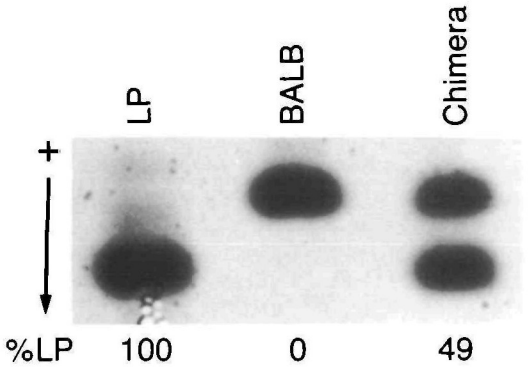

Fig. 3. GPI analysis of Chimera 811 neural retina. The electrophoretic gel contains neural retina homogenates: Loop-tail (LP), GPI-1BB; BALB, GPI-1AA; Chimera 811, GPI-1AA and BB. The proportion of mutant (LP) isozyme in each neural retina was determined by densitometry readings and are 100, 0 and 49%, respectively.

Analysis of neonatal chimeras

By analyzing the glucose phosphate isomerase isozyme status of the nineteen abnormal neonates, it could be determined whether the neonate was a chimera (summarized in Table 2) and the relative proportions of mutant and normal cells contributing to the chimera. An example is shown in Fig. 4. The overall genotypic composition of this *Lp*/*Lp*↔+/+ chimera (No. 4, Table 5) was approximately 70% *Lp*/*Lp* and 30% +/+. The specific proportions of *Lp*/*Lp* and +/+ cells for liver, intestines, eyes, skin and the neural axis are also shown. In this example, the brain tissue was not available for analysis. Six of the neonates with craniorachischisis could be shown to be chimeras by GPI analysis. In addition, one neonate with only exencephaly was determined to be chimeric. The remaining twelve neonates with craniorachischisis were either shown to be nonchimeric or their chimeric status could not be determined. Seven of these appeared to be 100% mutant. The remaining five neonates were composed of mutant (LP, HLP, or CLP) and normal (B6 or C3H) cells which express the same glucose phosphate variant, GPI-1B; therefore, no isozyme analysis was done.

The genotypic composition of the six neonatal chimeras with craniorachischisis by GPI analysis is shown in Table 5. These are presumed to be homozygous *Lp*/*Lp* chimeras because of their abnormal phenotype. Five of these chimeras (Chimeras 2 through 6) were LP↔BALB. The tissues analyzed were at least 20%

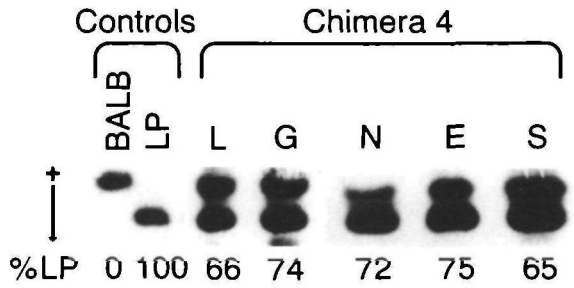

Fig. 4. Gel of GPI isozymes in mouse tissue homogenates from neonatal craniorachischisis Chimera 4. Lanes 1 and 2 are from adult liver of BALB (GPI-1AA) and LP (GPI-1BB) mice, respectively. Both isozymes, GPI-1AA and BB, are seen in various tissues of Chimera 4 (Lanes 3–7). The proportion of mutant (LP) isozyme in each tissue was determined by densitometer readings and is shown below each lane. The source of the GPI is as follows: L, liver; G, gut; N, neural axis; E, eyes; S, skin. Although the staining of the neural axis sample (N) may appear to be more than 72 % LP, we obtained similar measurements on repeated readings of this gel as well as from densitometric readings of the above photograph. Also, the 72 % LP was similar to that found in the other tissues of this chimera.

BALB, with the highest BALB proportion, 58 %, being in the gut and skin of Chimera 6. An exception was seen in the neural axis and gut of Chimera 3 which was 4 % and 9 % BALB, respectively. GPI analysis of Chimera 1, a HLP↔BALB chimera, showed a heterozygous band indicating it was A/B↔A/A. After correcting for this heterozygous band as described in Materials and methods, the resulting proportions of mutant and normal cells of Chimera 1 were similar to Chimeras 2 through 6. Overall, the proportions of mutant and normal cells are generally equivalent in all the tissues in each chimera. Five of the neonatal chimeras showed a difference of less than the 20 percentage points between the various tissues of each individual chimera.

