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Mini-mouse: phenotypic characterization of a transgenic insertional mutant allelic to pygmy

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Summary

A phenotypic analysis was performed on two alleles at the pygmy locus which arose by insertional mutagenesis in transgenic mice. Similar to the spontaneous mutant pygmy, the adult insertional transgenic mutants are 40% of the size of wild-type litter-mates whereas adult heterozygotes are 80% of wild-type litter-mates. An analysis of the various organs revealed that, in general, there was a reduction in weight of each organ commensurate with the overall reduction in body size. However, two organs did not follow this pattern, the brain being disproportionately larger and the adrenals disproportionately smaller in the mutant mice. In addition, mini-mice have less adipose tissue than their wild-type or heterozygous litter-mates. A developmental analysis determined that mutants could first be identified on the basis of reduced body weight at day 15·5 of gestation. The small size is not due to a growth hormone deficiency so these mice differ from other known dwarf mouse mutants. Therefore they should provide insight into the growth hormone-resistant human dwarfisms and help in furthering our knowledge of mammalian growth and development.

1. Introduction

A greater understanding of the complicated interactive biological processes of a multicellular organism will come from a genetic approach within the context of the whole organism. This has already given insights into the development of the invertebrates *C. elegans* (Kenyon, 1988) and *D. melanogaster* (Rubin, 1988), where the isolation of single genes and the elucidation of their roles have been extensively investigated. A similar analysis has not been possible in the mouse since the mammalian geneticist does not possess the range of sophisticated techniques required for a rapid genetic analysis as in the aforementioned organisms. To overcome this limitation, the advent of incorporating DNA into the germline of mice has provided a way of isolating mutants and their further molecular characterization (Palmiter & Brinster, 1986) with the foreign DNA, or transgene, being envisaged as an insertional mutagen. This approach has led to the isolation of a number of transgenic insertional mutants which express various aberrant developmental phenotypes (Reith & Bernstein, 1991; Meisler, 1992). These include mutations with effects ranging from limb deformities (e.g. legless; McNeish *et al.* 1990) to abnormal behavioral traits (e.g. chakragati; Ratty *et al.* 1990).

Because of the great potential of the technique, transgenic mice in our laboratory are routinely intercrossed to produce homozygosity for the disrupted locus so as to reveal any phenotypic aberration. Intercross matings in one transgenic mouse line consistently gave mice that were greatly reduced in size in comparison to both wild-type and heterozygous litter-mates, and were called mini-mice (Xiang *et al.* 1990). Further analysis determined that the founder transgenic mouse had two different integration patterns at the same locus, called A and B, and homozygosity for each resulted in the mini-phenotype (Xiang *et al.* 1990). Subsequently, the disrupted locus was cloned and mapped to mouse chromosome 10 (Xiang *et al.* 1990). Surprisingly, a known spontaneous mutant isolated in 1944 called pygmy mapped to a similar location (MacArthur, 1944; Green, 1989). Genetic and molecular studies confirmed that the spontaneous and insertional transgenic loci were allelic (Xiang *et al.* 1990). Since this is an insertional mutation at the pygmy locus, the two lines are designated $pg^{TgN40ACha}$ and $pg^{TgN40BCha}$. In order to gain insight

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into the function of the pygmy gene product, this study describes a phenotypic analysis of the mini-mouse mutant.

2. Materials and methods

(i) *Lines of mice*

The two transgenic insertional mutant lines $pg^{TgN40ACha}$ and $pg^{TgN40BCha}$ (formerly designated as sub-line A and B) originated from the same founder mouse (Xiang *et al.* 1990) which was generated from the microinjection of a fertilized mouse egg derived from an F_1 (CBA/J × C57BL/6J) × F_1 mating. Segregation of the $pg^{TgN40ACha}$ and $pg^{TgN40BCha}$ integrations was accomplished by mating the founder female with an F_1 male and then determining the genotype of the offspring by Southern blot, using the transgene as a probe (see below). Subsequent matings were between siblings resulting in mice of varying CBA/J and C57BL/6J hybrid backgrounds. Current backcross experiments of up to 12 generations of both $pg^{TgN40ACha}$ and $pg^{TgN40BCha}$ heterozygotes to the CBA/J and C57BL/6J strains individually have not revealed any difference in expression of the mini-phenotype (unpublished data).

