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The induction of tail malformations in trisomy 16 mouse fetuses heterozygous for the *curly tail* recessive gene

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Summary

The mouse mutant *curly tail* is thought to be inherited as an autosomal recessive (*ct*/*ct*) with incomplete penetrance so that approximately 60% of *ct*/*ct* individuals exhibit the *curly tail* (CT) phenotype. By outcrossing *ct*/*ct* with mouse stock carrying specific heterozygous combinations of Robertsonian (Rb) chromosomes, trisomy 16 (Ts16) and Ts19 mouse fetuses (and their chromosomally balanced littermates) were derived which were heterozygous for the *ct* gene. All of the Ts16 (*ct*/Rb;Rb) fetuses, studied between days 14–19 gestation had tail malformations, 86% of which were tail flexion defects (TFD) apparently very similar to the *curly tail* phenotype. Neither Ts19 nor any of the chromosomally balanced (*ct*/Rb) littermates from both experimental crosses showed any type of tail or other spinal malformation. At the 27–29 somite stage of development, Ts16 (*ct*/Rb;Rb) fetuses did not show any significant delay in the closure of the posterior neuropore (PNP) compared with their littermate controls, suggesting that the tail malformation observed in Ts16 (*ct*/Rb;Rb) occur as a result of mechanisms which differ significantly from those thought to be responsible to causing the *curly tail* malformation.

1. Introduction

Autosomal aneuploidy in most mammalian species is a lethal condition, which in the case of monosomies results in death usually before implantation (Baranov, 1983; Hassold & Jacobs, 1984), and in most trisomies leads to embryonic or fetal death mediated through profound development delay and/or an association with multiple and presumably lethal malformations. Because of the high spontaneous incidence of human aneuploidy (Hassold & Jacobs, 1984), in contrast to other mammalian species (Bond & Chandley, 1983), human trisomies were, until recently, the main focus of attention for understanding the relationships between chromosome imbalance, disruption of normal development, and the association between different trisomies and malformations syndromes (Warkany, 1971).

Experimental studies of the embryonic and developmental mechanisms associated with autosomal trisomy and malformations in another mammalian species, became possible with the development of a mouse breeding system which exploits the high spontaneous frequency of non-disjunction at the anaphase of the first meiotic division when Robertsonian (Rb) chromosomes are carried in heterozygous combinations (Tettenborn & Gropp, 1970). The introduction of Rb chromosomes from feral mouse populations onto laboratory mouse backgrounds (Gropp & Kolbus, 1974; Gropp *et al.* 1975; Gropp & Winking, 1981), means that it is possible to induce, experimentally, trisomy (and hence monosomy) for any of the 19 autosomes in the mouse complement (see reviews by Gropp, 1982; Epstein, 1985).

Specific phenotypes are associated with each of the murine autosomal trisomies (reviewed by Gearhart *et al.* 1986), but, following the pattern in human aneuploidies, some murine trisomies do not survive beyond the headfold stage of development (slightly less than the halfway stage of gestation). Morphological studies, therefore, have concentrated on those trisomies which are compatible with longer survival *in utero*, for example Ts12 which is associated with exencephaly (Gropp & Kolbus, 1974; Gearhart *et al.* 1986); Ts16 with cardiac and renal malformations and gross oedema (Miyabara *et al.* 1982) and Ts19 with severe growth retardation and liver hypoplasia (White *et al.* 1972). Unlike man, however, none of the murine autosomal trisomies is compatible with extended postpartum survival.

With the exception of Ts12 and Ts14 mouse fetuses

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(which are associated with exencephaly), no other murine autosomal trisomy has been reported to have a significant association with neural tube defects (NTD). Tail and lower spinal lesions (including spina bifida) occur rarely and sporadically (Gearhart *et al.* 1986). However, induction of Ts16 and Ts19 on the A Strong background had resulted in a small proportion of Ts16 fetuses with tail flexion defects [(TFD) 12/257; 4·7%; Crolla, unpublished results]. No tail malformations were observed in corresponding Ts19 fetuses nor in the chromosomally balanced littermates of both trisomic breeding systems. This pattern suggested a possible genetic susceptibility of Ts16 (in our mouse stock) to TFDs and in order to test this hypothesis we decided to test-cross two sets of Rb-heterozygotes (see Materials and Methods) to a strain homozygous for the *curly tail* malformation, the phenotypic expression of which is similar, although not identical, to the TFDs associated with the Ts16 fetuses described above.

