Identification of Quantitative Trait Loci Affecting Murine Long Bone Length in a Two-Generation Intercross of LG/J and SM/J Mice

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ABSTRACT:

Introduction: Study of mutations with large phenotypic effects has allowed the identification of key players in skeletal development. However, the molecular nature of variation in large, phenotypically normal populations tends to be characterized by smaller phenotypic effects that remain undefined.

Materials and Methods: We use interval mapping and quantitative trait locus (QTL) mapping techniques in the combined F₂-F₃ populations ($n = 2111$) of an LG/J $\times$ SM/J mouse intercross to detect QTLs associated with the lengths of the humerus, ulna, femur, and tibia.

Results: Seventy individual trait QTLs affecting long bone lengths were detected, with several chromosomes harboring multiple QTLs. The genetic architecture suggests mainly small, additive effects on long bone length, with roughly one third of the QTLs displaying dominance. Sex interactions were common, and four sex-specific QTLs were observed. Pleiotropy could not be rejected for most of the QTLs identified. Thirty-one epistatic interactions were detected, almost all affecting regions including or immediately adjacent to QTLs.

Conclusions: A complex regulatory network with many gene interactions modulates bone growth, possibly with integrated skeletal modules that allow fine-tuning of developmental processes present. Candidate genes in the QTL CIs include many genes known to affect endochondral bone growth and genes that have not yet been associated with bone growth or body size but have a strong potential to influence these traits.

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INTRODUCTION

ENDOCHONDRAL BONE DEVELOPMENT is a complex process controlled by many different genetic factors, including morphogens, hormones, transcription factors, and growth factors, as well as by environmental components such as biomechanical forces. These elements either initiate or directly function in intricate signaling pathways that modulate all aspects of longitudinal bone growth, which occurs primarily through the division and hypertrophy of growth plate chondrocytes. The study of mutants with abnormal limb lengths and other bone phenotypes has facilitated the identification of many major players in skeletal development and growth. Known molecular variants have been associated with a wide range of phenotypic effects ranging from slight shortening or elongation of long bones or digits to the formation of additional digits or even the complete loss of appendicular skeletal elements.⁽¹⁾ Whereas the paradigm of analyzing extreme pathological phenotypes with mendelian properties has yielded great insights into bone development and growth, the molecular basis of

“normal” bone length variation remains obscure. We expect these variations to arise from genes of small effect or from small changes to known genes, both of which allow the fine-tuning of normal developmental processes.

A quantitative genetic approach builds on information about known pathways by identifying and investigating genomic elements with more complex genetic properties that play smaller, modulatory roles (contributing 10% or less of the overall phenotypic variance) in the developmental process. Linkage analysis has successfully identified numerous quantitative trait loci (QTLs) associated with skeletal traits, including bone morphology and dimensions,^(2–8) BMD,^(9–12) bone mechanical properties,^(13–17) and trabecular bone architecture.^(18,19) Multiple QTLs affecting length are also known and frequently map to loci associated with BMD or total body size.^(2–6,8,20–22) In some cases, specific polymorphisms, such as the number of (GT) dinucleotide repeats in the first intron of the *Col1a2* gene^(23,24) or a biallelic restriction fragment length polymorphism in the first intron of the *CD38* gene,⁽²⁵⁾ have been established as the underlying bases for QTLs affecting bone. However, despite the ever-growing list of loci associated with bone phenotypes, few

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FIG. 1. SNP map. Locations of SNPs used for genotyping. The chromosomes are depicted with the centromere at 0 cM. The diamonds represent individual SNP positions.

instances of phenotype-altering polymorphisms within the translated regions of candidate genes have been reported.

QTL mapping studies are particularly useful for examining the complexity of the genetic architecture—the context of a genetic element. QTL mapping allows estimation of more global parameters influencing physical characters, including the number of loci underlying trait variation, effect sizes and specific dominance characteristics, and epistatic interactions between loci, as well as pleiotropic and sex-specific profiles.⁽⁸⁾ Genetic architectural elements may be thought of as statistical descriptions of physical phenomena and can be further characterized to gain a better understanding of how the genetic elements controlling phenotypes—including promoters, enhancers, and splice sites, as well as genes—are modulated by the genetic background. A detailed picture of the genetic architecture for a trait can provide crucial insight into the mechanisms by which the phenotype is generated. In the case of a pleiotropic bone QTL, where one locus contributes to multiple traits, the molecular mechanism may be a change in the regulation of a transcription factor necessary for endochondral bone growth in all long bones or just in serially homologous structures.⁽²⁶⁾ In the case of epistasis, where a trait varies because of conditional effects of multiple loci, it is possible to identify specific genetic backgrounds where one bone QTL has different effects depending on the alleles present at other loci, indicating potential direct or indirect interaction of two or more genomic elements or their products. In this study, we used the F_2 and F_3 generations of an LG/J $\times$ SM/J mouse intercross to map QTLs for long bone length.^(2,27,28) Future characterization of these QTLs will yield insights into the mechanisms controlling small variations in long bone elongation.