Mice were maintained in standard cages, illuminated 14 h (05.00–19.00) per day and food and water were provided *ad libitum*.

(ii) *Genotyping*

The genotype of the mice was determined by either Southern blot or quantitative dot blot. For quantitative dot blot analysis, genomic DNA was isolated from tails (Hogan *et al.* 1986) and DNA concentration determined by the diaminobenzoic acid fluorimetric assay (Thomas & Farquhar, 1978). Serial dilutions of genomic DNA were denatured and applied to nitrocellulose and hybridized with the nick-translated transgene (Sambrook *et al.* 1989). Hybridization was quantitated by scintillation counting. Homozygotes for the transgene integration event have twice the hybridization signal as their corresponding heterozygotes when normalized for DNA amount.

Southern blot analysis was performed by cleaving 10 μg of mouse tail DNA (Hogan *et al.* 1986) with the restriction enzyme *Eco*R I and electrophoresing in 0·8% agarose gels at 40 V for 16 h. DNA was transferred to nitrocellulose and baked at 80 °C for 2 h in a vacuum oven. Blots were then hybridized with the nick-translated transgene (Sambrook *et al.* 1989). Hybridization with the 2·8 kb transgene differentiates between the $pg^{TgN40ACha}$ and $pg^{TgN40BCha}$ heterozygotes and is based on the presence of a unique 6·0 or 8·6 kb hybridizing band respectively as previously described (Xiang *et al.* 1990).

(iii) *Growth curves*

Starting at one week, males from litters of both heterozygous $pg^{TgN40ACha}$ and heterozygous $pg^{TgN40BCha}$ intercross matings were weighed weekly on an analytical balance. Data were collected for 14–16 weeks at which time the mice were genotyped using quantitative dot blot analysis and the weight time point averages calculated. Average weights according to genotype were then plotted against time in weeks.

(iv) *Developmental analysis*

Initial appearance of the mutant phenotype based on reduced body weight was determined in the following manner. Heterozygous $pg^{TgN40ACha}$ intercrosses were established and the day of fertilization determined by daily inspection of females and recording of copulation plugs. Appearance of a copulation plug constitutes 0·5 days post coitum (d.p.c.). Females were killed by cervical dislocation at 14·5, 15·5 and 16·5 d.p.c. and litters dissected. Embryos were separated from the yolk sac and placenta, dried off with twisted pieces of tissue paper and weighed on an analytical balance. Tail tissue was removed from the embryos to make DNA for genotyping. Weights were grouped according to genotype and age and the Student's *t*-test statistical analysis performed.

(v) *Tissue analysis*

Analysis of adult tissues was performed by cervical dislocation of age-matched males and females of the three genotypes in line $pg^{TgN40ACha}$ followed by the dissection and weighing of the various tissues. The average tissue weight was calculated for each genotype and the heterozygote and homozygote values expressed as a percentage of wild-type value. Mice were genotyped by quantitative dot blot as previously described and statistical analysis carried out using Student's *t*-test.

