Mapping Modifiers Affecting Muscularity of the Myostatin Mutant (*Mstn*$^{Cmpt\text{-}dl1Abc}$) Compact Mouse

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ABSTRACT

The hypermuscular Compact phenotype was first noted in a line of mice selected for high body weight and protein content. A new line, based on mice showing the Compact phenotype, was formed and selected for maximum expression of the Compact phenotype. Previously we mapped and identified a 12-bp deletion in the myostatin gene, denoted *Mstn*$^{Cmpt\text{-}dl1Abc}$, which can be considered as a major gene responsible for the hypermuscular phenotype. Genetic analysis revealed that full expression of the hypermuscular phenotype requires the action of modifier loci in addition to *Mstn*$^{Cmpt\text{-}dl1Abc}$. To map these modifier loci, an interspecific F_2 population was generated between Comp9, an inbred line homozygous for *Mstn*$^{Cmpt\text{-}dl1Abc}$, and CAST/Ei, an inbred line generated from *Mus musculus castaneus*. Selective DNA pooling and genotyping, separately by gender, was carried out within a subpopulation of the F_2 consisting of individuals homozygous for *Mstn*$^{Cmpt\text{-}dl1Abc}$. Significant association with hypermuscularity at a false discovery rate (FDR) of 0.05 was found for markers on chromosomes 3, 5, 7, 11, 16, and X. In all cases, the marker allele derived from the Comp9 parent showed a higher frequency in the hypermuscular group and the CAST/Ei allele in the normal group. The modifier loci apparently exerted their effects on muscularity only in the presence of *Mstn*$^{Cmpt\text{-}dl1Abc}$.

THE Compact "hypermuscular" mutation arose during a selection experiment in Germany (Bunger *et al.* 2001). A "Compact" subline was formed and selected for maximum hypermuscularity. Genetic analysis of the Compact line indicated that a major gene, denoted *Cmpt*, and one or more modifier genes were involved in determining the degree of expression of the Compact phenotype. The putative major gene (*Cmpt*) was mapped to an 8.2-cM region on mouse chromosome 1 (Varga *et al.* 1997). After the discovery of the myostatin gene (McPherron *et al.* 1997), we identified the *Mstn*$^{Cmpt\text{-}dl1Abc}$ deletion in the propeptide region of the gene and mapped the mouse myostatin gene to a point 27.7 cM from the centromere on chromosome 1 (Szabó *et al.* 1998).

Myostatin, belonging to the transforming growth differentiation factor-β (TGF-β) superfamily, is a negative regulator of muscle growth. Myostatin was first identified when null-mutant knockout mice exhibited a large and widespread increase in skeletal muscle mass due to an increase of muscle fiber number (hyperplasia) and thickness (hypertrophy; McPherron *et al.* 1997). Subsequently, an 11-bp myostatin deletion in the Belgian Blue and a missense myostatin mutation in the Piedmontese cattle breeds were identified in the C-terminal coding region of *Mstn* and shown to be responsible for the double-muscled phenotype in these breeds (Grobet *et al.* 1997; Kambadur *et al.* 1997; McPherron and Lee 1997). *Mstn*$^{Cmpt\text{-}dl1Abc}$, however, was found in the propeptide region of the myostatin precursor (Szabó *et al.* 1998). In analogy with TGF-β1, we hypothesized that the propeptide region might play an important role in the proper folding, efficient secretion, processing, or targeting of the mature growth factor domain of myostatin. Although some of these steps may be seriously impaired by the *Mstn*$^{Cmpt\text{-}dl1Abc}$ mutation, the activity of mature myostatin is not zero in homozygous Cmpt/Cmpt mutants. Modifier genes may therefore have a significant influence on the phenotype (Szabó *et al.* 1998).

In the current study we report the mapping of modifier loci affecting the expression of the hypermuscularity in the Compact mouse. Our basic assumption is that due to selection for hypermuscularity, the Compact line, in addition to achieving homozygosity for the *Mstn*$^{Cmpt\text{-}dl1Abc}$ mutation, also accumulated modifier alleles that increased the expression of hypermuscularity in the Compact mouse. To map these modifier genes, a new intersubspecific cross (Cross4) was initiated, which produced a large F_2 population. A genome scan was carried out by selective DNA pooling within the F_2 progeny group homozygous for the *Mstn*$^{Cmpt\text{-}dl1Abc}$ deletion. Markers on several chromosomes showed linkage with the putative modifiers, and the strongest association was found for markers on chromosomes 16 and X. Mapping of the

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modifier loci and their identification by positional cloning might provide a route to additional upstream and downstream factors involved in muscle development (Varga *et al.* 1997).

MATERIALS AND METHODS

Mice: The hypermuscular Compact phenotype was first noted in a line of mice selected for high body weight and high protein content at the Technical University of Berlin. Mice showing the Compact phenotype were separated from the main selection line and a new COMPACT line, based on these individuals, was originated and subjected to selection for maximum expression of the Compact phenotype (F. Major and W. Schlote, personal communication). An inbred line, HCI, was generated within the HCR and used in our previous F_2 crosses (Szabó *et al.* 1998). HCI became extinct at generation 22, and a new inbred strain, Comp9, developed by 16 generations of full-sib mating, was used for the current intersubspecific cross, denoted Cross4. In the present study, five Comp9 males were mated with five CAST/Ei females to produce an F_1 generation. The F_1 progeny were intercrossed to produce 2373 F_2 mice.

Phenotypic analysis: At 6 weeks of age the F_2 mice were classified for muscularity by visual inspection using a five-grade scale for phenotypes of from 1 (normal) to 5 (most muscular). The classification was always done by G. Müller. Each animal was scored three times on the same day. An animal was characterized by the average of the three independent scores. In this way, the mice distributed across 13 phenotypic categories ranging from 1.00, 1.33, 1.67, . . . , to 5.00. The standard deviation among the three scores of the same mouse averaged 1.09 score units. A spread of 0, 1, 2, 3, and 4 within the three scores of the same mouse was found in 78, 9, 11, and 2% and in one individual, respectively. Thus, scoring on the whole was consistent and closely reflected the actual Compact status of the animal. Animals with a wide spread would have ended up with a moderate score and thus would not be included in either the high or the low pools. Thus, they represent lost information, but would not bias the results.

Genotypic analysis: DNA from tail tips was prepared using standard protocols. A total of 141 microsatellites distinguishing Comp9 and CAST/Ei were typed, using primers purchased from Research Genetics (Huntsville, AL). These microsatellites covered the genome with an average spacing of 10.6 cM between markers (Figure 1). The largest intermarker distance was 17.0 cM. PCR, allele separation, and silver staining were as described (Varga *et al.* 1997).

Notation: Genotypes for the $Mstn^{Cmpt\text{-}dl1Abc}$ deletion are denoted as follows: *K*, $Mstn^{Cmpt\text{-}dl1Abc}/Mstn^{Cmpt\text{-}dl1Abc}$; *H*, $Mstn^{Cmpt\text{-}dl1Abc}/+$; and *B*, $+/+$. Marker genotypes are denoted as follows: M_K, the marker genotype homozygous for the allele derived from the Compact line; M_B, the marker genotype homozygous for the allele derived from the CAST/Ei line; and M_H, the heterozygous marker genotype.

