Mouse Loci Associated With Life Span Exhibit Sex-Specific and Epistatic Effects

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We have looked for genetic predictors of life span in a sibship of mice created as a four-way cross among inbred grandparental strains BALB/cJ, C57BL/6J, C3H/HeJ, and DBA/2J. To minimize the potential confounding effects of loci that influence early-life illnesses only, we conducted two analyses: one involving all the mice, and the other using a data set from which the first 20% of the deaths were excluded. The two strongest associations reach experimentwise significance levels (p < .01) when tested on the 80% of the mice with the longest life spans. Surprisingly, three of the four strongest associations showed sex-specific effects, with an influence on life span of either male or female mice, but not both. Epistatic interactions among the loci were also identified. The life-span effect of a locus on chromosome 10 (D10Mit15) exhibited epistatic interactions with loci on chromosomes 9 and 16 (D9Mit10 and D16Mit182). In a second example, a locus on chromosome 12 (D12Mit167) depended on the specific combination of alleles inherited from both male and female parents. Our results show that the common laboratory mouse strains are polymorphic at loci that produce substantial differences in life span and that these effects can be sex specific and conditional on alleles inherited at other loci.

Very little is known about the number or position of loci with detectably large effects on life span in mammals. Heritability estimates (1) have indicated that genetic differences may account for as much as 25–30% of the variance in life span in populations of flies, rodents, and humans, but they give no inkling as to what portion of this variation is attributable to loci with major effects. Studies of recombinant inbred mouse lines (2) and of backcrosses between heterogeneous lines of mice selected for differences in antibody production (3) have suggested that longevity may be influenced by a fairly small number of loci. Quantitative trait locus (QTL) mapping in Drosophila (4) has provided evidence for loci whose effects on longevity are limited to male or female flies. To see if the QTL approach could also help enumerate and map mammalian loci with substantial (i.e., detectable) influence on longevity, we have carried out a genomewide scan for loci associated with differential survival in a four-way cross among four inbred mouse lines commonly used for studies of aging and disease. At the same time we have looked for evidence that loci with effects on life span might be sex specific in their action, and we tested for possible epistatic interactions among the loci with strongest effects.

METHODS

Mouse Handling
(BALB/cJ × C57BL/6J)F1 females and (C3H/HeJ × DBA/2J)F1 males were purchased from Jackson Laboratories (Bar Harbor, ME) and mated to produce the study population. Throughout the study, all mice were housed in a single suite of specific-pathogen free (SPF) rooms under identical environmental conditions (12:12 hour light:dark cycle, 23°C) and given ad libitum access to water and laboratory mouse chow; quarterly tests of sentinel mice showed that the facility remained SPF throughout the period of the study. The animals were maintained for their natural life span or sacrificed when judged by an experienced technician to be moribund, who used a set of criteria including rapid weight loss, lethargy, inability to eat or to drink, and/or the presence of a very large or ulcerated tumor mass. Unpublished data (David Harrison, written communication, 1991) have shown that a “sacrifice when moribund” strategy does not lead to significant underestimation of life span when the animal’s health is evaluated by skilled caretakers. In addition, our own previous work (5) has shown that the longevity of mice euthanized when moribund is not significantly less than that of animals in the same study that were found dead in their cages and that >95% of mice sacrificed when moribund were found to have advanced disease, such as malignant neoplasia, likely to be incompatible with their long-term survival. At necropsy, the animals exhibited a range of pathologies and presumptive causes of death, as expected for a genetically heterogeneous population (5).

Genotyping
Genotyping was performed by standard polymerase chain reaction (PCR) amplification of genomic DNA from each animal, using marker loci obtained from the Mouse Simple Sequence Length Polymorphism Database, Whitehead/MIT Center for Genome Research (carbon.wi.mit.edu:8000/ftp/distribution/mouse_ssslp_releases/may99) (6). Polyacrylamide
gels were scored by silver staining or by using the ALFExpress automated sequencer as described (7). Analyses at 78 marker loci were performed on 253 individuals (110 males and 143 females) with an average intermarker interval of 23 cM. Of the markers, 69 are fully informative for all four grandparental alleles; the other 9 are informative for only one of the two parents. Genotypes were 95% complete on average for each marker. A full listing of loci as well as the complete genotype and phenotype data sets used in this analysis are available at sitemaker.med.umich.edu/dtburke/files/253mice_78markers.xls.