(vi) *Determination of fat index*

The procedure described by Rogers & Webb (1980) was used to determine the fat index. Briefly, eight-month-old male mice from line $pg^{TgN40BCha}$ intercrosses were killed by cervical dislocation and their gonadal fat pads removed. The remaining carcass was dried for 24 h in a vacuum oven at 105 °C to constant weight. The gonadal fat pads were desiccated for 3 d to constant weight using calcium chloride under vacuum and the fat index determined by dividing the constant gonadal fat pad weight by the constant body weight. This ratio has been shown to be a reliable

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Table 1. *Segregation of mini-phenotype as an autosomal recessive trait*

Parental genotype	Phenotype			Tested			
	Total	Normal	Mini	Total	Wild-type	Heterozygote	Homozygote
$pg^{TgN40ACha}/+ \times +/+$	139	139	0	139	77	62	NA
$pg^{TgN40BCha}/+ \times +/+$	170	170	0	170	87	83	NA
$pg^{TgN40ACha}/+ \times pg^{TgN40ACha}/+$	546	424	122[a]	214	46	103	65
$pg^{TgN40BCha}/+ \times pg^{TgN40BCha}/+$	382	291	91[b]	91	25	42	24
$pg^{TgN40ACha}/+ \times pg^{TgN40BCha}/+$	149	115	34[c]	54	9	30	15*

Transmission of the mini-phenotype and co-segregation with the transgene. The various matings outlined in the table were established and the genotype of the mice determined as in Materials and methods. Mini-mice were identified by visual inspection.
NA = not applicable.
* Compound heterozygote ($pg^{TgN40ACha}/pg^{TgN40BCha}$)
[a] $\chi^2 = 55{\cdot}83$, $P > 0{\cdot}975$.
[b] $\chi^2 = 25{\cdot}77$, $P > 0{\cdot}950$.
[c] $\chi^2 = 12{\cdot}98$, $P > 0{\cdot}995$.

indicator of body fat content (Rogers & Webb, 1980). Mice were genotyped by quantitative dot blot and fat indices grouped and averaged, and statistical analysis was performed using the Student's *t*-test.

3. Results

(i) *Mutation segregates as an autosomal recessive trait*

Whilst breeding transgenic mice to homozygosity for the disrupted locus so as to identify recessive mutations, mice of reduced stature consistently appeared in one transgenic line. Closer analysis determined that this transgenic line consisted of two different integration patterns at the same locus. These were designated lines $pg^{TgN40ACha}$ and $pg^{TgN40BCha}$ based on the presence of a 6·0 or 8·6 kb hybridizing band in addition to the 2·8 kb transgene repeat unit, when DNA was digested with *Eco*R I and probed with the transgene (Xiang *et al.* 1990). This transgenic line was studied further by establishing heterozygote by wild-type matings for both lines to determine the mode of transmission and to rule out the possibility of a translocation (Mahon *et al.* 1988; Gordon *et al.* 1989; Francke *et al.* 1992) (Table 1). Heterozygotes from both lines were fertile, producing litters of normal size (unpublished data), with heterozygous and wild-type progeny appearing in equal proportions. Intercross matings within each line resulted in the production of homozygotes, all of which were small. In addition to homozygotes for either the $pg^{TgN40ACha}$ or $pg^{TgN40BCha}$ integration event being small, compound heterozygotes were also small. These compound heterozygotes were identified on the basis of their DNA containing both the 6·0 and 8·6 kb hybridizing bands. This demonstrated that the $pg^{TgN40ACha}$ and $pg^{TgN40BCha}$ insertional mutants were allelic. Analysis of mating data (Table 1) determined that the mutation segregated as an autosomal recessive trait since the χ^2 values indicate that the various genotypes appeared in the expected ratios.