MATERIALS AND METHODS

Mouse populations

We used the F_2 and F_3 generations of an intercross of LG/J and SM/J mice, obtained from The Jackson Laboratory (Bar Harbor, ME, USA). These strains maintain a

~20-g difference in adult body size: LG/J mice were selected for large body size (~40 g) at 60 days,^(29,30) and SM/J mice were selected for small body size (~20 g) at 60 days.⁽³¹⁾ The LG/J $\times$ SM/J intercross was initiated by mating 10 LG/J females with 10 SM/J males, producing 41 F_1 animals completely heterozygous at variable loci. An F_2 mapping generation ($n = 506$) was produced by randomly mating the F_1 animals and has subsequently been used to identify 18 long bone length QTLs.⁽²⁷⁾ To produce the F_3 generation ($n = 1605$), males and females from the F_2 mapping population were randomly mated to initiate an advanced intercross line from the LG/J $\times$ SM/J intercross.⁽³²⁾

All mice were weaned at 3 wk postpartum and were mated at a variable time after 10 wk. Males and females were maintained separately in an animal facility held at a constant temperature of 21°C and with a 12-h light-dark cycle. Animals were killed, weighed, and necropsied after the production of sufficient litters to continue the next generation. DNA was extracted from the liver tissue, and each individual was genotyped at 384 polymorphic SNPs (Fig. 1) using the Illumina GoldenGate platform. Skeletons were prepared with dermestid beetles and the right set of long bones (humerus, ulna, femur, and tibia) were measured to the nearest 0.01 mm with digital calipers. If right bones were missing or damaged, measurements were taken from left bones.

Statistical analysis

To minimize residual variation and increase our power to detect genetic effects, long bone measurements were corrected for sex, generation, age at necropsy, litter size at birth, and litter size at weaning using linear regression analysis to identify the residual effects after adjusting for each of these factors on each trait. After correction, the residual phenotypes and genotypes were combined and statistical analysis was performed using SYSTAT 11.0 (<http://www.systat.com>) and R (<http://www.r-project.org>). The X chromosome was not analyzed.

Corrected phenotypes were analyzed for the presence of single QTLs by interval mapping.^(33,34) Map distances were

TABLE 1. SKELETAL HERITABILITIES AND GENETIC CORRELATIONS

Trait	Mean $\pm$ SD	Humerus	Ulna	Femur	Tibia
Humerus	12.36 $\pm$ 0.387	0.84			
Ulna	14.14 $\pm$ 0.419	0.90	0.87		
Femur	15.75 $\pm$ 0.526	0.91	0.89	0.90	
Tibia	17.36 $\pm$ 0.556	0.91	0.95	0.91	0.92

Mean values and SDs are listed. Heritabilities (diagonal) and genetic correlations (off-diagonals) were calculated from the F_3 population using the full sibship method.⁽³⁵⁾

generated in R/QTL and are given in Haldane's cM units. Genotypic scores were imputed every 1 cM using a Perl script written by JP Jarvis. Missing data were calculated by imputing the genotypes between flanking marker regions using known genotypes. Additive genotypic scores (X_a) for each position were designated as the probability of being a SM/J homozygote multiplied by -1 added to the probability of being a LG/J homozygote multiplied by $+1$. Probabilities were obtained using recombination rates between the position of interest and the genotypes of the flanking markers.⁽³⁴⁾ Dominance scores (X_d) for each position were designated as the probability of heterozygosity multiplied by $+1$. The multiple regression analysis was performed using the SETCOR function in SYSTAT 11.0 to regress the phenotypes onto the genotypic scores every 1 cM along each chromosome. F statistics and associated p values were generated for each individual trait and for all traits combined in a multivariate test, under a null model of no gene effect at each position analyzed. We used the multiple regression coefficients as estimates of the additive (a) and dominance (d) genotypic values at the QTL.⁽³⁵⁾ The most likely position of the QTL is the position with the lowest probability of occurring by chance if there is no genotype-phenotype association. To allow easy comparison with the LOD scores generated by maximum likelihood analysis, our probabilities were translated to a linear scale using logarithmic transformation [$LPR = -\log_{10}(\text{Prob.})$].⁽³³⁾