Statistical Analysis

The initial genomewide search for QTL was performed by using a single-point locus scan. An analysis of variance (ANOVA) was performed for single loci by using Proc MIXED in SAS version 7.0 (SAS Institute, Cary, NC). For fully informative loci, the effects specified were maternal allele, paternal allele, and maternal allele by paternal allele interaction. For partially informative loci, only the informative allelic pairs were examined. The single-locus analysis for the male only, female only, and combined data used the following statistical model (8):

$$ y_{ik} = \mu + \alpha_{ij} + \delta_{ij} + \epsilon_k $$

for $i,j = 1,2$

where $y_{ik}$ is the phenotype for the $k$th individual; $\mu$ is the overall mean; $\alpha_{ij}$ and $\delta_{ij}$ are the additive effects of the maternal allele $M_i$ and the paternal allele $M_j$, respectively, of marker $M$; $\delta_{ij}$ is the maternal by paternal allelic interaction (dominance effect); and $\epsilon_k$ is the error term with $N(0,\sigma^2)$. For reasons described in the text, the linkage analyses were conducted twice: once on the entire data set and once on a data set (EDE, for early deaths excluded) that removed the 20% of the mice dying prior to 657 days of age. For linkage analysis, the data were transformed to satisfy the assumptions of the classical regression model, that is, normality and constant variance of residuals. For this to be accomplished, the data were logarithmically transformed for the EDE populations and for the male-only population, and they were raised to the power of 1.8 for the female-only and male-plus-female populations.

Following the single-locus genome scan, a two-locus search was performed by examining all of the possible pairwise allelic interactions in the data. The computational difficulty of the two-locus search necessitated a change in the analytical procedure. Rather than viewing the genome scan data set as 69 four-way informative loci (plus nine biallelic informative loci), the two-locus genome scan defined 147 informative biallelic genotypes per animal (77 maternally informative and 70 paternally informative). Each pairwise combination of biallelic genotypes was examined by using an ANOVA with the following statistical model:

$$ y_{ij} = \mu + g_{1i} + g_{2j} + X_{ij} + \epsilon_k $$

for $i,j = 1,2$

where $y_{ij}$ is the phenotype for the $k$th individual; $\mu$ is the overall mean; $g_{1i}$ and $g_{2j}$ are the effects of the biallelic informative markers $M1$ and $M2$, $X_{ij}$ is the marker by marker allelic interaction (epistatic effect) for $M1$ allele $i$ and $M2$ allele $j$, and $\epsilon_k$ is the error term with $N(0,\sigma^2)$. As with the single-locus analysis, the two-locus genome scan was performed on the male only, female only, and combined data sets, sequentially, and it was conducted on both the entire data set and then on the EDE data set.

Assessment of Statistical Significance

Permutation testing was the principal method used to estimate experimentwide statistical significance. Permutation tests were performed essentially as described by Churchill and Doerge (9). For each round of permutation, phenotypes were shuffled and distributed at random among the individual animals, with each animal retaining its complete genomewide genotype. The resulting synthetic data set disrupts the genotype–phenotype association. The reshuffling was performed 1000 times, and the genome search was performed by using the original statistical model on each synthetic data set. The maximum test statistic for each synthetic genome scan is then recorded, and the threshold test statistic to obtain $\alpha = 0.05$ is derived from the resulting null distribution.

In addition, for each of the single-locus hypotheses (male only, female only, and combined), a Bonferroni correction was used that multiplies the pointwise probability by $216 = 69 \times 3 + 9$. This correction reflects the use of 69 markers that were informative about maternal, paternal, and maternal by paternal interaction effects (i.e., $N = 3$ hypotheses for each marker), and 9 other markers that were informative about maternally inherited or paternally inherited alleles but not both. For the two-locus search, the Bonferroni correction multiplies the pointwise probability by $10,731 = 147 \times 146/2$, because the genotype information was derived from 147 informative biallelic genotypings in the genome scan.

A post hoc analysis of epistatic interactions used an ANOVA in which life span was modeled as a function of allele at each of the two interacting loci plus an interaction term; reported $p$ values refer to the significance of the interaction term.

