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Sex-specific modifiers of tail development in mice heterozygous for the brachyury (*T*) mutation

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Abstract. The brachyury, or *T,* locus encodes a transcription factor that plays a crucial role in the early development of all animals. In the mouse, animals heterozygous for a null mutation at this locus are born with a characteristic short tail. Expressivity of the short tail phenotype is greatly affected by genetic background. As a genetic entry into the identification of genes that interact with the Brachyury locus, we have performed a QTL analysis for modifiers of this phenotype. Surprisingly, we discovered that the major modifiers uncovered all act in a sex-limited manner. We have identified two QTLs—*Brm1* on Chr 9 and *Brm2* on Chr 15—that act only in female offspring(N_2) from female T/+ parents(F_1) and are responsible together for most, or all, of the genetic variance in phenotypic expression observed between C57BL/10 and C3H/HeJ animals.

Introduction

The brachyury (*T*) mutation was among the first to be discovered with a dominant effect on a developmental process by Dobrovolskaia-Zavadskaia and Kobozieff (1927). Animals heterozygous for the mutation (*T*/+) are born with a characteristic shortened, blunt-ended tail, while homozygous *T/T* embryos die in utero on the 11th day of gestation with defects in the formation of posterior mesoderm and notochord differentiation. In 1990, the *T* locus was cloned, and with this cloning came the tools required to investigate its gene product and its biochemical effect on development (Hermann et al. 1990). Studies along these lines have demonstrated that the *T* locus product is a transcription factor expressed specifically in nascent mesoderm and in the differentiating notochord (Herrmann and Kispert, 1994). The gene is strongly conserved across animal species from mammals to flies.

Molecular studies have also demonstrated that the original *T* mutation contains a deletion of approximately 200 kb that extends over the entire transcription unit. Thus, the *T* mutation is a true null allele, and its dominant effect on phenotype is almost certainly a consequence of haploinsufficiency. Dominant haploinsufficient phenotypes have also been demonstrated with null alleles at a number of other loci that encode transcription factors expressed during development, including various members of the Pax gene family (Schmahl et al. 1993). The accumulated data suggest that the developing embryo may be extremely sensitive to the precise concentrations of certain critical transcription factors, such that a twofold reduction can have drastic developmental consequences. In contrast, twofold reductions in the concentration of most other mammalian gene products have no visible effect on phenotype.

It has long been known that the amount of tail shortening caused by heterozygosity for a *T* mutation is greatly affected by genetic background. On certain inbred backgrounds, like those of the C3H family, *T*/+ animals tend to have tails that are reduced in length by 50% or less. On other backgrounds, like those of the C57 family, tail lengths of *T*/+ animals tend to be reduced by 90% or more. These results clearly demonstrate the existence of modifier genes with strong effects on the expressivity of the *T* phenotype.

With the easy recognition and quantitation of small changes in the effective concentration of the *T* gene product through the measurement of tail length, this phenotype provides an exquisitely sensitive assay for allelic differences at secondary loci that interact, at some level directly or indirectly, with the *T* locus gene or its product. Changes in gene products that lie upstream, downstream, or interact directly with the T protein or its binding site could all have an impact on the expression of the shortened tail phenotype. Thus, the identification of genetic modifiers of the *T*/+ phenotype could provide an entry into other genes of developmental importance.

This paper describes the first identification and mapping of modifier loci that act upon the expression of the *T* locus phenotype. Surprisingly, each of the loci identified operates in a sex-limited manner. In the case of females, we have identified two modifier loci that act equally and additively and appear to account entirely for the genetic variance in phenotypic expression observed between C57BL/10 (B10) and C3H/HeJ (C3H) animals heterozygous for a *T* mutation.

Materials and methods

Phenotypic and genotypic analyses. A pair of reciprocal two-generation crosses were used in this study. In cross D [(C3H × B10-T) × B10], C3H females were crossed with congenic *T*-bearing males on the B10 background. Female offspring that inherited the *T* mutation were backcrossed to B10 males (wild-type at the *T* locus). In cross M [B10 × (B10-T × C3H)], congenic *T*-bearing females on the B10 background were crossed to C3H males, and male offspring that inherited the *T* mutation were backcrossed to B10 females (wild-type at the *T* locus). Three hundred and twenty eight N_2 mice with a *T*/+ genotype were generated. Each N_2 animal was subjected to a measurement of tail length as a fraction of body length. Since development of the tail is not yet accomplished at the time of the birth, the measurement was performed on 6-week-old animals at the weaning period. The DNA from tailless animals, which were born with visible body abnormalities and could not survive till the weaning period, was taken at the day of birth.

