Maximum Life Spans in Mice Are Extended by Wild Strain Alleles

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The genes that control basic aging mechanisms in mammals are unknown. By using two four-way crosses, each including a strain derived from wild, undomesticated stocks, we identified two quantitative trait loci that extend murine life spans by approximately 10%. In one cross, the longest-lived 18% of carriers of the *D8Mit171* marker allele from the MOLD/Rk strain, *Mus m. molossinus*, outlived the longest lived 18% of noncarriers by 129 days ($P = 5.4 \times 10^{-5}$); in a second cross, carriers of the *D10Mit267* allele from the CAST/Ei strain, *Mus m. castaneus*, outlived noncarriers by 125 days ($P = 1.6 \times 10^{-6}$). In both crosses, $P < 1.0 \times 10^{-4}$ is considered significant. Because these life span increases required that all essential biological systems function longer than normal, these alleles most likely retarded basic aging mechanisms in multiple biological systems simultaneously. [Exp Biol Med Vol. 226(9):854–859, 2001]

Key words: anti-aging genes; maximum life span; aging; mice; DNA markers

Although the enormous variability of aging rates in different mammalian species is attributable to their genetic differences (1, 2), the specific genes involved remain largely unknown. Recent findings in *Caenorhabditis elegans* (3, 4) and *Drosophila melanogaster* (5) indicate that single genes may substantially increase the life spans of these organisms, but nematodes and flies are evolutionarily distant from and have very different life histories from mammals. The few studies to identify genes that increase life spans in mammals have been conducted using standard mouse strains, and have failed to identify genes that increase maximum life spans for the species. These studies could not distinguish between loci that affect basic mechanisms of aging and loci that only affect disease rates (6–9).

In this study we tried to overcome three obstacles that have traditionally thwarted finding "age-retarding" genes in mouse models. First, in order to minimize strain-specific effects and look for alleles that retard basic mechanisms of aging, and not simply affect disease, we crossed four, rather than two, unrelated strains. Each mouse strain carries a random set of deleterious alleles accidentally fixed in the process of making this particular inbred strain. For any combination of two inbred strains, there will be loci at which only one of the two strains has an allele causing early disease and death. In the traditional intercross or recombinant inbred strain designs, the nondisease allele will increase life span, but not by retarding aging. Alleles causing early disease and death are useful in studying pathology, but provide no information about the basic mechanisms of aging responsible for species differences in aging rates. We used the four-way cross design because several unrelated inbred strains are unlikely to have a fixed deleterious allele at the same locus. Therefore, an allele from one strain that increases life span compared with the three alleles from the other strains is likely to be a true "age-retarding" allele.

Second, to capture the genetic diversity and alleles that retard basic mechanisms of aging, we included in our crosses two genetically very distant inbred strains. Standard laboratory strains may not be optimal for genetic experiments in aging, because these strains were derived from mouse stocks that had been domesticated first, and inbred afterwards (10). During domestication, unintentional selective breeding for rapid reproduction, lower activity, and other aspects of domestication might eliminate many alleles that confer longevity (11). The two strains we included, MOLD/Rk and CAST/Ei, were inbred directly from wild undomesticated stocks. In addition, these strains greatly increase genetic diversity because they belong to two different *Mus musculus* subspecies, as detailed in the next section.

Third, to identify loci affecting basic mechanisms of aging, we focused on loci that increased maximal, rather than average life span. To increase maximal life span, all essential biological systems must function longer than normal, since life span is limited by the first system to fail (12).

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Thus, alleles that increase maximum life span must retard aging in multiple biological systems, suggesting that they retard basic mechanisms of aging. Another reason to look at the maximum, rather than average, life span is that an allele that retards aging may at the same time increase mortality at young ages (11, 13). This may be analogous to the effect of caloric restriction, which extends murine life span, but may actually increase early mortality when colony conditions are not optimal (14, 15).

Materials and Methods

Populations of Mice Used. We constructed two four-way cross populations (16), with the following eight strains obtained from The Jackson Laboratory: Experiment 1, LP/J (LP), MOLD/Rk (MOLD), NZW/LacJ (NZW), and BALB/cBy (BALB); Experiment 2, ST/bJ (ST), C57BL/6J (B6), CAST/Ei (CAST), and DBA/2J (D2). The MOLD and CAST strains are genetically distant from the other six strains because they were derived from *Mus m. molossinus* and *Mus m. castaneus*, respectively. These subspecies diverged about half a million years ago (10) from *Mus m. musculus*, the major genetic contributor to most standard laboratory strains. These two strains are also different from the rest in that they were inbred from undomesticated stocks. For Experiment 1, we crossed (LP × MOLD)F1 with (NZW × BALB)F1 to obtain S0 offspring. We then randomly mated S0 offspring to produce the S1 population on which our analyses were performed. For Experiment 2, we crossed (ST × B6)F1 with (CAST × D2)F1 to obtain S0 offspring. We then randomly mated S0 offspring to produce the S1 population on which our analyses were performed. In both experiments, 160 S1 animals (80 males and 80 females) were produced. Only those surviving past 500 days were genotyped (130 mice in Experiment 1 and 100 mice in Experiment 2) and used for further analyses.