Results

Genomewide Survey for Life-Span-Associated Loci

The genetic analysis was based on a large population derived from a set of four grandparental inbred strains: C3H/HeJ (C3), DBA/2J (D2), BALB/cJ (C), and C57BL/6J (B6). The test population was produced by a cross between CB6F1 females and C3D2F1 males, thereby producing the genetic equivalent of full siblings. All animals were housed in SPF conditions. Each cage initially contained four or five same-sex littermates. Life span was calculated based on date of death for those animals found dead (45%), or on date of euthanasia for mice sacrificed when clearly moribund (55%). The sacrifice of moribund animals allows high-quality necropsies to be performed yet provides life-span values accurate to within a few days of natural death. Genotyping analyses at 78 marker loci were performed on 253 individuals (110 males and 143 females), with an average intermarker interval of 23 cM. Of the autosomal markers, 69 are fully informative for all four grandparental alleles; the other 9 are informative for only one of the two parents (7). Genotypes were 95% complete on average for each marker.
The genomewide search for life-span-associated QTL was performed with two statistical analyses: once using all of the data, and then a second time using the EDE data set that excluded the first 20% of the mice to die (i.e., excluding all deaths prior to 657 days of age). We chose to conduct a separate examination of this truncated EDE data set in part because of earlier observations that the heritability of life span in mice increases with age at death (3). In addition, we considered that deaths at early ages are unlikely to reflect the effects of aging per se, and their inclusion in life tables thus can confound the search for genes that modify aging processes. In fact, necropsy data showed that many of the early deaths in males were due to a urinary syndrome (10,11) that is typically seen only in group-housed males and is thought to reflect stresses associated with adjustments in dominance hierarchy rather than an effect of the aging process. Indeed, this urinary syndrome was responsible for 58% of the male deaths prior to 657 days of age, but only 5% of the male deaths after this age.

The main analysis method was based on an ANOVA (8) to analyze single loci. The statistical strategy was also designed to detect loci whose effect on life span might be limited to males or to females, because loci with sex-specific effects on longevity have been documented in QTL studies in Drosophila (4). For these reasons we tested three hypotheses in each of two data sets (untruncated and EDE), first looking for loci that influence males alone, then for loci in females alone, and, last, loci in data pooled across genders. The combined data set provides improved power to detect QTL that are not sex specific. The combined data set was treated as independent for the purposes of Bonferroni correction, even though it pools data from the two single-sex data sets. Significance levels were based on permutation tests (9), using the same statistical model and experimental data to develop experimentwise confidence thresholds that adjust for the simultaneous evaluation of multiple nonindependent hypotheses.

No loci were found to be predictors of longevity in the entire population (using an experimentwise significance criterion of \( p < .05 \) to reduce the reporting of false positive associations; 12), perhaps because the genetic factors that influence mortality risk at early stages of the life span differ from those that modulate aging and late-life disease.

In the EDE data set, however, three loci were found to be predictors of life-span differences with comparisonwise (i.e., unadjusted) probabilities ranging between .0004 and .00002. These are summarized in Table 1. These pointwise probability estimates require adjustment for the simultaneous testing of multiple hypotheses, that is, simultaneous assessment of the 78 informative genetic polymorphisms used in the analysis. Our principal method of adjustment was to calculate an experimentwise probability by permutation analysis as suggested by Churchill and Doerge (7), and these values are included in Table 1. By these criteria, both \( D12Mit167 \) and \( D9Mit110 \) were significant at the \( p < .01 \) level in the EDE data set, and \( D10Mit15 \) met the \( p < .05 \) criterion. We also calculated the Bonferroni-corrected significance threshold for multiple independent tests and noted that two of the three loci met this conservative test (\( p < .05 \)).