Genomic DNA was prepared from animal tissues according to standard protocols (Maniatis et al. 1978). Primers were purchased from Research Genetics and PCR was performed as indicated by the manufacturer (Dietrich et al. 1994). Amplified DNA products were separated by electrophoresis on 7% acrylamide gel and stained with ethidium bromide.

Linkage analysis. All phenotypic and genotypic data were recorded and analyzed with the Microsoft Excel software package on the Macintosh computer. As a first step in the identification of potential QTLs, we performed genotype analyses on 24 animals combined from the two backcrosses with the most extreme expression of the short tail phenotype; all of these animals had no tail at all. Genotyping was performed on this set of

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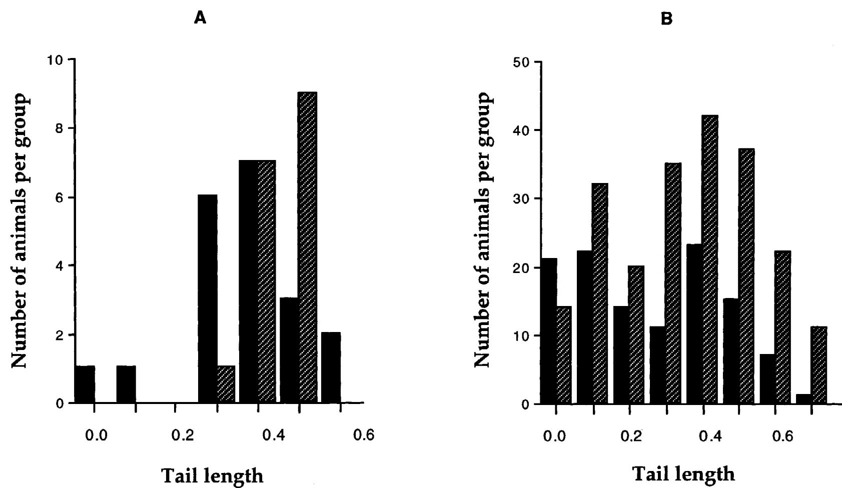


Fig. 1. Observed distribution of tail lengths for $T/+$ animals in **(A)** F_1 and **(B)** N_2 populations separated according to cross. Data from the M cross are shown with black bars. Data from the D cross are shown with hatched bars.

Table 1. Characteristics of Brachyury-modifies QTLs.

Modifier	Chr	Closest marker	Position (cM)	*P* value	Carrier sex	Contri-bution[a]
Brm1	9	*D9Mit48*	34.0	9.1 × 10 (−6)	F	52.3
Brm2	15	*D15Mit189*	44.3	4.9 × 10 (−5)	F	45.8
Brm1 + *Brm2*	—	—	—	1.5 × 10 (−10)	F	98.1
	X	*DXMit25*	27.8	2.5 × 10 (−3)	M	35.7

[a] The contribution to the total taillessness was calculated as the difference between the means of animals homozygous and heterozygous for that locus, divided by the difference between the means of the C57Bl10/*T* and F_1 females from D cross.

mice at 98 microsatellite markers distributed at ~25-cM intervals throughout the whole genome. Markers with potential linkage to Brachyury modifier loci were identified on the basis of an overrepresentation of B/B homozygote genotypes among members of the extreme group. When 17 or more animals were found to be B/B at a particular marker, a simple χ^2 test (with a null hypothesis of 12 B/B: 12 B/C Genotypes expected in the absence of linkage) yielded a *P* value of less than 0.05. Although this *P* value was far from sufficient for the demonstration of a true modifier, it provided candidate regions for further study.

As a scond test for potential modifier loci, 24 animals in total from both the shortest (tailless) extreme and the longest extreme were analyzed from each cross separately. Significance was estimated with the *t*-test with a self-imposed cutoff of $P < 0.005$.