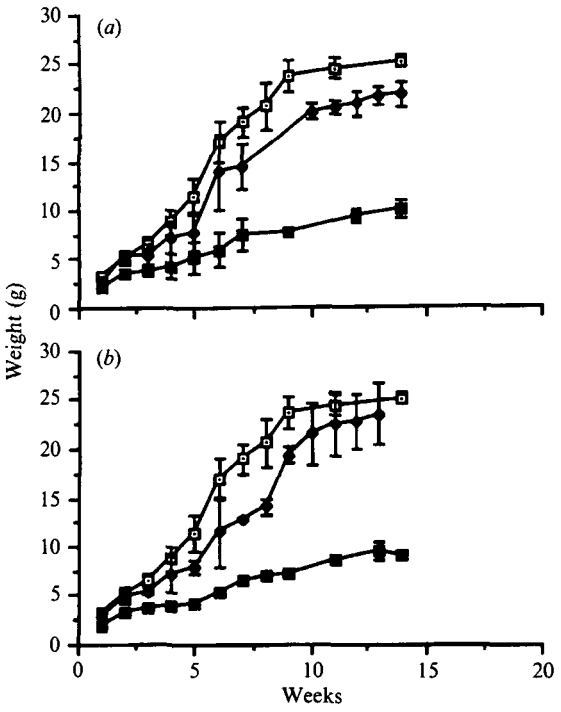

Fig. 1. Growth curves of male progeny of all genotypes (wild-type, heterozygous and homozygous) from matings between heterozygous mice in line $pg^{TgN40ACha}$ (*a*) and line $pg^{TgN40BCha}$ (*b*). All mice were analysed by quantitative dot blot hybridization as described in Materials and methods. Standard error bars are as indicated and data points lacking error bars have a standard error value equal to or less than the size of the marker. Time points are averages representing values from 3 to 6 mice. (*a*) ⊡—⊡, Wild-type; ◆—◆, heterozygous $pg^{TgN40ACha}$; ■—■, homozygous $pg^{TgN40ACha}$; (*b*) ⊡—⊡, wild-type; ◆—◆, heterozygous $pg^{TgN40BCha}$; ■—■, homozygous $pg^{TgN40BCha}$.

The most prominent feature of the mutation in the adult mini-mice is proportionate small stature. The growth curves of male mice (Fig. 1) demonstrate that

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Table 2. *Determination of initial onset of mini-phenotype*

Genotype	Age (d.p.c.)	No. of embryos	Body wt. (g)	*P*
Homozygous $pg^{TgN40ACha}$	14·5	7	0·240 ± 0·023	N.S.
Heterozygous $pg^{TgN40ACha}$		13	0·241 ± 0·027	
Wild-type		6	0·249 ± 0·047	
Homozygous $pg^{TgN40ACha}$	15·5	5	0·381 ± 0·015	< 0·001*
Heterozygous $pg^{TgN40ACha}$		5	0·458 ± 0·033	
Wild-type		3	0·434 ± 0·014	
Homozygous $pg^{TgN40ACha}$	16·5	4	0·567 ± 0·024	< 0·005
Heterozygous $pg^{TgN40ACha}$		3	0·683 ± 0·035	

N.S., not statistically significant.
* Values for heterozygous $pg^{TgN40ACha}$ and wild-type were combined since they are not significantly different.

within the first two weeks of age both $pg^{TgN40ACha}$ and $pg^{TgN40BCha}$ homozygotes are significantly smaller ($P < 0·01$, Student's *t*-test) compared to their heterozygous and wild-type counterparts and this is confirmed by visual inspection. There is no statistical difference between the weights of heterozygous and wild-type mice in either line during this time period. Growth is completed by 10 weeks and at this time, heterozygotes in $pg^{TgN40ACha}$ are also retarded in their overall growth ($P < 0·001$, Student's *t*-test) attaining about 80% the weight of their wild-type litter-mates. Most dramatically, of course, homozygotes of both lines are considerably smaller at 10 weeks of age than wild-type litter-mates (approximately 40%) and there is no statistical difference in the weights between homozygotes of line $pg^{TgN40ACha}$ and $pg^{TgN40BCha}$. Preliminary results with homozygous females in both lines and the compound heterozygotes, $pg^{TgN40ACha}/pg^{TgN40BCha}$, also suggest a similar growth curve as described above (unpublished results).