The \( D9Mit110 \) polymorphism was associated with life-span differences only in male mice (Figure 1). The top panel shows the survival curve for males in the EDE subpopulation in which the C3 allele at \( D9Mit110 \) promotes longevity. For \( D9Mit110 \), mean survival values for EDE males were as follows: genotype C/C3, 854 ± 137 (given as mean ± standard deviation); genotype B6/C3, 933 ± 120; genotype C/D2, 847 ± 106; and genotype B6/D2, 772 ± 68. The middle panel shows survival for all males. The data show a crossover in the survival plot, in that the C3 allele at \( D9Mit110 \) is associated both with a higher death rate in early life (caused in large part by an effect on the timing of the mouse urinary syndrome) and a lower death rate at later ages. Among mice dying of this urinary syndrome, those with the C3 allele at \( D9Mit110 \) died at an average age (442 ± 139 days, \( n = 14 \)) that is significantly younger than the age of those with the D2 allele (601 ± 8 days, \( n = 8 \); \( p = .03 \) by two-sided Student’s t test). Thus the C3 allele at \( D9Mit110 \) is associated with two effects: relatively early death in those males that die of urinary syndrome and relatively long survival in male mice surviving longer than 657 days. Survival of female mice, shown in the bottom panel of Figure 1, is not appreciably affected by \( D9Mit110 \) genotype at any age.

Figure 2 presents a similar analysis for the \( D10Mit15 \) locus. Among EDE males (top panel), those with the C/D2 genotype lived 879 ± 140 days, and those with the B6/D2 genotype 881 ± 105 days, compared with 835 ± 113 for the C/C3 genotype and 758 ± 88 for the B6/C3 genotype. The middle panel shows that the \( D10Mit15 \) genotype seems not to affect mortality risk in the 20% of mice that die prior to 657 days of age, and the bottom panel shows that this locus is not associated with longevity differentials in female mice. Two other loci, not shown in Table 1, showed suggestive but not fully compelling evidence for linkage to life-span-regulating alleles in the untruncated data set: \( D4Mit171 \).

Table 1. Loci Associated With Differential Life Span in UM-HET3 Mice Bred as the Progeny of CB6F1 Females and C3D2F1 Males

<table>
<thead>
<tr>
<th>Locus or Locus Pair</th>
<th>Sex Affected</th>
<th>Population Affected*</th>
<th>Pointwise†</th>
<th>Experimentwise‡</th>
<th>Bonferroni Method§</th>
</tr>
</thead>
<tbody>
<tr>
<td>( D9Mit110 )</td>
<td>Males</td>
<td>Life span &gt; 657 days (EDE)</td>
<td>0.000018</td>
<td>&lt;0.01</td>
<td>0.0039</td>
</tr>
<tr>
<td>( D10Mit15 )</td>
<td>Males</td>
<td>Life span &gt; 657 days (EDE)</td>
<td>0.000371</td>
<td>&lt;0.05</td>
<td>0.080</td>
</tr>
<tr>
<td>( D12Mit167 )</td>
<td>Both</td>
<td>Life span &gt; 657 days (EDE)</td>
<td>0.000076</td>
<td>&lt;0.01</td>
<td>0.016</td>
</tr>
</tbody>
</table>

*EDE = early deaths excluded.
†Probability by analysis of variation for the indicated sex and population; not adjusted for multiple comparisons.
‡Probability estimated by permutation analysis.
§For the single-locus situation, the Bonferroni correction multiplies the pointwise probability by 216.
Interaction Between Maternal and Paternal Genotypes

Figure 3 presents the survival curves, among EDE mice, for the four genotypes discriminated by alleles segregating at D12Mit167. For the longest lived 80% of the population, D12Mit167 genotypes differ in life span (experimentwise \( p = .01 \)), with a significant interaction between the maternal and paternal alleles (post hoc \( p = .0001 \) by two-factor ANOVA). Both males and females are equally affected (not shown). For D12Mit167, the mean longevity values (± SD) in the EDE data set were as follows: genotype B6/C3, 886 ± 111; genotype C/D2, 867 ± 110; genotype B6/D2, 828 ± 116; and genotype C/C3, 790 ± 105. It is noteworthy that superior longevity is not associated with any one allele at this locus, but instead with a particular combination of alleles: the combination B6 + C3 is associated with longer survival, though neither the B6 nor the C3 allele confers increased longevity in combination with the alternate counter-alleles. Further work will be needed to determine whether this pattern represents interaction among alleles of a single effector locus, or interaction among alleles of linked but distinct loci on chromosome 12.