Markers that passed this second test provided the identification of candidate chromosomal regions that were genotyped across multiple microsatellite loci in the entire N_2 population of 328 animals. The genotypic data obtained were subjected to interval mapping by linear regression (Martinez and Curnow 1992). The contribution to total variance exerted by individual QTLs was measured as the proportion of the total variance in linear regression that was not accounted for by the residual variance; the values obtained were likely to underestimate the true contributions of each QTL (Xu and Atchley 1995).

Results

The distribution of tail length measurements (as a fraction of total body length) for the F_1 and N_2 populations analyzed in this study is shown in Fig. 1. The most extreme form of the phenotype—taillessness—was expressed by 18% (21) of the N_2 animals of the M cross and 7% (14) of those generated in the D cross. Twenty-four of these extreme animals were genotyped in a first-level genome scan with 98 microsatellite markers. This scan identified seven genomic regions—on Chrs 1, 4, 9, 10, 15, 19, and the X—with an overrepresentation of the B/B genotype based on an uncorrected *P* value of less than 0.05. Five of these regions contained previously identified loci with mutations that cause skeletal or tail abnormalities.

All seven regions were subjected to a second-level analysis for linkage with independent sets of 24 animals from each cross from both extremes of the distribution. After this second-level screening, only three potential QTLs (on Chrs 9, 15, 19) were retained for further analysis on the basis of *P* values that were less than 0.005. All three showed evidence of a significant departure from the null hypothesis for the D cross only.

These results suggest a parent-of-origin or X Chr effect on the expression of the phenotype. According to the genetic and physical imprinting maps of the mouse (Beechey and Cattanach 1997) there are no imprinted regions on Chr 9 and 15 within the regions for *Brm* modifiers. The microsatellite *D19Mit11* is 10 cM from the imprinted gene *Insl* (49 cM). From this observation, we proceeded with X Chr analysis even though the *P* value for the best potential X Chr QTL-linked marker was only 0.032. Only the D cross allows an analysis of linkage on the X Chr.

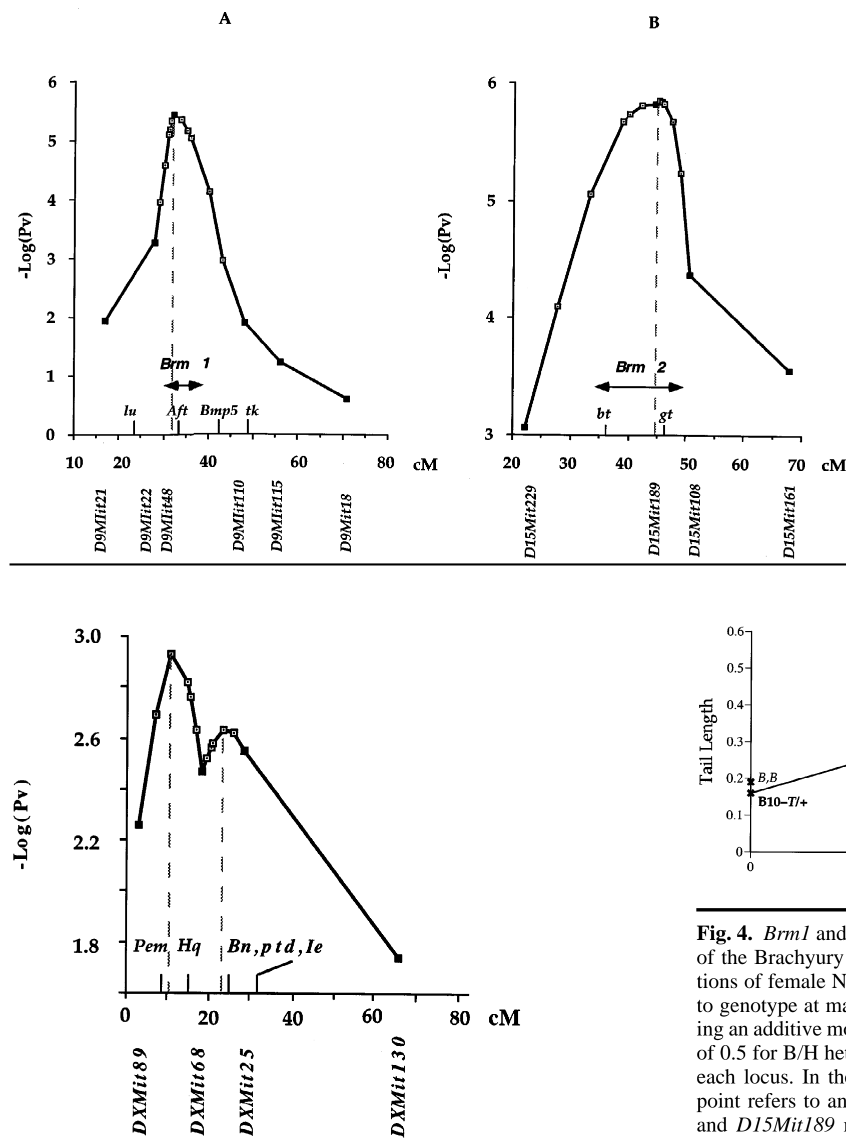
At the third level of analysis, *D19Mit11* was eliminated (data not shown) from further consideration with a *P* value greater than 0.5. The other two autosomal loci (on Chrs 9 and 15) continued to improve in significance. Analysis of both putative QTLs demonstrated a direct sex-limited effect on modification of the Brachyury phenotype expressed only in females. The X Chr locus, however, appears to act only in males (Table 1).