DNA pooling and individual genotyping: We applied the selective DNA pooling strategy (Darvasi and Soller 1994) for the initial genome scan to map quantitative trait loci (QTL) modifying the expression of the $Mstn^{Cmpt\text{-}dl1Abc}$ mutation. On the hypothesis that the expression of $Mstn^{Cmpt\text{-}dl1Abc}$ is strongly modulated by modifier genes, then in the F_2 population, the allele frequencies of particular modifier genes will differ between pools of homozygous $Mstn^{Cmpt\text{-}dl1Abc}$ animals with low compact score and those with high compact score. Taking into consideration the differences in phenotypic distribution of males and females among F_2 animals of *K* genotype, four pools were constructed on the basis of gender (male, female) and Compact score (high, low). There were sufficient male *K* mice of compact score 5.00, and female *K* mice of compact score 1.00, so that the high muscularity male pool (designated M5*K*) consisted of animals of score 5.00 only, and the low muscularity female pool (designated F1*K*) consisted of animals of score 1.00 only. There were only a few male *K* mice of very low compact score and a few female *K* mice of very high compact score. Consequently, the low muscularity male pool (designated M1*K*) had a final composition of 14 animals, score 1.00; 8 animals, score 1.67; 4 animals, score 2.00; and 3 animals, score 2.33. Similarly, the high muscularity female pool (designated F4*K*) had a final composition of 24 animals, score 4.00; 2 animals, score 4.33; and 1 animal, score 4.67. Total numbers of mice in the various pools were: M1*K*, 29; M5*K*, 40; F1*K*, 39; and F4*K*, 30. Adding less extreme animals to the M1*K* and F4*K* pools reduced the selection applied to the pools, but also reduced the effects of binomial sampling. Thus, net effect on the power of selective genotyping is negligible (Lebowitz *et al.* 1987).

At this stage of the cross, 138 mice were selected for the pools, comprising 5.8% of the entire F_2 population. DNA pooling analysis was performed using these mice. Later, as the cross was continued, we were able to choose an additional 51 extreme F_2 animals for the individual selective genotyping, so that a total of 189 animals, 8% of the entire F_2 population ($N = 2373$), was included in this study.

The pool experiments were carried out with these four pools, using a heterozygous (*H*) animal from the first generation of the cross as an intensity control. Intensities of the Comp9 and CAST/Ei alleles in these four pools were evaluated "by eye" and independently by two persons. Our mapping strategy was sequential: if we observed allele intensity differences between the pools, we tested an additional flanking marker on the pools. If this result supported the first observation, we individually genotyped the mice from the pools. If there was a significant frequency difference ($P < 0.05$) between individuals belonging to the high and low pools, subsequent markers along the chromosome were genotyped individually without pooling. This was continued until the significance of the frequency difference fell below the abovementioned threshold.

False discovery rate and proportion of false positives: The genome scan performed in the present study is a multiple-test procedure and hence the usual comparison-wise error rate (CWER) significance levels of $P = 0.05$ and $P = 0.01$ are not appropriate. Lander and Kruglyak (1995) suggested that in this situation CWER thresholds that control GWER (genome-wise error rate) as though based on a high-density marker map should be used. However, this leads to an exceedingly stringent significance level with much loss of power. As an alternative, Weller *et al.* (1998) proposed controlling the comparison-wise false discovery rate (FDR), developed by Benjamini and Hochberg (1995) in genome scan experiments. An extensive comparison of FDR and GWER methods in the context of interval mapping was carried out by Lee *et al.* (2002) and indeed showed that power was greater under an FDR criterion than under the Lander and Kruglyak (1995) GWER criterion. The FDR as defined by Benjamini and Hochberg (1995) is somewhat inappropriate for use in a genome scan (Weller 2000; Zaykin *et al.* 2000). A modified version, the adjusted FDR, equivalent to the proportion of false positives (PFP) criterion of Southey and Fernando (1998), was utilized by Mosig *et al.* (2001).

On the basis of chi-square CWER *P*-values, comparison-wise FDR and PFP for a given marker, M_i, were calculated as

$$\text{FDR} = P_i n/i; \quad \text{PFP} = P_i(n - n_L)/i,$$

where P_i is CWER for the *i*th marker; n is the total number of markers in the scan; n_L is the number of markers in linkage to QTL (since a number of markers will be in linkage to the

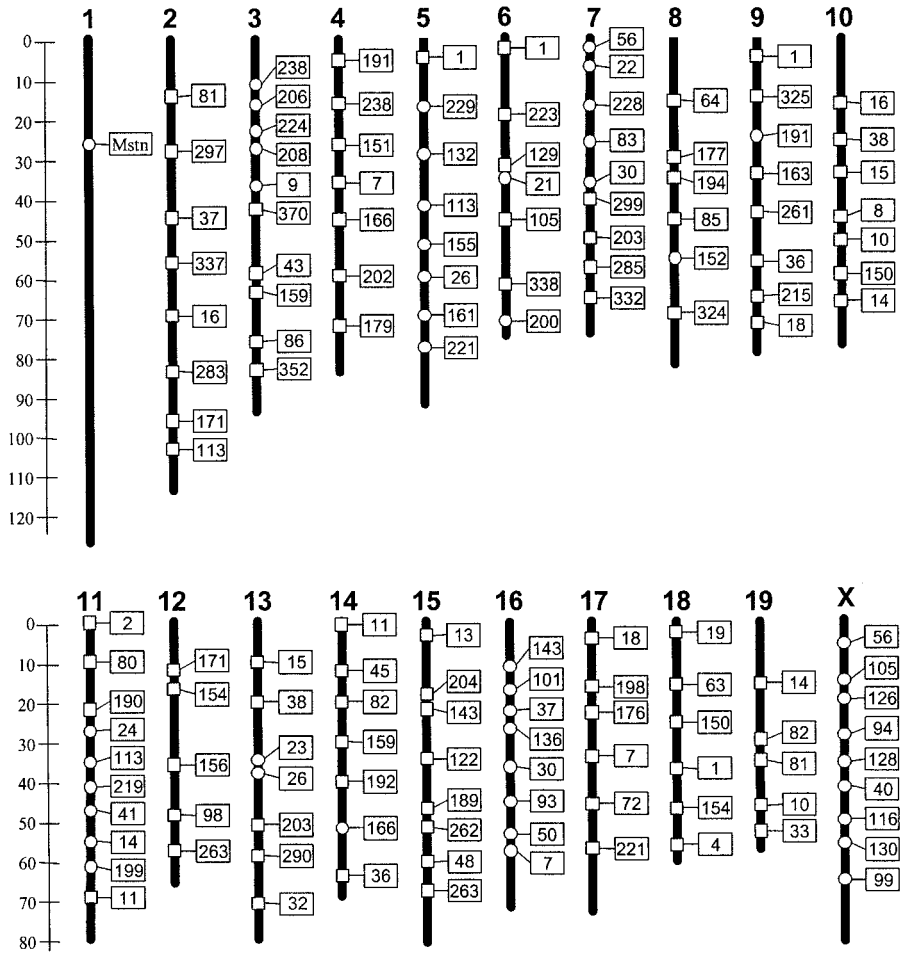


FIGURE 1.—Chromosomal location of the microsatellite markers used in this study. Formal microsatellite names have been abbreviated. Open squares indicate markers tested only on pools, while open circles represent markers that were individually genotyped. The centimorgan positions of microsatellites were obtained from the Mouse Genome Database, Mouse Genome Informatics Project (October 2002), The Jackson Laboratory, Bar Harbor, Maine (http://www.informatics.jax.org).

same QTL, it should be noted that n_L is not an estimate of the number of modifier QTL); and i is the ordinal number of the ith marker, when the markers are ranked according to ascending P-values.