Curly tail is thought to be inherited as an autosomal recessive but with incomplete penetrance (Grüneberg, 1954; Embury *et al.* 1979), and to date, the chromosomal location of the *ct* gene has not been determined. Approximately 60% of the offspring from matings between *ct* homozygotes have a curly tail (CT) phenotype, which has been shown experimentally to arise following a delay in posterior neuropore (PNP) closure (Copp *et al.* 1982; Copp, 1985). Affected *curly tail* fetuses (and newborns) most commonly have a curly tail, around 20% have open spina bifida and/or more rarely (approximately 1%) exencephaly. The remainder (roughly 40% have a normal straight tail (ST) phenotype (Embury *et al.* 1979).

2. Materials and methods

(i) *Mouse stock and breeding protocols*

Closed colony, random bred (CT and ST) *curly tail* homozygotes *ct/ct* (with acrocentric chromosomes only) were mated with either Rb(9.16)9Rma/Rb(11.16)2H or Rb(5.19)1Wh/Rb(9.19)163H monobrachially homologous heterozygotes for Ts16 and Ts19 production respectively. Reciprocal male and female matings were carried out with the Rb9Rma/Rb2H × *curly tail* crosses, but only male Rb1Wh/Rb163H heterozygotes were mated with *curly tail* (CT and ST) females. Males and females were paired overnight and the females examined the following morning for vaginal plugs. The day on which the plug was noted was designated day 1 of gestation. All animals were kept in a constant 12 h light, 12 h dark environment with food and water given *ad libitum*.

(ii) *Morphological assessment of fetuses*

Pregnant females were sacrificed on either day 11 (for neuropore studies, see below) or between days 14–19 gestation. Uteri were dissected from the abdominal cavity and a note taken of fetal positions and of the number and position of resorptions. Each fetus of 14–19 days was examined under a Wild stereo dissecting microscope for evidence of spinal and/or tail malformations. Ts16 fetuses (Fig. 1) were readily identified by gross oedema in the cephalic and thoracic regions (irrespective of tail anomalies), and Ts19 fetuses were considerably smaller than their chromosomally balanced littermates and had severely hypoplastic livers. In all the cases of suspected Ts16 or Ts19, but also in those fetuses with other atypical phenotypes, the chromosome constitutions were confirmed from air dried chromosome preparations made from fetal liver.

(iii) *Scoring of posterior neuropore size*

Pregnant females were culled at 06.00 h on day 11 and the fetuses dissected from the uterus. Following the removal of the amnion, which was used for chromosome analysis (see below), the crown-rump measurement of each embryo was taken using a Zeiss eyepiece with a moving micrometer scale attached to a Wild stereo-dissecting microscope at a standard magnification. The fetuses were fixed overnight in Bouin's and the somites then counted; in those with 27–29 somites, the size of the PNP was scored according to the method and categories described by Copp (see footnote to Table 2).

(iv) *Chromosome preparation and analyses*

The chromosomes of the morphologically abnormal day 14 or later fetuses were counted from air-dried preparations obtained from suspensions of fetal liver cells incubated for 1 h in Ham's F10 containing 10 μg/ml colcemid. For day 11 fetuses, chromosome preparations and counts were made from the amnion of each individual fetus following a 1 h incubation in Ham's F10 and colcemid, followed by chromosome spreading using a modification of Meredith's (1969) method. Metaphases were stained in orcein, and at least three were counted from each fetus. Those with 2 Rb and 37 acrocentrics (i.e. 41 chromosome arms) were therefore either Ts16 or Ts19 [i.e. following non-disjunction of *both* Rb in the monobrachially homologous Rb-heterozygotes and fusion of such gametes with acrocentric chromosome germ cells from the *curly tail* stock (*ct*/Rb;Rb)]. Fetuses with 1 Rb and 38 acrocentrics [(40 chromosome arms) were therefore balanced (*ct*/Rb)]. In one day 16 fetus, Ts16 had resulted following non-disjunction in the *curly tail* (i.e. all acrocentric) parent (see below), and this karyotype was confirmed using a standard G-banding analysis (Cowell, 1984).