(ii) *Mini-phenotype is apparent at day 15·5 of gestation*

Since the insertional transgenic mutants could be distinguished at birth, as previously noted for the spontaneous mutant (King, 1955), a developmental analysis was performed on mini-mice to determine when the small phenotype is first manifested (Table 2). Litters were dissected from intercross matings in line $pg^{TgN40ACha}$ at 14·5, 15·5 and 16·5 d.p.c. A statistical difference was observed between homozygotes and their respective heterozygous and wild-type litter-mates beginning at day 15·5 of gestation ($P < 0·001$) (Table 2) but not at day 14·5 of gestation ($P < 0·832$). There was no significant difference between heterozygous and wild-type mice at any of these stages.

(iii) *Specific tissue weight analysis*

The phenotypic analysis was extended by measuring the wet weight of various organs of wild-type, heterozygous $pg^{TgN40ACha}$ and homozygous $pg^{TgN40ACha}$ mice (Fig. 2). Most organs (kidneys, heart, lungs, liver) of the mini-mice weigh 40–60% of wild-type litter-mates, which is commensurate with the reduction in their overall weight. However, their brains were found to be nearly the same weight (90%) as that of their wild-type litter-mates. In contrast, the testes were found to be smaller than expected (17%) and the most severe reduction occurred in the adrenal gland, being 12 and 16% of the weight of wild-type litter-mates for male and female mini-mice respectively. The significance of this sex difference is not obvious. A similar pattern of tissue effect was reflected in the heterozygous $pg^{TgN40ACha}$ mice, resulting in larger than expected brain size (97%), slightly smaller testes (67%) and the most severe effect occurring in the adrenals (58 and 67% for males and females re-

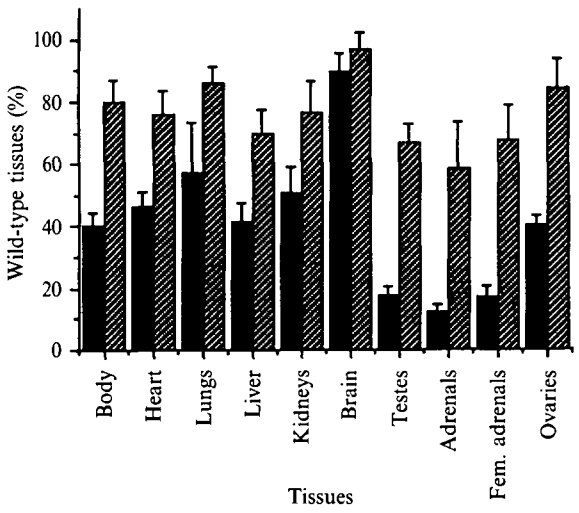

Fig. 2. The mean body weight and wet weight of indicated tissues of homozygous $pg^{TgN40ACha}$ (black) and heterozygous $pg^{TgN40ACha}$ mice expressed as a percentage of wild-type counterparts. Male mice were used with the exception of the female adrenal and ovary values. Using Student's *t*-test, all homozygous tissues were significantly different ($P < 0·001$) except brain. Heterozygous tissues were significantly different ($P < 0·005$) except ovaries and brain. The mean weight of each sample was obtained from 3–5 wild-type mice, 5–12 heterozygous mice and 5–12 homozygous mice.

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Table 3. *Body weights (BW), gonadal fatpad weights (GFPW) and ratios of gonadal fatpad weight to body weight (fat index) from eight-month-old male mice*

Genotype	No. of mice	BW (g)	GFPW (g)	GFPW/BW (mg g−1)
Homozygous $pg^{TgN40BCha}$	7	7·65 ± 2·26	0·078 ± 0·039	12·02 ± 8·61*
Heterozygous $pg^{TgN40BCha}$	7	16·47 ± 2·18	1·082 ± 0·206	66·96 ± 18·07**
Wild-type	6	17·28 ± 2·19	1·651 ± 0·666	94·50 ± 33·35

* $P < 0·001$ for both homozygous versus heterozygous and homozygous versus wild-type.
** Heterozygous versus wild-type was not significantly different.

spectively). All other tissues were 70–90% the weight of wild-type mice. Currently a detailed histological analysis of all organs of mini-mice described in this study is being carried out and to date light microscopy has not revealed any apparent abnormalities (unpublished data).