To account for the family structure introduced by combining F_2 and F_3 animals, simulations were performed in R to calculate significance thresholds under a null hypothesis of no QTLs present. Artificial genotypes were generated for each individual using the known population pedigree and recombination rates of the observed population. Observed phenotypes were paired with the simulated genotypes for analysis. Next, QTLs were mapped as described above, and the process was repeated 1000 times with the distribution of LPR scores used to obtain significance thresholds. Significance thresholds obtained in this fashion take multiple comparisons into account and maintain both the autocorrelation of genotypes and the overall heritability of the phenotypes, listed in Table 1. The genome-wide multivariate threshold is 8.32, and thresholds for each chromosome are given in Table 2. Chen and Storey⁽³⁶⁾ recommend chromosome-wise significance thresholds as a means of controlling the false-positive rate in genome scans. Typical CIs, described as a 1-LOD (or 1-LPR) drop on either side of a QTL peak, were not useful for the extremely large peaks generated here. Because our significance thresholds

were inflated relative to the F_2 population, the chromosome-wide significance thresholds of the F_2 - F_3 were divided by those of the F_2 ,⁽²¹⁾ and the resulting scaled values were used to determine the CIs (Table 2).

To test for the presence of two QTLs on a chromosome, X_a and X_d of two positions were considered jointly, with every chromosomal marker or imputed position tested against all others.⁽³⁴⁾ The 2-QTL model was accepted if it fit better than the 1-QTL model at the 5% significance level, determined by a χ^2 test with two degrees of freedom. We also examined the data for the presence of sex-specificity in the QTL by analyzing each chromosome for interactions between sex and the genotypic values (X_a and X_d) for individual traits. Significant results ($p < 0.05$) indicated sex-based differences in genotypic effects. Chromosomes with significant sex differences were reanalyzed with the sexes separated, and visual inspection was used to resolve whether a QTL was sexually dimorphic.

To test for pleiotropy, the multivariate peak position was compared with the peaks for individual traits using a linkage versus pleiotropy test.⁽³⁷⁾ Determinants of the multivariate sum of squares and cross-product (SSCP) matrix for a model with all traits at a common position and the SSCP matrix for a model with each trait mapped to its own peak LPR position were calculated, and the log of the ratio of multivariate to individual location determinants was considered. A χ^2 test was performed. Non-significant results ($p > 0.05$) indicate that the pleiotropic model, the null hypothesis in this analysis, could not be rejected.

Epistatic interactions for each trait were identified in R by pairwise scanning for each combination of positions in the genome, including SNPs and imputed positions. Epistatic loci within 10 cM of an established QTL were considered to be part of that QTL. The epistasis model includes the effects of four types of interactions: additive-by-additive ($X_{a1} \times X_{a2}$), additive-by-dominance ($X_{a1} \times X_{d2}$), dominance-by-additive ($X_{d1} \times X_{a2}$), and dominance-by-dominance ($X_{d1} \times X_{d2}$). Bonferroni thresholds for QTL-by-QTL, QTL-by-genome, chromosome-by-chromosome, and genome-by-genome interactions were estimated using trait heritabilities calculated using the among-litter variance method⁽³⁵⁾ and the effective number of comparisons. The QTL-by-QTL LPR thresholds were 1.86 for the humerus, 1.88 for the ulna, 1.91 for the femur, and 1.92 for the tibia. QTL-by-genome LPR thresholds ranged from 5.41 to 5.64, whereas chromosome-by-chromosome LPR thresholds ranged from 4.39 to 6.08. Genome-by-genome LPR thresholds were 8.59 for the humerus, 8.70 for the ulna, 8.81 for the femur, and 8.88 for the tibia.

Candidate genes, listed in Table 3, were identified using Ingenuity Pathway Analysis (<http://www.ingenuity.com>). The relevance of all genes to bone or body size phenotypes was confirmed using the Online Mendelian Inheritance in Man (<http://www.ncbi.nlm.nih.gov/sites/entrez?db=omim>) or PubMed (<http://www.pubmed.gov>) databases. Not all candidate genes listed are immediately relevant to bone length phenotypes.