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3. Results

(i) *Incidence of Ts16 and Ts19 on the* curly tail *background and breeding studies*

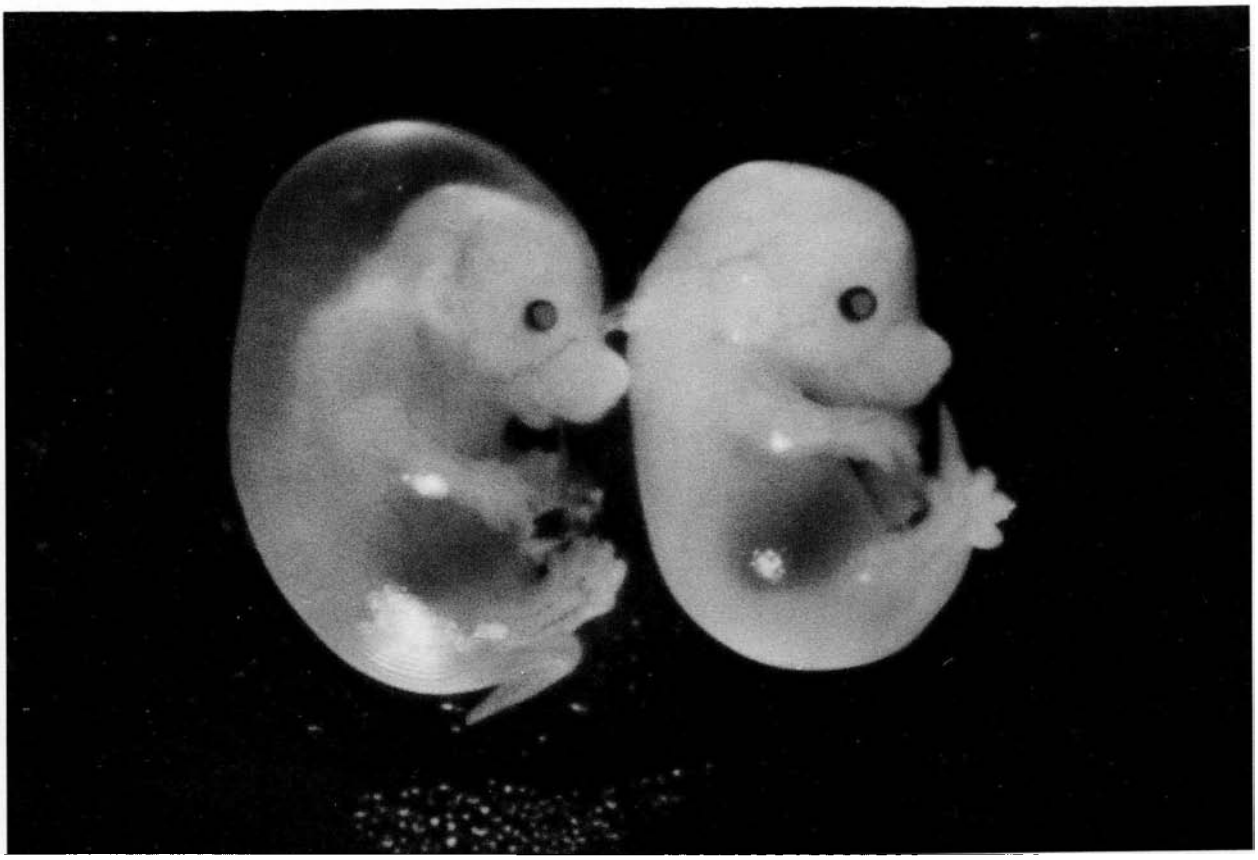
The overall incidence of viable Ts16 (*ct*/Rb;Rb) fetuses ranged from 17·6% on day 11 to 12·1% on days 17–19, and this frequency did not differ significantly when male or female Rb heterozygotes were used in the matings. Ts19 (*ct*/Rb;Rb) fetuses were only examined on day 15 gestation and a frequency of 18% viable trisomics was observed (data not shown). The incidence of *curly tail* newborns when the F_1 daughters (*ct*/Rb) were backcrossed to their *curly tail* father, was 29/195 (15%). This frequency of expression of *curly tail* individuals using the Rb9Rma/Rb2H stock was higher than observed in A Strong, but lower than BALB/c (Embury *et al.* 1979).

(ii) *The incidence of tail malformations in Ts16* (ct/*Rb*;*Rb*) *and Ts19* (ct/*Rb*;*Rb*) *fetuses*

All of the 31 viable Ts16 fetuses examined between days 14–19 gestation had a tail malformation of which 81% were TFDs in which the flexion exceeded 90° (Fig. 1); in two others, the angle of TFD was slightly less than 90° (Table 1). In this context, the day 16 Ts16 fetus derived by non-disjunction in the *curly tail* parent (i.e. *ct*/Rb, Ts16) was morphologically indistinguishable from the Ts16 fetuses derived from non-disjunction in the Rb heterozygote parents (*ct*/Rb;Rb). The tails of four further Ts16 (*ct*/Rb;Rb) fetuses were approximately half the normal length. By contrast, none of the 13 Ts19 (*ct*/Rb;Rb) fetuses had TFDs (or other tail malformations), and all the chromosomally balanced littermates (*ct*/Rb) from the Ts16 and Ts19 breeding systems had normal tails.