Chimera 7 was an abnormal neonate that was found dead *in utero* upon Caesarian section. Instead of craniorachischisis, this neonate had exencephaly, with exposed eyes and a short tail. The spinal cord appeared grossly normal. Contrary to the other chimeras with craniorachischisis, the GPI analysis indicates that this mouse was predominantly BALB with a small proportion (≥10 %) being LP (Table 5).

Discussion

The results of this progeny testing of adult chimeras revealed that 2 normal-appearing chimeras were in fact *Lp/Lp*↔+/+. These chimeras expressed a normal phenotype without any overt evidence of neural tube defects. Mutant cells were known to be present in the neuroectoderm of these chimeras as evidenced by the presence of pigmented melanocytes, derivatives of the neural crest, in their coats. In addition, Chimera 811 revealed by GPI analysis that its neural retina was also composed of mutant and normal cells. Thus, the normal phenotype can be expressed in the presence of mutant cells indicating that the homozygous Loop-tail phenotype can be rescued.

Examination of neonatal chimeras with craniorachischisis, *Lp/Lp*↔+/+, by GPI analysis revealed that their tissues were predominantly mutant with most tissues being more than 60 % mutant. An exception is Chimera 6 which was on average 45 % mutant. More significantly, these results show that the full mutant phenotype can be expressed even when one-third to one-half of the cells are genotypically wild-type.

The striking result of this research is that an all-or-none craniorachischisis is observed. This suggests that some type of 'threshold' mechanism underlies the Loop-tail mutant phenotype. In some chimeras that threshold is not reached and the neural tube remains open, whereas in other chimeras the threshold is reached and the neural tube closes completely. Five of the neonatal chimeras that had craniorachischisis were at least 63 % mutant in their neural tissue (i.e. brain, eyes and neural axis). Chimera 6 (Table 5) had only about 47 % mutant cells present in its eyes, with 42 % mutant cells in its skin and gut. This would suggest that

Table 5. *Proportion of mutant cells in abnormal neonatal chimeras based on densitometer readings of GPI-1 isozymes*

	% Mutant					
	Liver	Gut	Brain	Eyes	Neural* Axis	Skin
Presumed *Lp/Lp* chimeras						
1†	88	79	63	89	NA	NA
2	81	73	71	NA	NA	NA
3	72	91	63	72	96	81
4	66	74	NA	75	72	65
5	67	65	64	NA	NA	84
6	46	42	NA	47	NA	42
Presumed *Lp*/+ chimera						
7‡	10	7	4	8	NA	7

NA, tissue is not available.
* Neural axis-spinal cord, vertebral column, and adjacent tissue.
† Hybrid chimera, HLP↔+/+.
‡ This chimera has only exencephaly, the spinal cord appeared grossly normal.

a certain percentage of mutant cells, in this case usually greater than 50–60 % mutant, is necessary for the defective phenotype. However, the two normal appearing *Lp/Lp*↔+/+ chimeras were also predominantly mutant. By coat color Chimera 811 was 60 % mutant (though only 49 % in neural retina) and Chimera 809 was 80 % mutant. These results suggest that there might not be a distinct proportion of mutant cells that would always result in the craniorachischisis phenotype.

However, that interpretation should be qualified. If the craniorachischisis is due to a defect in a single cell type, and we could determine the proportion of mutant cells of that type in chimeras, we might find that when the proportion exceeded a certain percentage, the mutant phenotype would be expressed. For example, if the defect were in the notochord and we could determine the genotype of the notochord cells, we might find that the proportion of mutant cells would always exceed 60 % in chimeras with craniorachischisis.

The 'threshold mechanism' that leads to all-or-none craniorachischisis could involve the level of a normal factor such that if a certain level is reached the neural tube will close, whereas if that level is not reached the tube will remain open. The term 'factor' is used in the very broadest sense to include circulating or diffusible substances, cellular components such as enzymes, and structural molecules such as membrane components. Conversely, the *Lp* mutation could result in the production of an abnormal and harmful factor, in which case the neural tube would remain open if the level of the aberrant factor exceeded the threshold. The fact that *Lp* is a semi-dominant mutation lends support to a model of a harmful factor being produced. A threshold mechanism might also be a temporal phenomenon where the tube must either begin to close by a particular time in development or it will remain completely open. Finally, it could be a spatial phenomenon whereby certain tissues or populations of cells must be in particular positions to initiate tube closure. Malpositioning of these tissues would result in the tube remaining open.

Other characteristics of Loop-tail also seem to involve thresholds. For example, the Loop-tail gene shows incomplete penetrance in heterozygous *Lp*/+ mice, with only 70 % of these mice having loop-tails. In the remaining 30 %, the normal threshold is apparently reached, and the mice, therefore, have normal tails. Threshold mechanisms can also be used to explain the presence of imperforated vaginas (Strong and Hollander, 1949) and blocked oviducts in heterozygous females (personal observations).