Application of FDR and PFP in the present experiment required some adjustment due to the sequential nature of the scan. As noted above, only markers that showed qualitative indications of a frequency difference between the high-compact-score pools and the low-compact-score pools were individually genotyped. Thus, only 50 of 141 markers employed in the scan were individually genotyped and provided CWER P-values. The FDR and PFP analyses were then carried out on the assumption that all markers that would have given a low P-value had been identified by the pool scan. Thus, the markers were ranked on the basis of their P-value on individual genotyping, but $n = 141$ was taken as the total number of markers in the scan. This assumption is conservative. If any such markers were not included among the individually genotyped markers, they would (if included) have increased the rank score (i number) of the individually genotyped markers following them in the ranking and hence reduced their FDR or PFP values. Because of the way in which FDR and PFP were calculated, markers with very high P-values obtained calculated FDR and PFP values >1.0. Since this is not possible, these were adjusted to a value of 1.0 and are shown as 1.0* in Table 1.

The number of markers in linkage to QTL, n_L, was estimated as follows. On the null hypothesis, the expected number of P-values in the range 0.00–0.099 is $n/10$. Let n_D be the number of markers analyzed by individual genotyping and having CWER P-values in this range. On the conservative assumption that all markers having a P-value in this range would have been flagged by pool genotyping and individually genotyped, n_L will equal the excess of n_D over $n/10$; *i.e.*, $n_L = n_D - n/10$. This, too, is a conservative estimate, since any markers that were not individually genotyped that would have been included in this class and would have increased the estimate of n_L.

Chi-square test for linkage of markers with QTL modifying the Compact phenotype in homozygous *Mstn*$^{Cmpt\text{-}dl1Abc}$ animals: Let $NM_K(\mathrm{M1}K)$ be the number of presentations of genotype M_K in the M1K phenotypic group, and let $NM_K(\mathrm{M5}K)$, $NM_K(\mathrm{F1}K)$, etc., be defined accordingly. Then, under the null hypothesis of no linkage, and assuming equal numbers of progeny in each phenotypic group,

$$\begin{aligned} E[NM_K(\mathrm{M1}K)] &= E[NM_B(\mathrm{M1}K)] = E[NM_K(\mathrm{M5}K)] \\ &= E[NM_B(\mathrm{M5}K)] = E[NM_K(\mathrm{F1}K)] \\ &= E[NM_B(\mathrm{F1}K)] = E[NM_K(\mathrm{F4}K)] \\ &= E[NM_B(\mathrm{F4}K)], \end{aligned}$$

while under linkage we have

$$\begin{aligned} E[NM_K(\mathrm{M5}K)] &= E[NM_B(\mathrm{M1}K)] = E[NM_K(\mathrm{F4}K)] \\ &= E[NM_B(\mathrm{F1}K)] > E[NM_K(\mathrm{M1}K)] \\ &= E[NM_B(\mathrm{M5}K)] = E[NM_K(\mathrm{F1}K)] \\ &= E[NM_B(\mathrm{F4}K)]. \end{aligned}$$

Then, letting

$$U = NM_K(\mathrm{M5}K) + NM_B(\mathrm{M1}K) + NM_K(\mathrm{F4}K) + NM_B(\mathrm{F1}K)$$

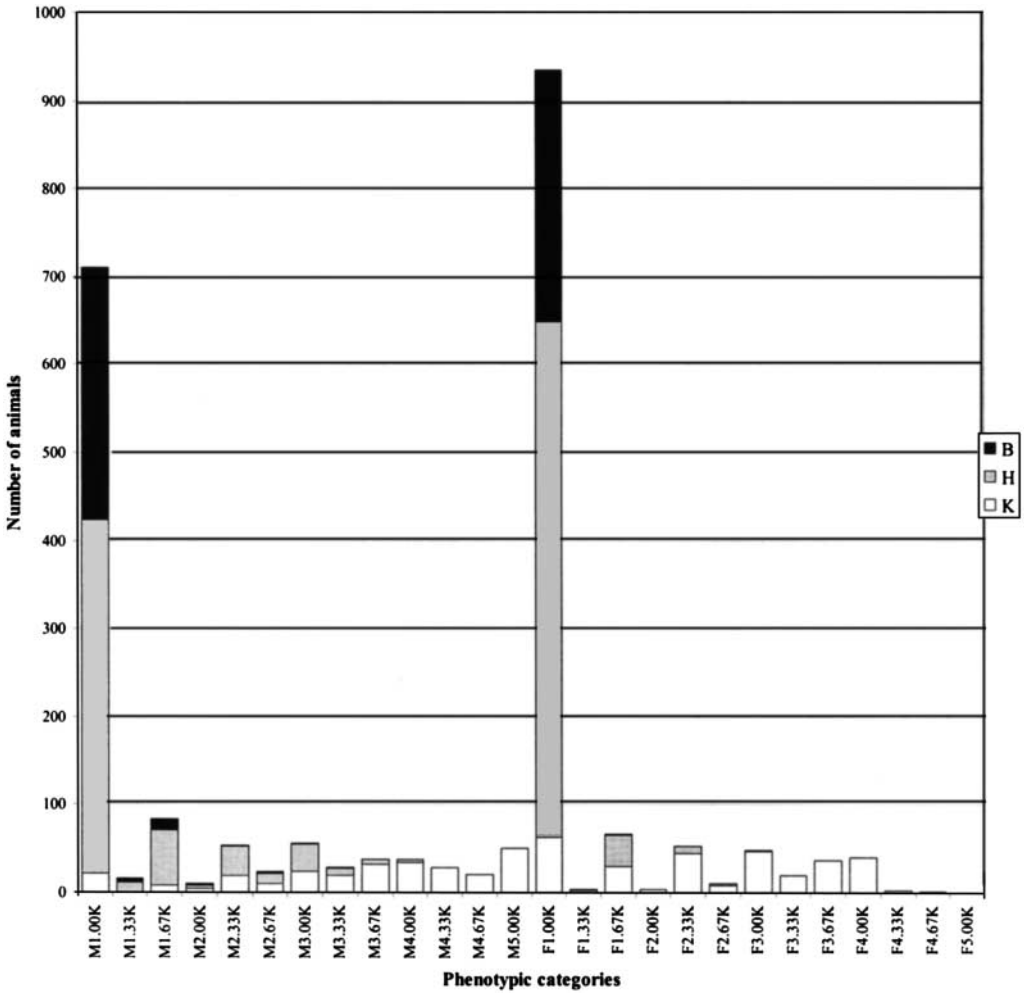


FIGURE 2.—Distribution of the three genotypes (*K*, $Mstn^{Cmpt\text{-}dl1Abc}/Mstn^{Cmpt\text{-}dl1Abc}$; *H*, $Mstn^{Cmpt\text{-}dl1Abc}/+$; *B*, +/+) for F_2 males and females within the phenotypic categories. The 13 phenotypic categories derived from the average of three independent visual classifications using a five-grade scale (1, normal muscularity; 5, extreme hypermuscularity).

and

$$L = NM_K(\mathrm{M1}K) + NM_B(\mathrm{M5}K) + NM_K(\mathrm{F1}K) + NM_B(\mathrm{F4}K),$$

under the null hypothesis $E(U) = E(L)$, we can test for deviation from expectation by $\chi^2 = (U - L)^2/(U + L)$ with 1 d.f.