(iii) *Timing of PNP closure in Ts16 and balanced fetuses*

Fifty-one fetuses from *Curly tail* × Rb9Rma/Rb2H crosses were studied on day 11 gestation at the 27–29 somite stage of development. Fetuses were initially scored for PNP size and crown–rump length and subsequently the results of chromosome analyses were correlated with these measurements. The difference in mean crown–rump lengths of 9 Ts16 (*ct*/Rb;Rb) fetuses compared with the 41 chromosomally balanced *ct*/Rb controls was not significant at the 5% level. Ten Ts16 (*ct*/Rb;Rb) fetuses (including one in which the cephalic region was damaged during dissection and therefore could not be scored for crown-rump length) and their 41 chromosomally balanced littermates were subsequently re-classified into the three categories of PNP size defined by Copp (1985). Table



Two day 15 gestation mouse fetuses from female Rb9Rma/Rb2H × male *ct*/*ct* cross. The male fetus on the left (*ct*/Rb;Rb) shows the characteristic gross oedema associated with Ts16 together with a tail flexion defect. A chromosomally balanced, phenotypically normal male litter mate (*ct*/Rb) is shown for comparison.

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Table 1. *Incidence of tail malformations in* ct/ct × *Rb9Rma/Rb2H and* ct/ct × *Rb1Wh/Rb163H crosses.*

Phenotype of *ct/ct*	Rb heterozygote	Number of viable Ts16	Tail Malformations			
			Flexion > 90°	Flexion < 90°	Short tail	Total
CT	Rb9Rma/Rb2H	22	17	2	3	22
ST	Rb9Rma/Rb2H	9	8	—	1	9
CT+ST	Rb9Rma/Rb2H	31	25	2	4	31
		Number of viable Ts19				
CT	Rb1Wh/Rb163H	3	—	—	—	—
ST	Rb1Wh/Rb163H	10	—	—	—	—
CT+ST	Rb1Wh/Rb163H	13	—	—	—	—

Table 2. *Posterior neuropore size*[a] *and karyotype at day 11 gestation in fetuses from* ct/ct × *Rb9Rma/Rb2H crosses.*

	Balanced Number of somites					Ts16 Number of somites			
PNP category	27	28	29	Total (%)	PNP category	27	28	29	Total (%)
Closed	2	9	3	14[b] (34)	Closed	2	3	1	6[b] (60)
1/2	11	8	2	21[c] (51)	1/2	1	2	—	3[c] (30)
3	5	1	—	6[d] (15)	3	1	—	—	1[d] (10)
4/5	—	—	—	—	4/5	—	—	—	—
	18	18	5	41 (100)		4	5	1	10[e] (100)

[a] The PNP categories 1 and 2 are restricted to expanded distal region of tail bud. Category 3 extends beyond the distal expansion, but does not reach level of hindlimb rudiment. Categories 4 and 5 extends into the hindlimb bud (4) and somite (5) regions respectively.

[b,c] The distribution of closed *v.* category 1/2 PNP between Balanced and Ts16 fetuses was significant [χ^2 (with Yates' correction) $P < 0{\cdot}05$].

[b,d] The distribution of closed *v.* category 3 PNP in Balanced and Ts16 fetuses was not significant at the 5% level (χ^2 with Yates' correction).

[e] One fetus damaged in caudal region. Scored for PNP and somites but not crown–rump length.

2 shows that the Ts16 fetuses do not show any evidence of delayed PNP closure.

4. Discussion

Murine Ts16 has been proposed as an animal model for human Ts21 (Gropp, 1982; Epstein, 1985) not only because a number of genes are syntenic on mouse chromosomes 16 and human 21 (Polani & Adinolfi, 1980), but also because of the similarities of phenotypic effects (particularly congenital malformations) which correlate strongly, but not exclusively, with both trisomies. In this respect, approximately 50% of liveborn human Ts21 (Down syndrome, DS) have congenital heart anomalies (Shapiro, 1983), and 90% of Ts16 mouse fetuses have cardiac malformations similar in type and distribution to those observed in DS patients (Miyabara *et al.* 1982; Bacchus *et al.* 1987). The range of congenital malformations associated with Ts16 does not generally include those affecting the neural tube, and such anomalies in the trisomic mouse are restricted to the very high patterns of incidence of exencephaly in Ts12 (Gropp & Kolbus, 1974) and Ts14 (Gearhart *et al.* 1986) respectively. Amongst all murine trisomies, tail and lower spinal lesions occur rarely and with a sporadic pattern of incidence (Gearhart *et al.* 1986) and open spina bifida is seen infrequently in DS, although spina bifida occulta is approximately 100 times more common in DS than in the general population (Shapiro, 1983).