The genotypic composition of mutant and normal cells contributing to the neural axis, which included the spinal cord, vertebral column and adjacent tissue, of craniorachischisis neonates, was examined by GPI analysis. Ideally, only the neural tissue of the spinal cord should have been examined; however, it was difficult to isolate a pure sample since it was thin and adhered to the vertebral column. Since previous studies suggest that the proportion of genotypes in the tissues of a single chimera are usually similar (Forsthoefel *et al.* 1983; Gearhart and Oster-Granite, 1981), it seemed reasonable that the composition of the neural axis would reflect the composition of the spinal cord. We examined the neural axis in two of the neonatal chimeras. In Chimera 4 (Table 5) the genotypic composition of the neural axis was similar to the other tissues from that chimera. In contrast, Chimera 3 was observed to have a higher percentage of mutant cells in its neural axis in comparison to other tissues. This could be due to the normal component being below the 20th percentile, which can result in inaccurate quantitation (see Materials and methods), or the ratio is skewed by one of the tissues in the sample being overwhelmingly mutant. For example, Peterson (1979) showed that with certain strain combinations, one strain would predominate in muscle. Alternatively, it is conceivable that the observed proportions of the neural axis reflects the true proportions of the two cell populations. The remaining neural axes were also saved for possible histological analysis.

Chimera 7 is problematic as it might be an exception to the all-or-none craniorachischisis we observed in all the other chimeras. By GPI analysis, this exencephalic chimera was predominantly wild-type (BALB) with an average of only 7 % mutant cells in its tissues. This result suggests that the proportion of mutant cells may dictate the severity of the defect. Instead of an all-or-none mutant phenotype, occasionally a partially mutant phenotype, in this case exencephaly, might be produced. An alternative explanation for this exencephalic fetus is that it is simply a congenital defect and has nothing to do with being a Loop-tail chimera. It must be kept in mind that the GPI analysis only proves that it is a chimera, not that it is a homozygous *Lp/Lp* chimera. In fact, Chimera 7 was probably a heterozygous *Lp*/+ chimera, and the following results support this conclusion. Stein and Mackensen (1957) reported that 22 % of their heterozygotes had exencephaly. Smith and Stein (1962) found nine exencephalic mice from six *Lp*/+×+/+ matings, three of which had straight-tails. In this study, Chimera 7 was observed to have exencephaly and a straight-tail. With these observations in mind, our bias is that this chimera is an *Lp*/+↔+/+.

If intermediate expression was observed in these chimeras, it might have been possible to determine the defective cell type of the mutant Loop-tail. Chimeras show a series of transverse pigmented and albino stripes down and on either side of the dorsal midline of the animals' bodies. Since the neural crest cells that give rise to the melanocytes are derived from the lateral edge of the neural plate, we believed that alternating areas of predominantly mutant and wild-type populations might also be observed in the neural tube. This would have allowed us to determine if there was a correlation between open neural tube areas with the proportion of mutant cells. This type of analysis could have shed light on where the defective gene in the Loop-tail mutant is primarily acting.

Instead, an all-or-none craniorachischisis phenomenon was observed. It is possible that no intermediate expression, i.e. open and closed areas of the neural

tube, was observed due to cell mixing or cell interactions in the spinal cord itself. Previous reports demonstrate that there is extensive cell mixing in the central nervous systems of chimeras (photoreceptor cells, Mullen and LaVail, 1976; Purkinje cells, Mullen, 1977; inferior olive, Wetts and Herrup, 1982; facial nerve nucleus, Herrup *et al.* 1984; cortical somatosensory barrels, Goldowitz, 1987). Although this suggests that cell mixing occurs throughout the CNS, only recently has the spinal cord been examined. Our laboratory and others have reported extensive cell mixing of cells in the spinal cord, and that the proportions of cells with like genotypes are similar throughout the spinal cord (Peterson *et al.* 1988; Cichocki (Musci) and Mullen, 1989; and in preparation). These observations suggest that if the Loop-tail defect is intrinsic to the spinal cord then perhaps cell mixing prevents intermediate expression of neural tube closure in the spinal cord since all regions of the cord would have similar proportions of mutant and wild-type cells. In addition, cell mixing would contribute to the threshold mechanism whether if it be a factor, or a temporal or spatial phenomenon, because all regions of the cord would have a similar genotypic composition.

The results presented in this paper demonstrate that an all-or-none craniorachischisis usually occurs in Loop-tail mutant mouse chimeras. This study did not determine which tissue is primarily responsible for the defective neurulation in this mutant, but suggests that a threshold mechanism may be involved in the pathogenesis of this disorder.

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