The results of interval analysis for Chr 9 are shown on Fig. 2A. The most likely position for a QTL is centered on the microsatellite marker *D9Mit48,* which is 34 cM from the centromere. The *P* value at this marker is 9.1×10^{-6}, which is almost an order of magnitude below the standard threshold value suggested for evidence of linkage with the backcross design recommended by Lander and Kruglyak (1995). We name this locus *Brm1* (Brachyury modifier 1).

Figure 2B shows the results of interval analysis for Chr 15. The most likely position for a QTL is centered on the microsatellite *D15Mit189,* which is 44 cM from the centromere. The *P* value at this marker is 4.9×10^{-5}, which also passes the stringent test for significance recommended by Lander and Kruglyak. We name this locus *Brm2* (Brachyury modifier 2).

Interval analysis data for the X Chr are shown in Fig. 3. The lowest *P* value score obtained—2.5×10^{-3} at position 28 cM—provides suggestive evidence for the presence of a QTL according to the threshold standard suggested by Lander and Kruglyak. As shown in the figure, there are two X Chr peaks separated by 12 cM, which suggests the possibility of two X Chr QTLs.

An analysis of potential genetic interactions between the two modifying QTLs identified for females in the D cross is presented in Fig. 4. The results are consistent with a model of simple additivity between two loci having equivalent contributions to phenotypic expression. This model is supported with a *P* value of 1.5×10^{-10}. When the two modifying loci *Brm1* and *Brm2* are consid-



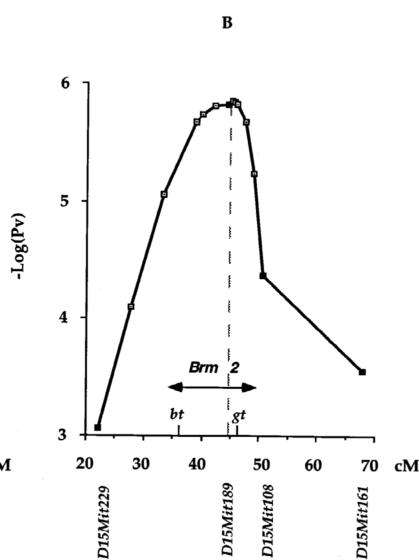


Fig. 2. Localization of the *Brm1* (**A**) and *Brm2* (**B**) loci on mouse Chr 9 and 15 respectively. *P* values associated with the individual microsatellite marker loci shown along the X axes were determined by the *t*-test. *P* values for chromosomal points between marker loci were determined by simple regression. Results are presented in $-(\log_{10})$-converted form. The 90% confidence intervals for *Brm1* and *Brm2* are indicated with double-arrowhead lines. Centimorgan distances from the centromere are shown below the X axes. Positions of previously characterized genes that affect tail or skeletal phenotypes are shown above the X axes.