Genomewide Survey of All Pairs of Loci for Life-Span Association

We also carried out an analysis by using pairs of tested loci instead of single loci. This genome scan was, as for the
single-locus scan, conducted in both the EDE and untruncated data sets, and, as before, male and female mice were analyzed separately. The strongest association seen in this analysis is illustrated in Figure 4. Among female mice, those that inherit both the C allele at D16Mit182 and the C3 allele at D2Mit58 had an average longevity of $924 \pm 116$ (SD) days, substantially greater than that of the other genotypes ($805 \pm 144$ for genotype C3 + B6, $803 \pm 155$ for D2 + C, and $750 \pm 170$ for D2 + B6). The comparisonwise probability for this difference was $p = .000013$. Experimentwise significance thresholds were again calculated by permutation analysis, across all locus pairs for 600 permutations, and the association of longevity with D16Mit182 + D2Mit58 approached the conventional significance criteria at $p = .06$. An ANOVA was calculated to see whether the effects of the D2Mit58 and D16Mit182 loci were independent. In this post hoc analysis, each of the two loci had an independent effect ($p < .001$) on life span, but there was no evidence for significant epistatic interaction ($p = .20$). The effects of D2Mit58 and D16Mit182 on longevity in females thus seem to be approximately additive.

Epistatic Interactions

The five QTL shown (Figures 1–4) to have strong effects on life span were examined in all pairwise combinations as a way to look for possible genetic interaction effects. This analysis revealed dramatic interactions among three of the five genes, and these are illustrated in Figure 5. The first, shown in the top panels, involves interaction between alleles of D9Mit110 and D10Mit15. Each of these loci was associated (see Table 1) with differential survival in males of the longest-lived 80% of the mice. The survival plot shows, however, that the increased longevity associated with the C3 allele at D9Mit110 is seen only in the presence of the D2 allele of D10Mit15, and vice versa; neither allele has an association with longevity except in the presence of the other one. An ANOVA showed a significant ($F = 8.44, p = .005$) interaction term, confirming the impression that the effects of the D9Mit110 and D10Mit15 genotypes were not independent. The post hoc probability of the difference between the means is $p(F) = .00003$ by $t$ test and .0003 by Tukey’s honest significant difference for unequal $N$, and it reflects a difference of 162 days of mean survival between the longest- and shortest-lived genotype. The genotype associated with the longest survival among male EDE mice (C3 at $D9Mit110 + D2$ at $D10Mit15$) does not seem to influence survival among females.

Similarly, the effect of the paternal allele of D10Mit15 on survival of male mice was conditioned on the inheritance of specific maternal alleles at D16Mit182 (Figure 5). The increased longevity associated with the D2 allele at D10Mit15 was apparent only in the presence of the B6 allele at D16Mit182 ($F = 4.5, p = .036$ for the interaction term). The shortest- and longest-lived genotypes differ by 186 days ($p = .004$ by $t$ test, and $p = .04$ by Tukey’s honest significant difference test for unequal $N$). It is noteworthy that the B6 allele at D16Mit182, which is associated with shorter life span in females, is associated both with long life span in males that inherit the D2 allele at D10Mit15 and with exceptionally short life span in males inheriting the C3 allele at D10Mit15. Again, the genotype associated with longest survival among male EDE mice seems not to be associated with exceptional longevity among females.

Discussion

In principle, complex quantitative traits such as life span might be influenced by large numbers of polymorphic loci, each with effects so small as to be undetectable in moderately sized mouse populations. Our results show, on the contrary, that mouse life span can, at least in this particular genetic cross, be influenced by inheritance at a small number of segregating loci each of which produces an effect of substantial size, that is, 5–10% of the mean life span. It will be of interest to see if the QTL mapped in our current study influence life span in other crosses among standard laboratory stocks, and it may be particularly helpful to carry out similar studies in crosses whose progenitors include mice of sister species (e.g., Mus spretus) or from wild-trapped Mus musculus populations that have not been subjected to many generations of selection for laboratory adaptations (13).

We found no genes that had significant effects on longevity in males, or in both sexes combined, until we reanalyzed our data set by excluding the first 20% of mice to die. We considered a number of alternate strategies, including excluding larger proportions of the entire population, but we
found that the statistical significance of our candidate loci diminished when the number of mice included in the study group became too small to provide adequate statistical power. The decision to exclude the first 20% is thus to some extent arbitrary, based in part on empirical considerations and in part on a desire to separate early-life deaths from those deaths, at later ages, which we think are more likely to be timed by aging. When in follow-up studies we accumulate sufficient numbers of mice, we will be able to test more thoroughly the idea that these (or other) QTL influence mortality risks in the oldest-surviving groups of mice.