(iv) *Mini-mouse has greatly reduced body fat*

Whilst performing the dissections of mice for the tissue study it became apparent that the mini-mice had much less body fat than their heterozygous and wild-type litter-mates. In order to investigate this further, the fat index (gonadal fat pad weight versus body weight) which is an indicator of total fat content, was determined for mice of the $pg^{TgN40BCha}$ line using the procedure of Rogers & Webb (1980) (Table 3). Fat content was significantly reduced in the mini-mice as compared to heterozygous and wild-type mice ($P < 0·001$) which did not differ significantly ($P < 0·085$).

4. Discussion

This study describes the phenotypic characterization of two allelic transgenic insertional mutants which were found by genetic and molecular analysis to be allelic to a previously described spontaneous mouse mutant, pygmy (Xiang *et al.* 1990; Green, 1989). Mating data demonstrate that this is an autosomal recessive trait and presence of the transgene in either line results in normal segregation and production of all genotypes in the expected ratios.

The original observations on the spontaneous mutant (King, 1955) state that the phenotype is apparent at birth and the six-week weight of homozygotes is about 50% that of normal litter-mates (wild-type and heterozygous combined). Our analysis is in agreement with this finding. Growth curve data determined that adult mini-mice are 40% the size of their wild-type litter-mates. In addition, there is an effect on growth in the heterozygote which only attains 80% the size of its wild-type litter-mates. Although this effect on the spontaneous pygmy heterozygote was previously noted (Warwick & Lewis, 1954; King, 1955) it was not quantified. This suggests that the pygmy gene is not a 'true' recessive. Disruption of one allele is sufficient to elicit an effect on growth, with the absolute amount of the pygmy gene product produced determining the extent of this effect.

To extend further the original observation that the small phenotype is apparent at birth in the spontaneous mutant (King, 1955), a developmental analysis was initiated on the insertional mutant which established the initial appearance of the small phenotype at day 15·5 of gestation. This result leads to the conclusion that the pygmy gene must be expressed before day 15·5 of gestation.

The next step was to determine what effect this reduction of growth had on specific tissues in the adult mini-mouse. In general, tissues were 40–60% reduced in weight as compared to wild-type but two unexpected observations were made. First, the lack of a significant effect of the mini-mouse mutation on brain size suggests that growth regulation for this organ may differ from the rest of the body. Interestingly, in hypophysectomized rats, brain growth is not altered, in contrast to all other tissues, with the adrenals and gonads being most severely effected (Glasscock *et al.* 1990, 1991). Additional evidence for independent regulation of brain growth can be derived from studies of transgenic mice expressing rat growth hormone (GH) or human insulin-like growth factor I (IGF-I) under the control of the mouse metallothionein promoter (Palmiter *et al.* 1982; Matthews *et al.* 1988). In IGF-I transgenic mice, the increased local synthesis of IGF-I in the brain results in increased brain growth (Quaife *et al.* 1989; Behringer *et al.* 1990), whereas excess GH in GH transgenic mice, which only produces increased circulating levels of IGF-I, does not give rise to an increase in brain size (Shea *et al.* 1987).

The second finding is the enhanced reduction in the size of the adrenals in the homozygous insertional

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mutant. This result conflicts with the original description of pygmy which states that adrenal size is normal (King, 1955). However, a study by Chubb & Nolan (1985) on the fertility of genetically small mice includes data on the smaller size of pygmy adrenals. In addition, the adrenals of three spontaneous pygmy mutants were analyzed and found to be greatly reduced in weight (unpublished data). It is interesting to note that in humans, impaired adrenal function can result in reduced fertility, high incidence of cryptorchidism (undescended testes) and lipodystrophy (absence of subcutaneous fat) (Kannan, 1988), which are also seen in the pygmy mouse.