RESULTS

Phenotypic data: A similar pattern of recessive hypermuscularity was found in both sexes (Figure 2). In spite of their general similarities, however, the two sexes showed some differences in their overall F_2 phenotypic distributions. The female distribution is skewed to the lower categories, while the male distribution is skewed to the higher categories. This tendency is in agreement with the phenotypic difference between males and females observed in the inbred Comp9 strain, where males were uniformly 5.00, while 31% of the females were 3.67 and 69% were 4.00. Similarly, in the F_2, female category F5.00 is completely missing and only a single animal was in F4.67 and two animals in the F4.33 category.

In the F_1, only 1 female out of 59 (1.69%), and 9 males out of 67 (13.43%) presented a compact score >1.0. In the F_2, 23.11% of females and 38.6% of males presented a compact score >1.0. Thus, in both F_1 and F_2, the males show a greater proportion of higher value scores than the females. Among the F_2 males the proportion of individuals presenting a compact score >1.0 (38.63%) is significantly greater than the expected proportion of individuals with *K* genotype, indicating that some individuals from the other two genotypic categories (*H* and *B*) must achieve compact scores >1.00 (see below). This is supported by the presence of an appreciable number of F_1 males presenting compact scores >1.00. Among the F_2 females the proportion of animals presenting a compact score >1.0 (23.11%) is equal to the expected proportion of *K* individuals.

Distribution of the $Mstn^{Cmpt\text{-}dl1Abc}$ genotype among the Compact phenotypic categories: $Mstn^{Cmpt\text{-}dl1Abc}$ was genotyped across the complete F_2 population of Cross4. The proportion of the three genotypic categories (*B* = 25.3%, *H* = 50.7%, and *K* = 23.9%) was in good agreement with the expected 1:2:1 Mendelian ratio.

Among F_2 animals showing the *B* genotype, only 25 of 312 males (8.01%) and 2 of 289 females (0.69%) presented phenotypic categories >1.0 (Figure 2). There was a certain oscillation in those categories where a score of 2 occurred more frequently, because this score

was used much less frequently than at the classification of the previous crosses. This uneven classification distorted the phenotypic distribution to some extent, but the trend is clear. With the exception of a single *B* genotype male presenting a compact score of 2.33, all of these were limited to compact scores of 1.33 or 1.67. Among F_2 animals showing the *H* genotype, the corresponding proportions were 30.02 and 7.29%, respectively. It is noteworthy that there was a greater proportion of higher compact scores among the heterozygous (*H*) F_2 male and female individuals (30.02 and 7.29%, respectively) than among the F_1 male and female individuals (14.93 and 1.69%, respectively), all of which are heterozygous. The differences between phenotypic distribution of *H* animals in F_1 and F_2 are highly significant by chi-square contingency test for the F_2 males, but somewhat under the significance threshold for the F_2 females. The greater proportion of higher Compact categories among F_2 individuals with *H* genotype as compared to F_1 individuals with *H* genotype is consistent with the notion that recessive modifier genes that come to a homozygous state in the F_2, but not in the F_1, contribute significantly to the increased compact score. Among F_2 animals showing the *K* genotype, the proportions showing compact scores >1.0 were 91.91 and 78.72%, for males and females, respectively. In both males and females having *K* genotypes, there was a broad, virtually uniform distribution of compact scores (Figure 2). The variable expressivity of the *K* genotype in the F_2, not seen in the Comp9 parent line (data not shown), is also consistent with the presence of modifier genes affecting *K* genotype expression.

Mapping of *Mstn*$^{Cmpt\text{-}dl1Abc}$ modifier genes: Since only the *Mstn*$^{Cmpt\text{-}dl1Abc}$ homozygous animals were involved in the experiment, chromosome 1 where *Mstn* resides was omitted from the mapping process. This chromosome will be explored by individual genotyping in a future experiment. In all, a total of 141 markers were genotyped on the four phenotypic pools (Figure 1). Thus, there were a total of 564 marker-by-phenotypic-group pool genotypings. On visual inspection of the stained gels, markers on chromosomes 2, 3, 5, 6, 7, 8, 11, 14, 15, 16, 17, and X showed apparent allele-frequency differences between *K* and *B* genotypes within one or more marker-by-group combinations. For these markers all four phenotypic groups were individually genotyped. When this was done, the tested combinations for markers on chromosomes 2, 6, 8, 14, 15, and 17 did not show appreciable frequency differences in any of the four groups. Therefore, these chromosomes were not investigated further by individual genotyping. For chromosomes 3, 5, 7, 11, 16, and X, one or more of the individually genotyped marker-by-group combinations were significant at the $P < 0.05$ level by chi-square (test data not shown). For these chromosomes, all four groups were then individually genotyped for the flagged marker, and additional markers were individually genotyped in both directions along the chromosome and tested for significance by chi-square, as described in MATERIALS AND METHODS, until a clear decrease in significance level was obtained.

Table 1 shows the results of individual genotyping by chromosome and marker. The frequency of M_K and M_B genotypes for the individually genotyped markers on chromosomes 3, 5, 7, 11, 16, and X were examined in the four phenotypic groups (M1*K*, F1*K*, M5*K*, and F4*K*). With two minor exceptions and two major exceptions, the frequency of M_K genotype was greater than the frequency of M_B genotype in the M5*K* and F4*K* phenotypic groups, and the frequency of the M_B genotype was greater than the frequency of the M_K genotype in the M1*K* and F1*K* phenotypic groups. For markers on the X chromosome, the frequency of the M_H genotype was greater than that of the M_B genotype in the F4*K* phenotypic group, while the reverse was true for the F1*K* phenotypic group. Thus, the modifier genes appear to have equivalent effects in males and females.