An earlier report (14) summarized preliminary evidence for QTL associations with longevity in a subset of the same mouse population presented in this paper, using a slightly different set of genotypic markers. The earlier report listed seven candidate QTL with pointwise \( p < 0.01 \), but none of these met experimentwise significance criteria. Two of the seven candidate QTL are now supported, with stronger evidence, in the present report. The C allele of D16Mit182 was found, in the initial report, to be associated with increased survival in female mice, and it is now shown to reach experimentwise \( p < 0.06 \) when considered with D2Mit58. The D2 allele of D10Mit40 was, in the initial report, found to be associated with increased life span in male mice; D10Mit40, at position 21 cM, is closely linked to D10Mit10 (position 25 cM), which is in the present report shown to be associated with increased life span in males with experimentwise \( p < 0.05 \). (The initial paper excluded male mice dying with MUS; the current paper, instead, examined mice of either sex dying after 657 days.) The current report cannot be taken as an independent replication of the earlier report, because the mice examined are a superset of the initial population. It is noteworthy that six of the seven initially reported QTL were also apparently sex specific in their effects. An analysis of larger numbers of mice will be needed to determine whether any of the other markers reported in the initial publication will eventually prove to be associated with life span to a significant degree.

Our new data support the surprising conclusion that QTL may influence life span through sex-specific pathways. Of the three individual loci with significant effects on life span (Table 1), two influence life span in males but not in females, and the pair of loci illustrated in Figure 4 influence survival only in females. Only one locus, D12Mit167, has equivalent effects in mice of both sexes. Most models of genetic influences on life span propose mechanisms in which these hypothetical polymorphisms act on pathways such as oxidant resistance, tumor suppression, mitochondrial function, or other processes thought likely to influence disease resistance equally in males and females. It is noteworthy that QTL analyses in Drosophila have also produced evidence for alleles with effects on life span limited to males or females only (4). Genome scans have also been successful in revealing QTL that affect life span in Caenorhabditis elegans (15–18).

Our data also led to a second interesting finding: alleles whose effects on life span are conditional on the alleles inherited at another locus (see Figure 5). Analyses of additional mice will be needed to address the question of whether the interaction between maternal and paternal alleles at D12Mit167 (Figure 3) reflects epistatic effects between two distinct but closely linked loci or an interaction...
among alleles at a single locus. The paired locus scan that produced evidence for the additive effect illustrated in Figure 4 also produced suggestive evidence (not shown) for epistatic interactions among other pairs of loci, but the numbers of mice tested are at this stage too small to allow detection of most paired-locus effects at high statistical power. It is striking that three (and possibly four, if one includes D12Mit167) of the five loci detected in the single and paired-locus genome scans have effects on life span that are conditional on inheritance at other loci. We therefore suspect that the significant interactions documented in Figure 5 will eventually prove to be a minimal estimate of the importance of interlocus effects on life span. A recent study of QTL with effects on life span in C. elegans (17) also found evidence for two cases of epistatic interactions between pairs of loci, with suggestive evidence for several others, and epistatic interactions among life span QTL have also been noted in studies of Drosophila melanogaster (19).

It will be highly informative to determine whether the genetic differences that influence life span among these siblings can also influence the pattern of progression of age-sensitive traits, such as changes in immune function, muscle atrophy, protein glycation, and cataract progression, and to see whether the loci influence either the frequency or the rate of progression of specific neoplastic and nonneoplastic diseases. Higher resolution mapping of the loci that influence life span in this system—which will require the analysis of larger numbers of animals—may be able to narrow the interval of interest to the point where it becomes feasible to identify the genes that influence life span by a candidate gene approach. It should be possible to use the QTL mapping data to identify, at birth, cohorts of mice that are very likely to live longer and age more slowly than their siblings. These genetic predispositions may then be exploited to test specific, mechanistic hypotheses about the connections that link genotype, aging, disease, and life span.

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