TABLE 2. PLEIOTROPIC QTLs

<i>QTL</i>	<i>Traits affected</i>	<i>Locus distance (cM)</i>	<i>Locus distance* (Mbp)</i>	<i>Distance to SNP (cM)</i>	<i>Nearest SNP</i>	<i>SNP position (Mbp)</i>	<i>LPR</i>	<i>CI (cM)</i>
Chromosome 1 (6.31)							CI LPR Drop: 2.98	
Lbn1.2	HUT	19	41.03	+2.53	rs13475837	43.4	9.41	5–25
Lbn1.1	HUFT	41	89.08	–0.60	petM22381–169–2	87.0	20.34	38–43
Lbn1.3	F	56	152.21	–0.11	rs3688042	151.1	19.30	55–60
Chromosome 2 (3.51)							CI LPR Drop: 1.72	
Lbn2.1	HUFT	45	77.35	–0.51	rs3713848	75.7	13.78	42–49
Lbn2.2	HFT	76	156.29	+0.23	gnf02.161.674	158.2	13.20	71–81
Chromosome 3 (4.30)							CI LPR Drop: 2.17	
Lbn3.1	HFT	29	60.39	+1.32	rs13477154	63.3	9.00	25–32
Lbn3.2	UF	60	115.92	+0.71	rs13477364	117.4	6.42	54–69
Lbn3.3	U	81	145.80	+1.69	rs3722447	147.9	10.46	74–86
Chromosome 4 (3.65)							CI LPR Drop: 1.85	
Lbn4.1	HUFT	29	63.67	+0.93	gnf04.062.327	65.8	18.39	25–31
Lbn4.2	UFT	64	133.39	–2.33	rs13477996	130.5*	6.06	58–72
Chromosome 5 (2.97)							CI LPR Drop: 1.47	
Lbn5.1 _M	HF	64	128.12	+3.00	rs6377710	131.6	6.88	53–71
Chromosome 6 (4.31)							CI LPR Drop: 2.27	
Lbn6.1	HUFT	37	85.33	+1.73	rs13478882	90.1	13.14	25–42
Lbn6.2	HUFT	72	144.01	–1.82	rs6199136	141.2	21.12	67–74.9
Chromosome 7 (3.35)							CI LPR Drop: 1.62	
Lbn7.1	FT	25	50.42	+2.70	gnf07.056.997	56.3	10.45	19–30
Chromosome 8 (3.55)							CI LPR Drop: 1.96	
Lbn8.2	T	2	9.80	–2.00	rs6273176	7.2	25.89	0–11
Lbn8.1	HUFT	39	71.85	–1.84	rs13479811	66.2	15.21	30–43
Chromosome 9 (2.83)							CI LPR Drop: 1.55	
Lbn9.1	U	13.5	29.85	0	CEL-9_29909656	29.9	12.73	3–18
Lbn9.2	H	42.4	73.42	0	rs13480273	73.4	11.34	38–47
Lbn9.3	HUFT	74	119.74	+1.77	rs8254378	120.8*	9.78	70–75.8
Chromosome 10 (4.48)							CI LPR Drop: 2.27	
Lbn10.1	H	33	82.11	+0.41	rs13480678	84.6	14.26	28–38
Lbn10.2	UT	66	127.64	+0.22	rs6290842	128.9	5.06	61–66.2
Chromosome 11 (4.57)							CI LPR Drop: 2.26	
Lbn11.1	UT	7	16.57	–1.52	rs3724116	14.6	9.37	0–12
Chromosome 13 (3.28)							CI LPR Drop: 1.79	
Lbn13.1	HUFT	48	98.97	+1.58	rs13481990	101.3	27.51	44–51
Chromosome 14 (4.06)							CI LPR Drop: 2.21	
Lbn14.1	HU	43	93.07	–3.83	rs3718262	88.0	4.82	26–51
Chromosome 15 (3.15)							CI LPR Drop: 1.73	
Lbn15.1 _F	U	25	60.88	–0.96	rs4139476	62.9	3.83	18–31
Lbn15.2 _M	HU	43	87.73	+1.82	rs3720931	89.55	5.82	27–52
Chromosome 17 (3.75)							CI LPR Drop: 2.25	
Lbn17.1	HUFT	17	41.06	+0.63	CEL-17_40073719	39.4	23.00	14–20
Chromosome 19 (3.76)							CI LPR Drop: 2.04	
Lbn19.1 _F	T	4	10.79	+1.03	rs6307076	13.8	4.53	0–30

Pleiotropic QTLs are listed by name, trait(s) affected (H, humerus; U, ulna; F, femur; T, tibia), the distance of the locus from the centromere (in cM and Mbp), the distance to, name, and position of the nearest SNP, the LPR score for the peak, and the CI. Nonitalics indicate QTLs significant at the chromosome-wide level (thresholds listed in parentheses after chromosome number). LPR drop for CI listed for each chromosome. Subscripts M and F denote male- and female-specific QTLs.

* Physical positions calculated with the flanking marker physical positions and the interval map.