While there is no evidence to suggest that the small size of pygmy mice is due to a primary defect in the adrenal, small adrenals could be a secondary effect resulting from a defect in the pituitary–hypothalamic axis. The evidence for this comes from the inability of spontaneous pygmy mutant mice to elicit an increase in the release of prolactin when injected with perphenizine (Sinha *et al.* 1979). However, another pituitary hormone, growth hormone, appears to be unaffected by the mutation since there are normal levels of circulating growth hormone in both the spontaneous mutant (Sinha *et al.* 1979) and the insertional mutants (M. Low, K. Benson & K. Chada, unpublished data). This is in contrast to three other mouse mutants of small size; Ames dwarf (*df*), Snell's dwarf (*dw*) and little (*lit*), where reduced growth can be directly attributed to a lack of circulating growth hormone (Green, 1989). In addition, pygmy pituitary implants are capable of rescuing the Snell's dwarf (*dw*) mutation (King, 1955). Finally, while the pygmy phenotype may be due to a subresponsiveness to growth hormone, it is noteworthy that the pygmy mutation is manifest prior to the time when growth hormone elicits its main effect, since total lack of growth hormone does not affect size until two to three weeks after birth (Eicher & Beamer, 1976; Girard, 1989).

The nature of the phenotype suggests the possibility of a growth factor, growth factor receptor or binding protein being a product of the pygmy locus. However, because of its localization on chromosome 10, the locus cannot be for the structural genes coding for somatostatin, growth hormone releasing factor or receptor, growth hormone, growth hormone receptor (and binding protein), insulin-like growth factor (IGF) I or II or their corresponding receptors (Lalley *et al.* 1988; Encyclopedia of the Mouse Genome III, 1993). Previously, IGF-I was a likely candidate since it mapped to mouse chromosome 10, but it has been localized to approximately 25 centiMorgans proximal to pygmy (Justice *et al.* 1990; Taylor & Grieco, 1991). Nonetheless it is interesting to note the similarities in phenotypes between pygmy and the recently created IGF-I null mouse mutants which have their IGF-I gene disrupted by gene targeting (Jeh-Ping *et al.* 1993). These include intrauterine growth deficiency, infertility, craniofacial appearance and delayed bone ossification (Baker *et al.* 1993). The similarities suggest that the pygmy gene product may interact directly or indirectly in the IGF-I pathway. This is an interesting corollary since IGF-I has only recently been shown to play an important role in embryonic growth (Baker *et al.* 1993; Jeh-Ping *et al.* 1993; Powell-Braxton *et al.* 1993).

One of the major applications of isolating mouse mutants is the possibility of developing models of human genetic diseases. There are many human dwarf syndromes and one which we became interested in is the Russell–Silver syndrome (RSS) (Silver *et al.* 1953; Russell, 1954; McKusick, 1990), since it has many phenotypic manifestations in common with the pygmy mouse model. These include intrauterine growth retardation, high bossing of the forehead, lack of subcutaneous fat, normal levels of growth hormone and non-responsiveness to growth hormone therapy, high incidence of cryptorchidism, and delayed bone ossification (King, 1955; Tanner *et al.* 1975; Marks & Bergeson, 1977; Escobar *et al.* 1978). Variability of expression of the RSS phenotype suggests that it may be due to a multigenic effect. In a similar way, the pygmy phenotype can be influenced by genetic constitution since placing the pygmy mutation on a larger mouse background results in restoration of fertility in homozygotes (King, 1955).

This study elucidates the initial appearance of the pygmy phenotype and characterizes the effect of this mutation on various organs and tissues. This should provide valuable information for defining the function of the pygmy gene in normal growth and development and the mice could be possible models for human dwarf growth hormone-resistant syndromes such as Russell–Silver.

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