The minor exceptions noted above, which we attribute to sampling variation, were markers D5Mit229 in F4*K* and D7Mit30 and D7Mit299 in M1*K* and M5*K*. The major exceptions, which appear to be too large to be due to sampling and are unexplained, were a large excess of M_K genotypes for markers D5Mit229–D5Mit221 in F1*K* and a marked excess of M_H genotype for markers D7Mit56 and D7Mit22 in M5*K*. Consequently, the F1*K* phenotypic group was not included in linkage tests for all chromosome 5 markers, and the M5*K* phenotypic group was not included in linkage tests for all chromosome 7 markers. With these exceptions, the overall consistency of marker effects in all four phenotypic groups and across all markers in a chromosome argues strongly that the observed frequency differences among marker genotypes within phenotypic groups are due to a common factor exerting its effect in all groups and on all markers within the same chromosome. In all cases, the marker derived from the Compact parent was associated with a greater compact score, and the marker derived from the CAST/Ei parent was associated with a lower compact score. This is consistent with the presence of one or more modifier QTL with positive alleles derived from the Compact parent in each of the tagged chromosomes. The consistency of effect across phenotypic groups justifies the test procedure of pooling genotype data across all four phenotypic groups for the chi-square analysis. CWER *P*-values resulting from the chi-square analysis for the markers on the tagged chromosomes are also shown in Table 1.

Of the total of 141 markers tested by selective DNA pooling, 50 markers were further analyzed by individual genotyping. On the null hypothesis, the expected number of *P*-values in the range 0.00–0.099 is 14. Of the markers analyzed by individual genotyping, 39 had CWER *P*-values in the range 0.00–0.099. On the conservative assumption that all markers having a *P*-value in

TABLE 1

Distribution of *Mstn*$^{Cmpt\text{-}dl1Abc}$ deletion genotypes and significance levels according to marker and phenotypic class

	M1*K*			M5*K*			F1*K*			F4*K*						
Marker	*K*	*H*	*B*	*K*	*H*	*B*	*K*	*H*	*B*	*K*	*H*	*B*	*P*-value	Rank	FDR	PFP
D3Mit238	7	17	11	16	25	9	11	28	23	13	18	9	6.66E-03	29	3.24E-02	2.66E-02
D3Mit206	6	15	13	20	20	10	11	27	24	11	23	8	1.15E-03	19	8.52E-03	7.01E-03
D3Mit224	7	14	13	16	25	9	8	29	24	13	22	6	2.39E-04	12	2.80E-03	2.31E-03
D3Mit208	8	16	11	16	22	12	13	26	23	12	25	5	1.64E-02	33	7.01E-02	5.76E-02
D5Mit229	7	16	12	12	23	15	0	0	0	8	24	10	1.0000	41	1.0*	1.0*
D5Mit132	6	17	12	17	20	12	0	0	0	7	30	5	9.06E-02	39	3.27E-01	2.69E-01
D5Mit113	5	16	14	16	25	9	0	0	0	8	27	7	2.69E-02	37	1.02E-01	8.43E-02
D5Mit155	6	12	17	14	29	7	0	0	0	9	27	5	3.87E-03	27	2.02E-02	1.66E-02
D5Mit26	4	15	16	15	28	7	0	0	0	12	25	5	4.40E-04	15	4.13E-03	3.40E-03
D5Mit161	8	11	16	16	27	7	0	0	0	9	28	5	7.17E-03	30	3.37E-02	2.77E-02
D5Mit221	8	13	14	18	25	7	0	0	0	12	21	9	1.53E-02	32	6.74E-02	5.54E-02
D7Mit56	7	17	11	0	0	0	15	28	19	11	21	10	2.92E-01	40	1.0*	8.47E-01
D7Mit22	7	16	12	0	0	0	14	28	20	13	23	6	3.39E-02	38	1.26E-01	1.03E-01
D7Mit228	6	20	9	0	0	0	12	28	22	14	25	3	3.13E-03	24	1.84E-02	1.52E-02
D7Mit83	9	17	9	0	0	0	11	28	23	14	26	2	3.61E-03	26	1.96E-02	1.61E-02
D7Mit30	7	23	5	0	0	0	11	30	21	15	22	5	2.44E-02	36	9.58E-02	7.88E-02
D7Mit299	7	22	6	0	0	0	11	30	21	16	22	4	9.19E-03	31	4.18E-02	3.44E-02
D11Mit190	7	16	12	17	27	6	14	28	17	9	25	6	1.90E-02	34	7.89E-02	6.49E-02
D11Mit24	5	19	11	19	28	3	13	28	21	10	25	7	4.69E-04	16	4.13E-03	3.40E-03
D11Mit113	5	19	11	19	22	8	14	24	24	10	24	7	2.44E-03	23	1.50E-02	1.23E-02
D11Mit219	4	21	10	18	22	9	12	26	24	9	27	6	1.76E-03	21	1.18E-02	9.73E-03
D11Mit41	5	20	10	18	21	11	11	27	24	12	25	4	7.10E-04	18	5.56E-03	4.57E-03
D11Mit14	5	20	10	17	23	9	15	22	25	13	21	7	3.91E-03	28	1.97E-02	1.62E-02
D11Mit199	5	22	8	15	27	8	14	21	26	14	19	7	3.23E-03	25	1.82E-02	1.50E-02
D16Mit143	4	19	12	21	23	6	8	36	18	9	27	6	8.57E-05	9	1.34E-03	1.10E-03
D16Mit101	5	16	14	21	22	7	9	34	18	9	26	7	3.38E-04	14	3.41E-03	2.80E-03
D16Mit37	5	16	14	20	24	6	11	34	17	9	25	8	1.57E-03	20	1.10E-02	9.08E-03
D16Mit136	5	17	13	21	25	4	11	29	22	12	21	9	7.50E-05	8	1.32E-03	1.09E-03
D16Mit30	7	14	12	19	24	5	11	27	23	11	24	7	3.30E-04	13	3.57E-03	2.94E-03
D16Mit93	8	14	13	20	27	2	13	29	20	13	23	6	1.47E-04	11	1.88E-03	1.55E-03
D16Mit50	8	15	12	22	25	3	16	25	21	13	22	6	4.97E-04	17	4.12E-03	3.39E-03
D16Mit7	9	14	12	24	23	3	17	26	19	14	20	8	1.88E-03	22	1.21E-02	9.93E-03
DXMit56	16	—	19	30	—	20	—	28	33	—	28	14	1.96E-02	35	7.90E-02	6.50E-02
DXMit105	12	—	22	37	—	12	—	26	35	—	25	17	1.37E-04	10	1.94E-03	1.59E-03
DXMit126	13	—	22	42	—	8	—	24	37	—	25	17	3.05E-06	5	8.59E-05	7.07E-05
DXMit94	12	—	23	43	—	7	—	27	34	—	25	17	6.13E-06	6	1.44E-04	1.19E-04
DXMit128	11	—	24	44	—	6	—	24	38	—	26	16	4.88E-08	1	6.89E-06	5.67E-06
DXMit40	10	—	25	40	—	10	—	24	38	—	27	15	2.41E-07	3	1.13E-05	9.32E-06
DXMit116	10	—	25	41	—	9	—	24	38	—	27	15	1.10E-07	2	7.73E-06	6.36E-06
DXMit130	10	—	25	41	—	9	—	24	38	—	26	16	2.41E-07	4	8.50E-06	6.99E-06
DXMit99	16	—	19	37	—	13	—	21	41	—	28	14	9.12E-06	7	1.84E-04	1.51E-04

Phenotypic classes: M, males; F, females; 1, 4, 5, Compact category; *K*, *Mstn*$^{Cmpt\text{-}dl1Abc}$ deletion genotype. Genotypes: *K*, *Mstn*$^{Cmpt\text{-}dl1Abc}$/*Mstn*$^{Cmpt\text{-}dl1Abc}$; *H*, *Mstn*$^{Cmpt\text{-}dl1Abc}$/+; *B*, +/+. Significance levels: *P*-value, comparison-wise error rate; Rank, rank of marker according to ascending *P*-value; Calculated FDR and PFP values >1.0 were entered as 1.0* (see MATERIALS AND METHODS).

this range would have been flagged by pool genotyping and individually genotyped, there is an excess of 25 markers over the expected in this class. The excess is highly significant by chi-square test. This is consistent with the presence of some factor, presumably linked segregating modifier QTL, which caused a greater-than-chance deviation of observed-to-expected marker genotype classes within some of the marker × group combinations. Thus, the excess of 28 markers represents a minimum estimate of n_L, the number of markers in linkage to modifier QTL.