RESULTS

QTLs

Long bone lengths were highly heritable, ranging from 0.84 to 0.92 (Table 1). The genetic correlations between skeletal traits (values off the diagonal) were also high, ranging from 0.89 to 0.95. All of these estimates were calculated using the F₃ population.⁽³⁵⁾ Heritabilities and genetic cor-

relations calculated using the combined F₂–F₃ population⁽³⁵⁾ resulted in similar values, except that humerus and ulna heritabilities were slightly lower (0.65 and 0.64, respectively).

Interval mapping analysis showed the presence of seventy single-trait QTL (55 significant at the genome-wide level [QTL] and 15 significant at the chromosome-wide level only [suggestive QTL]) affecting murine long bone

TABLE 3. CANDIDATE GENES

QTL	Candidate genes	Genes in CI
Lbn1.1	<i>Col3a1, Col9a1, Fhl2, Il17a, Il1r1, Stat1, Zap70</i>	140
Lbn1.2	<i>Col6a3, Dgkd, Hdac4, Inpp5d, Nppc, Ramp1, Ssp2, Twist2</i>	101
Lbn1.3	<i>Cacna1e, Lamc2, Pla2g4a, Prg4, Ptgs2, Rgs2, Rnf2</i>	37
Lbn2.1	<i>Acp2, Atf2, F2, Frzb, Hoxd3, Hoxd8, Hoxd10, Hoxd11, Hoxd12, Hoxd13, Lrp4, Lnp, Mdk, Nfe2l2, Prkra, Sp3</i>	110
Lbn2.2	<i>Ada, E2f1, Gdf5, Ghrh, Id1, Sox12, Src, Tcf15, Wisp2</i>	142
Lbn3.1	<i>Foxo1, Postn, Shox2</i>	78
Lbn3.2	<i>Casp6, Coll1a1, Egf, F3, Kcna3, Pitx2, Vav3</i>	129
Lbn3.3	<i>Bmpr1b, Nfkb1, Ppp3ca</i>	87
Lbn4.1	<i>Hmgb1, Slc31a1, Tlr4, Tnc</i>	54
Lbn4.2	<i>Cdc42, Cnr2, Ephb2, Grhl3, Hdac1, Hspg2, Phc2, Runx3, Stmn1, Wnt4</i>	199
Lbn6.1	<i>Cyp26b1, Eif2ak3, Ghrhr, Mthfd2, Nr2c2, Stambp, Tacrl, Wnt7a</i>	260
Lbn6.2	<i>Itpr2, Pthlh, Sox5</i>	53
Lbn7.1	<i>Atf5, Bax, Ccne1, Clec11a, Fgf21, Myod1, Nell1</i>	200
Lbn8.1	<i>Col4a1, Efnb2</i>	57
Lbn8.2	<i>Cln3, Comp, Gab1, Gdf15, Hhip, Smad1</i>	175
Lbn9.1	<i>Acp5, Barx2, Bmper, Carm1, Cdkn2d, Cdon, Ecsit, Epdr, Ets1, Fli1, Icam1</i>	134
Lbn9.2	<i>Adam10, Aldh1a2, Bmp5</i>	90
Lbn9.3	<i>Cdc25a, Gpx1, Mst1, Plxnb1, Pthr1, Rhoa, Tgfb2</i>	116
Lbn10.1	<i>Col18a1, Col6a1, Col6a2, Igfl, Itgb2, Rfx4</i>	244
Lbn11.1	<i>Aebp1, Egfr, Ewsi1, Grb10, Igfbp1, Igfbp3, Lif, Meis1, Osm, Spred2</i>	112
Lbn13.1	<i>Arsb, Ocln, Slc30a5</i>	61
Lbn17.1	<i>Adams10, Ager, Apom, Cbs, Ccnd3, Ck2b, Coll1a2, Cul7, Notch4, Rab11b, Runx2, Srf, Tnf, Vegfa</i>	298

Selected candidate genes in the CI of the indicated QTLs that have been shown to affect bone growth or body size are listed, with the total number of genes in the CI given in the final column. This list is not inclusive of all 2877 candidate genes in the regions.

length on all chromosomes except for 12, 16, and 18 (Table 2; full list available from author). On chromosomes 1, 2, 3, 4, 6, 8, and 9, the 2-QTL model fit better than the 1-QTL model, which indicated the presence of at least two QTLs for a trait on those chromosomes. Sex interactions were found on 10 of the 16 chromosomes (chromosomes 3, 4, 8, 9, 13, and 14 had no sex interactions) harboring bone length QTLs, and all long bones participated roughly equally in sexually dimorphic QTL. Four sex-specific QTLs were identified. Suggestive QTL Lbn5.1 showed a sex-specific effect on humerus and femur and was present only in males. Similarly, suggestive QTL Lbn19.1 affected tibia only in females. Another type of sexual dimorphism occurs when a peak is at the same position in both sexes but has a stronger effect in one sex than in the other. Suggestive QTL Lbn15.1, which affects ulna length in females, is marginally significant ($LPR = 3.83$), whereas suggestive QTL