Fig. 3. Localization of a suggestive brachyury-modifier locus on the X Chr. See legend to Fig. 2 for all details.

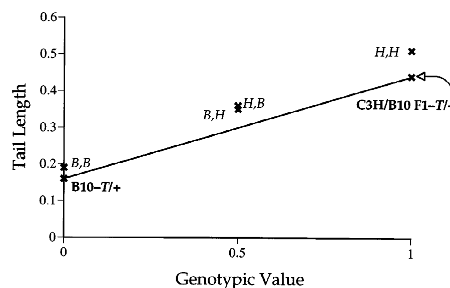


Fig. 4. *Brm1* and *Brm2* act in an additive manner to modify the expression of the Brachyury phenotype. Tail length values are shown for subpopulations of female N_2 mice derived from the D cross and separated according to genotype at markers closely linked to the *Brm1* and *Brm2* loci. Assuming an additive model of inheritance, we assigned additive genotypic values of 0.5 for B/H heterozygosity genotype and 0.0 for a B/B homozygosity at each locus. In the figure, the B,B data (additive genotypic value of 0.0) point refers to animals homozygous for the B allele at both the *D9Mit48* and *D15Mit189* marker loci, which define the most likely positions for *Brm1* and *Brm2* respectively. The B,H and H,B data points (additive genotypic values of 0.5) refer to animals heterozygous at one marker locus and homozygous at the other (with the Chr 9 locus indicated first). The H,H data point (additive genotypic value of 1.0) refers to animals heterozygous at both marker loci. Mean tail length values observed for *T*/+ animals in the two parental genetic backgrounds used to generate the N_2 population {B10-*T*/+ and (C3H/B10) F_1*T*/+} are also shown and are connected by a line to demonstrate the validity of the simple additive model.

ered together, they account for essentially 100% of the genetic variance in tail length observed between B10 and F_1 parental animals that carry the Brachyury mutation.

Discussion

We have identified two strong modifiers of the *T* locus phenotype—*Brm1* on Chr 9 and *Brm2* on Chr 15—that distinguish B10 and C3H mice. Both modifiers are expressed only in females. They act equally and additively and account for most, if not all, of the genetic variance in expressivity observed between female *T*/+ animals from the two inbred strains.

We have identified one suggestive modifier of the *T* locus phenotype that operates in males only. It is possible that other major male-limited modifiers of expressivity have been missed in our study. On the other hand, it is also possible that the modification of the phenotype in males is more complex, with a larger number of minor loci involved. Further genetic analysis will be required to distinguish between these two possibilities.

The demonstration of sex-limited action in the modification of a tail phenotype caused by an autosomal mutation was unexpected. This finding suggests that sexual dimorphism in development may be much greater and earlier-acting than previously assumed.

Although the B10-C3H allelic differences at the *Brm1* and *Brm2* loci identified in this study have no direct effect on phenotypic expression in wild-type C3H and B10 animals, it seems reasonable to suggest that mutations in these loci could have visible effects that were similar to those caused by *T* mutations. In this light, it becomes possible to identify potential candidate mutations that could lie within one of the *Brm* loci.

One candidate mutation at the *Brm1* locus is called abnormal feet and tail (*Aft*) (Lane 1987). The *Aft* mutation is dominant with poor penetrance. It produces syndactyly of digits 3 and 4 in the hind feet and a kinky tail on the 129/Sv-c(ch)/c(ch)-w(54J) genetic background. No obvious candidates for the *Brm2* locus are suggested by currently available linkage data. But mapping close to the suggestive *Brm* locus on the X Chr is the mutation bent tail

(*Bn*), which has a dominant effect on tail formation, with incomplete penetrance (Garber, 1952).

Further genetic studies will be required to determine whether any of the candidate mutations is actually equivalent to a *Brm* locus. And ultimately, further molecular studies will be required to identify the actual genes and products that exert on effect on the *T* locus phenotype. Only when this work has been completed will it be possible to know whether our original hypothesis was correct—that modifier loci will represent genes that play crucial roles in development, just like brachyury.

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