Calculating FDR and PFP gave a value of $P = 0.0092$ as cut-off CWER significance levels for both FDR and PFP = 0.05. On this basis, significant markers were found on chromosomes 3, 5, 7, 11, 16, and X. Chromosomes 3, 5, 11, 16, and X had 1, 1, 2, 6, and 8 markers, respectively, significant at $P < 0.001$; chromosomes 3, 5, and 7 each had three markers, significant at $P \leq 0.0092$. Thus, the results of this analysis support the presence of modifier genes on these six chromosomes (Figure 3).

Figure 3 shows plots of the chi-square values of the individual markers along the chromosomes. In these

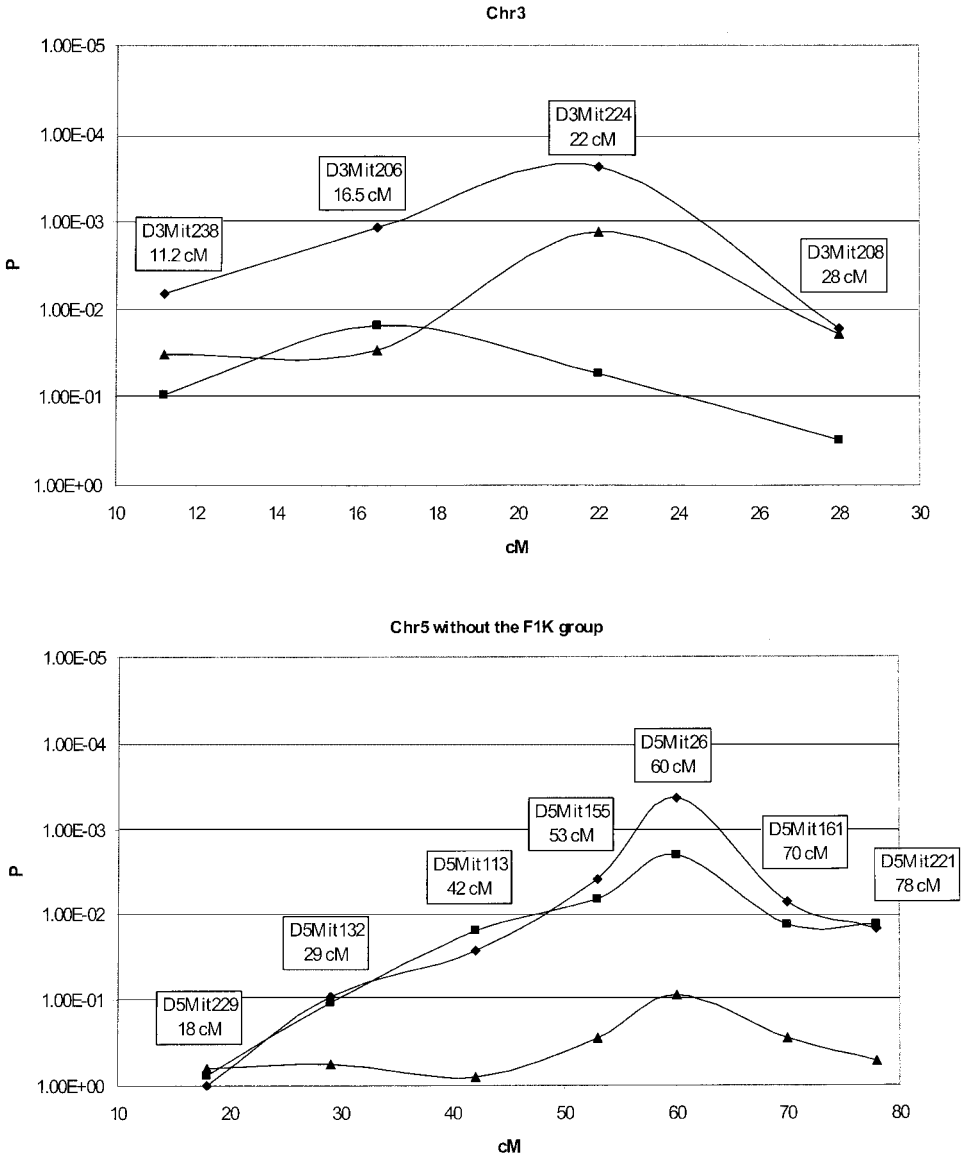


FIGURE 3.—Chi-square test for mapping modifier loci affecting hypermuscularity in F_2 animals homozygous for the *Mstn*$^{Cmpt\text{-}dl1Abc}$ deletion. Significant association at an FDR of 0.05 was found for markers on chromosomes 3, 5, 7, 11, 16, and X. Similar results were obtained using the Map Manager QTX program (http://mapmgr.roswellpark.org/mmQTX.html; our unpublished results). The results of the individual genotypings were evaluated separately for males (■), for females (▲), and also for the gender-merged data set (◆). *P*-values are plotted on the *y*-axis; centimorgan distances from the centromere are plotted on the *x*-axis, together with the position of the microsatellite markers used for individual genotyping. Note that peak values represent the marker with lowest CWER *P*-value and are not point estimates of QTL location, as given by interval mapping programs.

plots the peak is at the marker with highest chi-square value. This is not the best estimate of the QTL location, which would ordinarily be located, by interval mapping programs, between the two highest peaks. However, the plots do show the general regions within which the QTL is to be found. The point estimates of QTL location given by interval mapping come with wide confidence intervals, so the actual loss of information is negligible. Considering the chromosomes individually, narrow regions of strong effect were found on chromosome 3 (markers D3Mit206 and D3Mit224 from centimorgan 17 to 25); on chromosome 5 (markers D5Mit239 and D5Mit26 from centimorgan 56 to 64); and on chromosome 7 (at marker D7Mit83 from centimorgan 23 to 26). Male and female peaks on chromosome 3 are separated by only 6 cM and hence almost certainly represent a single modifier gene. Broad regions of effect were found on chromosome 11 (markers D11Mit24–D11Mit199 from centimorgan 26 to 61) with peaks at D11Mit24 (from centimorgan 26 to 32) and D11Mit41 (from centimorgan 46 to 51) and on chromosome 16 (all markers from centimorgan 11 to 58) with peaks at D16Mit143 (centimorgan 10.8) and D16Mit136 (centimorgan 28.2). Chromosome X showed a broad and exceptionally strong region of effect extending from DXMit126 to DXMit99 (from centimorgan 19 to 66), with a single peak at DXMit128 (centimorgan 34.7). In chromosomes 3 and 7, female effects were more powerful than male effects. In the remaining chromosomes, male effects were more powerful than female effects. Significant effects for both males and females were found in chromosomes 3, 11, 16, and X. Effects in males only were found on chromosome 5; effects in females only were found on chromosome 7.