Lbn15.2, which affects humerus and ulna length in males, is more strongly significant ($LPR = 5.82$). We considered these QTLs as two separate loci, although they are located near each other on chromosome 15, 25 cM for Lbn15.1 and 34 cM for Lbn15.2, and have overlapping CIs. Because they are sex specific, QTL mapping in later generations with more recombination will help resolve whether these QTLs reside at separate positions. Thus, all four long bones also participated in sex-specific QTLs to roughly equal extents.

Most gene effects identified were additive, with almost all QTLs presenting significant additive genotypic values. Significant standardized additive values ranged from -0.079 to 0.370 SD units, with a normal distribution. These effect sizes are similar to those previously estimated and individually account for a relatively small portion of trait variance. Typically, the *LG/J* allele resulted in longer bone lengths than the *SM/J* allele. At Lbn1.3, Lbn3.3, Lbn4.2, Lbn9.2, and Lbn9.3, however, the *SM/J* allele led to longer bone lengths, as indicated by their negative additive genotypic values. Roughly one third of the QTLs identified in this study showed significant dominance values, including 3 cases of *SM/J* dominance ($-1.5 < d/a < -0.5$), 5 cases of co-dominance ($-0.5 < d/a < 0.5$), and 11 cases of *LG/J* dominance ($0.5 < d/a < 1.5$). One case of underdominance ($d/a < -1.5$) and two cases of overdominance ($1.5 < d/a$) were also observed. Additionally, the 48 QTLs without significant dominance values were all considered co-dominant.

Pleiotropy

Frequently, QTL peaks were located in the same chromosomal region for multiple traits. This situation can be caused by pleiotropy, where one locus contributes to multiple individual traits, or by linkage disequilibrium, where the individual traits have contributions from separate, linked loci. Pleiotropy was accepted in all tested cases except for sex-specific QTL and on chromosome 10, where the large peak for humerus at position 33 cM dwarfed the two suggestive QTL for ulna and tibia at position 66 cM. When pleiotropy was considered, the 70 individual-trait QTL were condensed into 28 QTLs affecting long bone lengths.

Overall, the results of this analysis have validated the presence of over half of the 52 single-trait QTLs identified in previous mapping studies performed on the F_2 generation of the *LG/J* $\times$ *SM/J* intercross. Lbn1.1 (Sk11), Lbn2.1 (Bod2.1, Sk12), Lbn4.1 (Bod4.1), Lbn5.1_M, Lbn6.1, Lbn6.2 (Bod6.1, Adip2, Wt2), Lbn7.1, Lbn8.1 (Bod8.1, Adip4), Lbn10.1, Lbn13.1 (Bod13.1, Sk16), Lbn15.1_F and Lbn15.2_M (Bod15.1), and Lbn 17.1 were all validated in this study, as determined by overlap in single-trait CIs (alternate names in parentheses).^(2,8) Twenty single-trait QTLs from the previous study were not replicated (single-trait QTLs were present for 12 chromosomes but their CIs did not overlap), and 14 of these were only significant at the chromosome-wide level in the original study.⁽²⁾ Additionally, 26 new single-trait QTLs were found in the F_2 - F_3 population. Differences in population size ($n = 1045$ versus $n = 2111$ here) and marker density (96 microsatellites versus 384 SNPs here) account for changes in QTL number, the slight shifts in

FIG. 2. Epistatic long bone QTL network. Long bone QTLs (numbers in boxes) that have significant interactions are shown. Thick black lines depict interactions affecting four bones, dark gray lines indicate interactions affecting two bones, and thin, light gray lines indicate interactions affecting one bone.

position, and the altered effect sizes between the studies, especially given that the previous study was unable to detect any significant 2-QTL effects for long bones because of sample size and recombination detection limitations.⁽²⁾

Epistasis

The epistasis analysis suggested the presence of 31 significant epistatic interactions between 24 separate loci (Fig. 2; epistatic interaction table available from author). Several interactions were observed multiple times, surpassing QTL-by-QTL, QTL-by-genome, and chromosome-by-chromosome significance thresholds. Some interactions involving novel regions were observed at the chromosome-by-chromosome significance threshold, but the majority of these interactions involved established QTLs or regions immediately adjacent to them. One new region with epistatic activity is the proximal half of chromosome 18.