The mechanisms by which autosomal trisomy can give rise to characteristically abnormal phenotypes are not understood, but hypotheses have generally explored either effects mediated through the genetic imbalance caused by triplicated autosomal genes or developmental instability caused by the disruption of the normal regulatory pathways inherent with the trisomic condition (for review see Dyban & Baranov, 1987). In this latter context, Shapiro (1983) proposed that the range of abnormal phenotypes (including malformations), and of biochemical and physiological anomalies associated with DS occur as a direct result of the trisomy *per se* causing a 'generalized disruption of evolved genetic balance', which in turn decreases the trisomic organism's capacity to withstand environmental and genetic infuences.

The experiments described in this paper may offer some support for the latter part of Shapiro's (1983)

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hypothesis. Tail flexion defects and other lower spinal anomalies (Table 2) were only noted in Ts16(*ct*/Rb;Rb) heterozygotes, and backcross matings of *curly tail* with the Rb9Rma/Rb2H strain (Table 1) excluded increased penetrance of the *curly tail* gene on this genetic background as a possible explanation of the manifestation of TFDs. Overall, therefore, with very rate exceptions, *curly tail* and its more extreme phenotypic manifestations (spina bifida and exencephaly) are observed only in *ct*/*ct* individuals. From our experimental crosses, none of the *ct*/Rb chromosomally balanced littermates of either the Ts16 or Ts19 cohorts had tail malformations; nor, indeed did any of the Ts19 (*ct*/Rb;Rb) fetuses. The presence of the extra chromosome 16, therefore, remains the only variable which can account for the manifestation of a tail malformation in the Ts16 (*ct*/Rb;Rb) fetuses.

The TFDs observed in the Ts16 (*ct*/Rb;Rb) cohort, although very similar to the *curly tail* phenotype, were not completely or consistently analogous. None of the affected experimental fetuses had a complete 'curl' and the flexion, unlike *curly tails*, was very consistent in shape and position between individuals. Furthermore, four animals, without flexions, were seen with apparent tail shortenings, a phenotypic variant not seen in *curly tail* litters. Grüneberg (1954) proposed from his original studies of the *curly tailed* mouse that the characteristic kinked or curled phenotype arises because of a delay in the closure of the posterior neuropore (PNP). This hypothesis was confirmed experimentally by Copp (1985) who, using a whole embryo *in vitro* technique, was able to predict, by examining the size of the PNP at the programmed time of closure (day 11 gestation), whether or not any individual *curly tail* fetus would go on to develop the *curly tailed* phenotype.

By using the same scoring and analysis methods for PNPs of Copp (1985), we could not demonstrate any delay in PNP closure in those fetuses destined to develop TFDs (i.e. Ts16). Indeed, the data suggested that the PNPs of the Ts16 (*ct*/Rb;Rb) fetuses closed slightly before the chromosomally normal littermates. We conclude from this that the TFDs we observed in the Ts16 (*ct*/Rb;Rb) fetuses were not true *curly tails* but a modified expression of the *curly tail* gene caused by the specific effect of trisomy 16 and the more general effects this trisomy has on growth rates and cell kinetics within specific tissues.

Recent experimental evidence in the *curly tail* mouse has demonstrated that the rescheduling of growth rates within the tissues involved with neurulation (Copp *et al.* 1988) can cause significant reductions in the incidence of *curly tail* fetuses. We speculate that the inclusion of Ts16 on the *curly tail* genetic background may further increase the susceptibility of the *curly tail* gene to environmental and/or genetic effects and that the genetic effects are more marked in different tissues at varying times in development. Chromosome 16, therefore, may be the location of a regulatory or modifier gene for normal tail development, and the presence of one copy of a recessive mutant gene (in this case *curly tail*) together with the triplication of the proposed chromosome 16 gene, is sufficient to disrupt normal tail development.

In summary, we have induced tail flexion defects in Ts16 mouse fetuses heterozygous for the *curly tail* recessive gene and suggest that the tail malformation may have arisen because of the increased genetic susceptibility of Ts16 [as an analogy with DS-Shapiro (1983)] to mutant gene expression. The TFDs observed were not, however, true *curly tails* and may have arisen due to a modification of the complex interaction of many gene products responsible for normal cell growth during neurulation.

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