DISCUSSION

Previous genetic analysis of the Compact trait through crosses of Compact lines to normal phenotype BALB/c

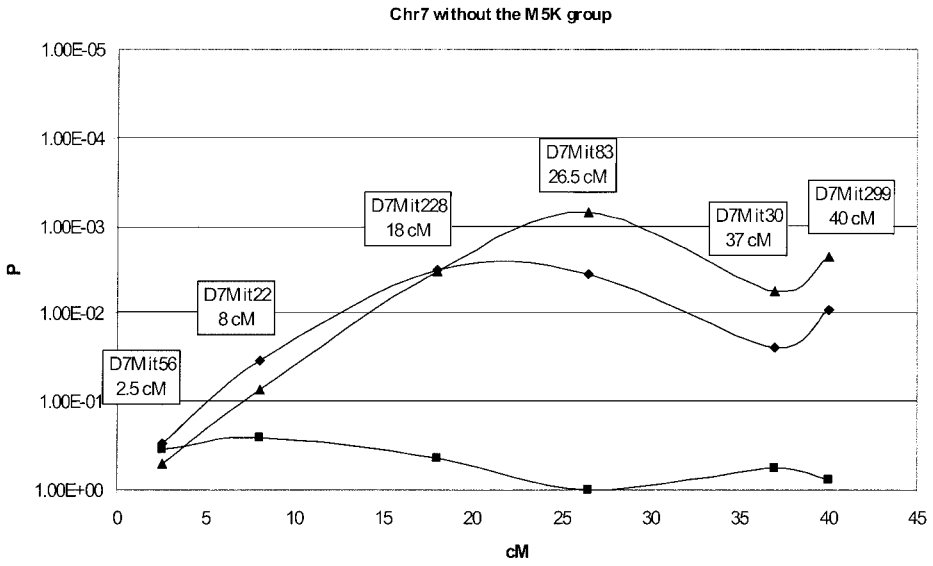
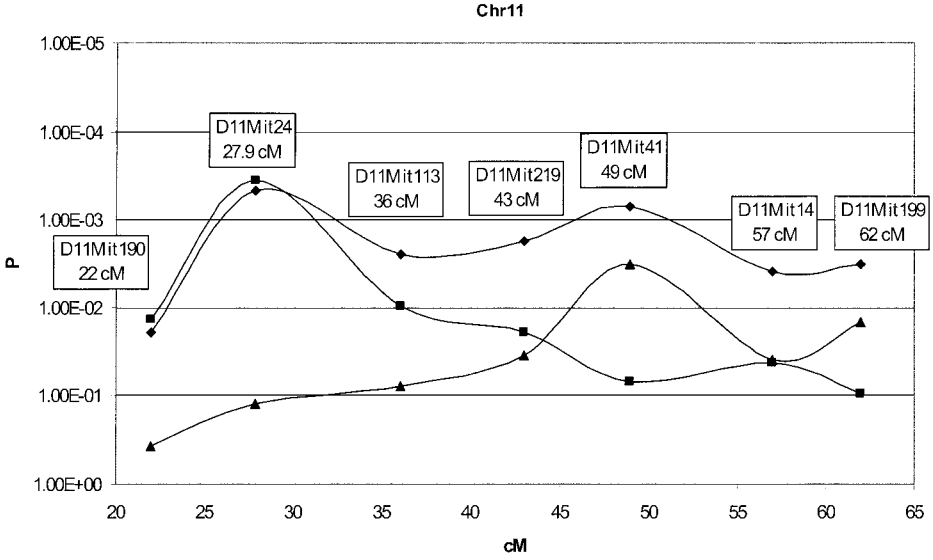


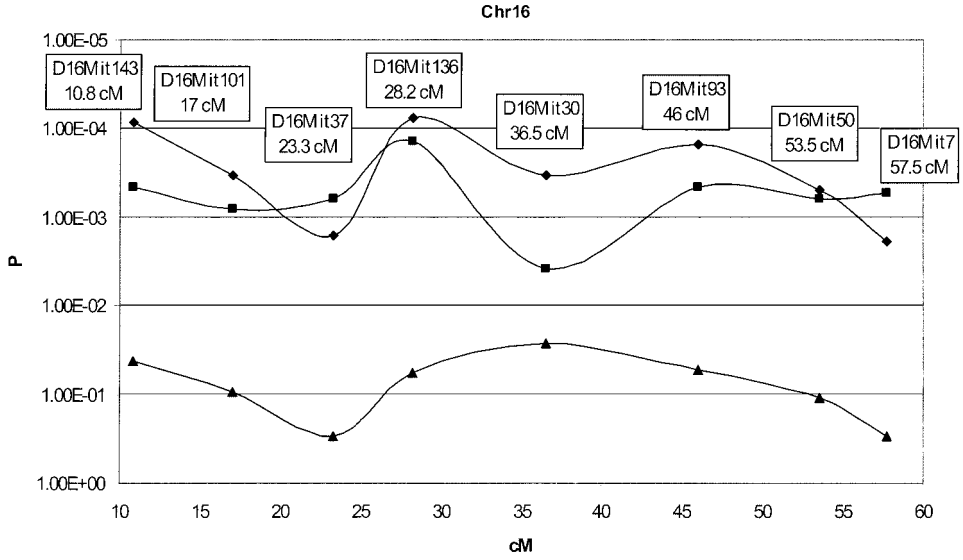
FIGURE 3.—*Continued.*



mice indicated that a major gene and one or more modifier genes were involved in determining the degree of expression of the Compact phenotype. On this basis we speculated that the Compact phenotype derives primarily from a major mutation, which arose *de novo* or was already present in the selection line, and that the selection line was also segregating at one or more modifier loci, which came to phenotypic expression only in conjunction with the major Compact mutation (VARGA *et al.* 1997; SZABÓ *et al.* 1998). In subsequent work, the putative major gene (*Cmpt*) was mapped to a small region on chromosome 1 very close to the D1Mit237 marker and syntenic to the segment of bovine chromosome 2 containing the double-muscle *mh* gene.

After the discovery of the myostatin gene (MCPHERRON *et al.* 1997), we identified the *Mstn*$^{Cmpt\text{-}dl1Abc}$ deletion in the propeptide region of the mouse myostatin (*Mstn*) gene in homozygous *Cmpt* animals and mapped the mouse *Mstn* gene to the same small region on chromosome 1 containing the putative *Cmpt* gene. On this basis, we identified the *Mstn*$^{Cmpt\text{-}dl1Abc}$ deletion as the causative mutation responsible for the hypermuscular Compact phenotype (SZABÓ *et al.* 1998).