All four long bones were subject to epistatic interactions, with the humerus interacting at 9 locus pairs, the ulna and femur interacting at 14 locus pairs, and the tibia interacting at 10 locus pairs. Four interactions involved all four long bones, and four interactions involved two long bones, including two affecting ulna and tibia, one affecting femur and tibia, and one affecting ulna and femur. The general paucity of significant epistatic interactions in this population may be partially caused by strictly applying the extremely stringent significance thresholds. Epistatic interactions accounted for roughly 3% of the phenotypic variance for all four traits (humerus, 3.3%; ulna, 3.5%, femur, 3.4%; tibia, 2.6%). Thus, the epistatic interactions detected were not major contributors to the phenotypic variance observed. However, this may be at least partially explained by the extremely high heritabilities observed for the traits (Table 1).

Twenty-four significant epistatic gene effects were observed in this population. Half were significant for additive-by-additive effects and had normalized values in the range of -0.15 – 0.15 SD units, whereas 10 were significant for additive-by-dominance or dominance-by-additive effects and had normalized values ranging from -0.19 to 0.24 SD. Only two were significant for dominance-by-dominance interactions and had normalized values of 0.20 and -0.25 SD. The proportions of these epistatic inter-

actions are similar to those reported for tail length,⁽⁸⁾ and the epistatic genotypic values are similar in magnitude to the additive and dominance genotypic values observed in the single-locus analysis. Interestingly, two locus pairs were significant for the multivariate but had no significant individual epistatic effects (Lbn1.3 $\times$ Lbn8.2 and Lbn8.1 $\times$ Lbn9.2); further analysis of additional data will be necessary to confirm these interactions. Interactions between Lbn6.1 and Lbn6.2 were present for humerus and ulna, with significant additive-by-additive and dominance-by-dominance effects for humerus and significant additive-by-additive and additive-by-dominance effects for ulna. The only other intrachromosomal interaction was for Lbn1.1 and Lbn1.3, which displayed additive-by-dominance effects for the femur.

DISCUSSION

In our previous analysis of the F_2 generation of an LG/J $\times$ SM/J intercross, we discovered 52 single-trait QTLs for long bone lengths.⁽²⁾ By including the F_3 generation in this analysis and using a much denser genetic map, we were able to detect 18 additional single-trait QTLs. Several of these QTLs overlapped with other known QTLs for adiposity (Adip2, Wt2, Adip4) or skeletal traits (Sk11, Sk12, Sk16) that have previously been detected in the F_2 population alone.^(2,8) Four new sex-specific QTLs were identified in the F_2 - F_3 generations, and five QTLs that showed sexual dimorphism in the F_2 alone were detected in both sexes in the F_2 - F_3 generations. The QTLs were also mapped with greater precision here, with an average CI of 27 cM in the analysis of the F_2 generation⁽²⁾ being reduced to 12.8 cM for the combined F_2 - F_3 analysis. The increased amount of information gained from these analyses shows the advantages of using a much denser marker map and combining data across generations.

Many previously reported bone length QTL map to positions close to the ones reported here. There is some correspondence between our QTLs and those from other studies, but it is hard to judge whether the overlap with QTL confidence regions from other studies is greater than that expected by chance, making it difficult to provide a meaningful comparison of our QTLs with previously reported QTLs for bone-related phenotypes. In general, the QTL mapping studies in different mouse strains suggest that, whereas some regions of the genome may contain elements that universally affect long bone length, the different genetic backgrounds of the strains can drastically alter the numbers, positions, and effect sizes of bone length QTLs. This finding underscores the importance of understanding the genetic architecture underlying complex traits and in considering multiple strains. Additionally, most of the studies described used less dense marker maps and smaller population sizes. Both of these factors influence the power to detect QTLs and could have caused some of the differences in QTL number and positions between studies.

Of the 28 pleiotropic long bone length QTLs identified in this study, 23 had CIs that overlapped with the QTLs for F_2 - F_3 body weight components, including individual organ

weights, body weight at necropsy, and tail length (G Fawcett, personal communication). This may indicate either the presence of QTLs that affect total body size or the presence of linked QTLs for body size and skeletal traits. Further analysis of advanced generations with accumulated recombination will be necessary to determine whether any of these loci are indeed pleiotropic with body size QTLs. Five QTLs—Lbn2.2, Lbn3.1, Lbn4.1, Lbn5.1, and Lbn9.2—do not overlap with any other nonskeletal loci previously identified in this population, suggesting that these loci may be specific for long bone length.