To map the putative modifiers, we produced a large F_2 population from a Comp9 × CAST/Ei intersubspecific cross, Cross4. Within the F_2 progeny, mice homozygous for the normal myostatin allele were almost exclusively of normal phenotype. Although in one of the three scorings of individual mice a few male mice and even fewer female mice of this genotype were occasionally given compact scores or 2 or even 3, these were almost always accompanied by two scores of 1.0 and hence can be attributed to phenotyping error. Thus, there is no indication in these data that the *Cmpt* mutation was



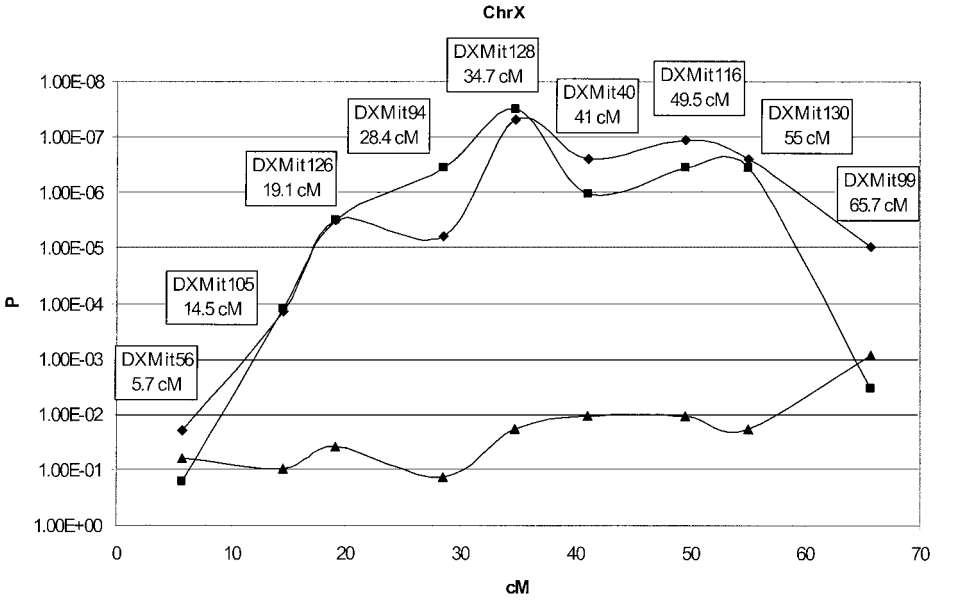


FIGURE 3.—*Continued.*

acting as a "sensitizing mutation" (MATIN and NADEAU 2001) in which a major gene (*Mstn*$^{Cmpt\text{-}dl1Abc}$) increases the expression and penetrance of loci affecting the trait, but which normally has only small effects that are difficult to detect. Rather, the modifier loci appear to act only in the presence of *Mstn*$^{Cmpt\text{-}dl1Abc}$.

In the Compact crosses reported previously, inheritance of the Compact phenotype showed intermediate dominance in males and was recessive in females (VARGA *et al.* 1997). In the present cross (Cross4) where the new Comp9 strain was employed, a similar pattern of recessive hypermuscularity was found in both sexes. A comparable pattern of similar F_1 and F_2 male and female phenotypic distributions was observed in a previous Comp9 × BALB/c intercross (Cross3; data not shown). This indicates that differences in the pattern of inheritance of the Compact phenotype in Cross4, as compared to the previous crosses, can be attributed to the different genetic character of the new Compact strain, Comp9, rather than to the genetic character of the CAST/Ei parent line in Cross4.

F_2 mice homozygous for the *Mstn*$^{Cmpt\text{-}dl1Abc}$ deletion distributed over all the phenotypic classes, including normal muscularity. Thus, using the terminology of GREENBERG (1993), the *Mstn*$^{Cmpt\text{-}dl1Abc}$ deletion in the myostatin gene is necessary, but not sufficient, for the expression of extreme hypermuscularity. It is plausible that the observed wide variation in mutant expression was due to the influence of the putative modifier genes that are also segregating in this F_2 population. To map these modifier QTL, we carried out a whole genome scan, using only F_2 mice that were homozygous for the *Mstn*$^{Cmpt\text{-}dl1Abc}$ deletion. Significant association at an FDR of 0.05 was found for markers on chromosomes 3, 5, 7, 11, 16, and

X. With a few exceptions, markers examined in these regions showed a higher frequency of the Comp9-derived allele in the high-compact-score group and a higher frequency of the CAST/Ei-derived allele in the low-compact-score group. Thus, our mapping results support the hypothesis that modifier alleles with positive effect on hypermuscularity were present in the Compact lines and were exposed for selection by the presence of the $Mstn^{Cmpt\text{-}dl1Abc}$ deletion, and in this way increased in frequency by the continued selection for hypermuscularity in this line. In the present study, we utilized only part of the potential mapping power of our large F_2 population. By identifying additional recombinant *K* individuals in the regions containing putative QTL and by genotyping all recombinants for multiple markers across the putative QTL-containing regions, it should be possible to increase map resolution up to fourfold (Ronin *et al.* 2003) to define more precise locations for the modifier loci.

Although it may be premature to speculate on the identity of the modifier genes located on these chromosomes, we note with interest that a number of attractive candidate genes are located near some of the sharper peaks uncovered in the present mapping effort. Recent articles have shown that myostatin is a downstream target of MyoD1 (Spiller *et al.* 2002), which is a key regulator of skeletal myogenesis on chromosome 7 (23.5 cM), while myostatin can downregulate the activity of MyoD1 through Smad3, thus inhibiting myoblast differentiation, which results in hyperplasia and hypertrophy (Langley *et al.* 2002). Another candidate gene in the same region of chromosome 7 (28.5 cM) is the gene *Pcsk6* (for the *proprotein convertase subtilisin/kexin type 6*), an enzyme known to be involved in the proteolytic processing of precursors of TGF-β family members (Akamatsu *et al.* 1999, 2000; Constam and Robertson 1999, 2000). Chordin (chromosome 16 at 14 cM) is known to bind the TGF-β-like bone morphogenetic proteins and sequester them in latent complexes (Abreu *et al.* 2002), raising the possibility that it may also have a role in myostatin regulation. Androgen receptor (Ar) is found 1.3 cM distal to the strongest peak detected on chromosome X at 35.7 cM. The direct androgen regulation mediated by Ar might also influence the expression of myostatin (Ma *et al.* 2001), as in the case of TGF-β (Chipuk *et al.* 2002).

Innumerable experiments document the importance of modifier genes among the causes of variable expression and penetrance of spontaneous, engineered, and induced mutants in mice. A number of studies have gone on to map the modifier loci. Most of these involved modifiers affecting penetrance of genetically determined abnormal phenotypes (Neumann *et al.* 1994; Letts *et al.* 1995; Cryns *et al.* 2002). Only two, as in the present study, considered effects of modifiers on expressivity (Alfred *et al.* 1997; Woo *et al.* 1997). Both were successful in mapping modifiers of this nature. Determining and classifying the effects of modifier genes is of great importance in understanding the range of phenotypes shown by Mendelian disease traits in humans (Nadeau 2001) and the effects of engineered or spontaneous mutants in agricultural species. Positional cloning of modifiers can also be expected to expose new elements involved in the regulation of major or engineered genes that may be of physiological significance in their own right.

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