Several different pleiotropic patterns were observed in our data. In 13 cases, a pleiotropic QTL affected three or four long bones, which may indicate the presence of a general bone growth factor. Eight QTLs affected individual bones, perhaps indicating the presence of bone-specific factors. Additionally, there were three cases where a QTL affected only upper or lower limbs; Lbn14.1 and Lbn15.2 affected the humerus and ulna and Lbn7.1 affected the femur and tibia. There were also three cases where the QTLs showed an effect on serially homologous elements, affecting either the stylopodia (humerus and femur), as with Lbn5.1, or the zeugopodia (ulna and tibia), as with Lbn10.2 and Lbn11.1. The cases where QTLs affect different sets of limb bones may indicate the presence of factors that function in different regions of the developing embryos or during different developmental stages.⁽²⁶⁾ Finally, Lbn3.2 only affected the ulna and femur. This rare and unexpected combination may be the outcome of restricting chromosomes to only two QTLs per trait when there may be more than two QTLs.

The number of candidate genes in our QTL CIs ranged from 37 to 298, for a total of 2877 candidates for the 22 pleiotropic QTLs significant at the genome-wide level. Candidates associated with bone phenotypes or changes in body size are listed in Table 3. Like all somatic cells, chondrocytes are subject to the effects of normal cellular processes, which could result in bone length phenotypes. Proteins associated with DNA transcription, RNA translation, intracellular trafficking, extracellular transport, the unfolded protein response, programmed cell death, and immune system function were frequently found among the candidate genes (not listed). Fine-mapping later generations of the LG/J $\times$ SM/J intercross will help resolve which candidates persist in falling under our QTL peaks and should reduce the number of genes in the confidence regions by a factor of between 5 and 20, depending on the advanced intercross generation considered.

Further complicating the issue is the possibility that either coding genes or regulatory elements could be the basis of QTL effects. In the latter case, the affected gene product(s) could be located at a distance from the QTL peak, perhaps even outside of the CI. For example, the genes *Hoxa1–13* are located proximal to the confidence interval of Lbn6.1. If dysregulated genes *Hoxa1–13* are at the root of Lbn6.1, a regulatory element under the Lbn6.1 peak could have an effect on the distant structural genes or their expression. Future work will examine such situations in light of the genetic architecture influencing the QTLs and the sequence of fine-mapped QTL CIs.

The results of the epistasis analysis indicate that a complex network of interactions occurs to regulate long bone growth (Fig. 2). Almost all QTLs identified here are involved somewhere in the pathway, and most interact with more than one partner. The larger question now is how the candidate genes fit into this network: what is the physical nature of the observed interactions? There are several direct interactions that are already known to occur between the candidate genes listed in Table 3, including a known interaction between *Tgfb β 2* (Lbn9.3) and *Sp3* (Lbn2.1)⁽³⁸⁾; known interactions between *Egfr* (Lbn11.1) and either *Stat1* (Lbn1.1)⁽³⁹⁾ or *Lamc2* (Lbn1.1)⁽⁴⁰⁾; and known interactions between *Myod1* (Lbn7.1) and either cyclin D3 (*Ccnd3*; Lbn17.1)⁽⁴¹⁾ or *Col4a1* (Lbn8.1).⁽⁴²⁾ These interactions suggest possibilities for the types of gene interactions underlying our QTLs. However, roughly 77% of the genes listed in Table 3 (119 of 155) can be linked to another candidate gene through secondary or tertiary interaction partners, indicating the necessity of understanding the genetic context of a gene's function.

The complex nature of endochondral bone growth leads to an inherent plasticity in the regulation of long bone growth. Developmentally, there are several different paths that can lead to identical end phenotypes, a concept known as developmental homoplasy.⁽⁴³⁾ This phenomenon has been observed for adult body size in mice selected either for rapid early growth or rapid late growth.⁽⁴³⁾ Similarly, mechanisms to increase the length of adult long bones can range from producing larger cartilage templates during embryonic development to increasing the proliferation rate or hypertrophy of chondrocytes in the postnatal growth plate. Because epistatic interactions indicate that the effect of any given long bone length QTL is dependent on the genotype at other loci, this can lead either to indistinguishable phenotypes despite functional genetic variation at a given locus or to multiple phenotypes despite genetic uniformity at a given locus, both of which underscore the importance of understanding the genetic context of a QTL, gene, or genetic element. Creating reciprocal congenic mouse lines, where a QTL can be placed in a new genetic background, will allow us to perform histomorphological analysis of growth plates to identify the specific developmental stage(s) in which the QTL functions and determine how the genetic architecture influences QTL function. Once the stage at which a QTL function is known, future studies can be aimed at characterizing the mechanism(s) that cause small variations in long bone length.

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