Because they were similar, we pooled the maximum life spans of S1 males and females. All S1 mice used in our analysis were virgins, introduced into our research colony when they were weaned at 4 weeks old, housed four per cage. The mice had free access to acidified water and an NIH 31 diet with 4% fat, and they were free of known pathogens (as detailed in quarterly Jackson Laboratory reports). Use of the animals has been approved by the Institution's Animal Care and Use Committee and animal studies followed the guidelines of the Jackson Laboratory and The American Association for Accreditation of Laboratory Animal Care.

Genetic Analysis by Permutation Test. To identify QTL (Quantitative Trait Loci) involved in the regulation of the maximum life span, we looked for linkage between DNA MIT marker loci (17) that were polymorphic among the four inbred mouse strains used in each four-way cross and the mean life span of the 18% longest-lived mice in those crosses. In Experiment 1 we used the following 28 unlinked markers (cM from the centromere in parentheses): *D1Mit216* (49.7), *D1Mit425* (81.6), *D2Mit83* (16), *D2Mit287* (91.7), *D3Mit19* (87.6), *D4Mit312* (69.8), *D5Mit332* (8), *D5Mit233* (29), *D6Mit25* (65), *D7Mit211* (27.8), *D7Mit40* (53), *D8Mit171* (8), *D9Mit208* (36), *D9Mit20* (61), *D10Mit20* (35), *D10Mit180* (64), *D11Mit260* (34.4), *D12Mit58* (6), *D12Mit60* (16), *D13Mit250* (35), *D13Mit202* (47), *D15Mit208* (29), *D15Mit14* (56.8), *D15Mit41* (58.8), *D17Mit28* (18.4), *D17Mit68* (24.5), *D18Mit184* (41), and *D18Mit33* (44). After identifying an association between maximum life span and the *D8Mit171* marker in Experiment 1, we tested three additional markers, *D8Mit332* (6), *D8Mit290* (11), and *D8Mit125* (19.5), closely linked to that marker. In Experiment 2 we used the following 30 unlinked markers (cM from centromere in parentheses): *D1Mit170* (19.5), *D1Mit419* (63.1), *D1Mit221* (102), *D2Mit32* (11), *D2Mit92* (41.4), *D2Mit412* (78.7), *D3Mit65* (23.3), *D3Mit110* (64.1), *D4Mit41* (10.5), *D4Mit308* (57.4), *D5Mit80* (26), *D5Mit239* (58), *D6Mit236* (3.1), *D6Mit183* (26.5), *D7Mit211* (27.8), *D7Mit323* (50), *D8Mit238* (38.1), *D9Mit31* (35), *D10Mit206* (7), *D10Mit267* (67.5), *D11Mit227* (2), *D12Mit46* (16), *D12Mit263* (58), *D13Mit283* (36), *D13Mit290* (59), *D14Mit233* (19.5), *D15Mit241* (50.2), *D17Mit28* (18.4), *D18Mit184* (41), and *D19Mit11* (41). After identifying an association between maximum life span and the *D10Mit267* marker in Experiment 2, we tested three additional markers, *D10Mit134* (59), *D10Mit14* (65), and *D10Mit103* (70), closely linked to that marker. The chromosome locations for these markers were obtained from the Mouse Genome Database, Mouse Genome Informatics Web Site, The Jackson Laboratory (http://www.informatics.jax.org/).

To test for the linkage with a gene involved in the regulation of the maximum life span, at each MIT marker locus we tested four hypotheses that carriers of each of the four marker alleles had life spans significantly longer than those of noncarriers. For both 18% longest-lived carriers and noncarriers, we calculated mean life spans (LS_{car} and LS_{noncar}, respectively) and variances (σ^{2}_{car} and σ^{2}_{noncar}, respectively). Then we calculated pooled variance, σ^{2}_{pool}, by the standard formula (18):

$$\sigma^2_{pool} = (\sigma^2_{car}(N_{car} - 1) + \sigma^2_{noncar}(N_{noncar} - 1)/(N_{car} + N_{noncar} - 2),$$

where N_{car} and N_{noncar} are the numbers of carriers and noncarriers, respectively. The t-measure was calculated by the standard formula (18):

$$t = (LS_{car} - LS_{noncar})/\sqrt{(\sigma^2_{pool} * (1/N_{car} + 1/N_{noncar}))}.$$

Because life spans were not distributed normally, and because group sizes were relatively small, the *t* test could not be applied directly. We therefore used the permutation test and generated the actual distribution of the *t* measure by randomly reassigning life spans of carriers and noncarriers, without replacement (19). These simulations were conducted up to one million times for Experiment 1 and five million times for Experiment 2. To determine the *P* values,

actual t measures for each allele at each locus were compared with the experimentally generated distribution. We used the nominal significance threshold, $P < 0.0001$, which is an indication of a genome-wide significant ($P < 0.05$) linkage for a backcross (20). The four-way cross is a similar experimental design, since only one of the four inbred strains carried the allele that increased maximum life span.

Model Fit of Mortality Data. By using a maximum likelihood analysis (21), we determined that Gompertz models provided good fits for our data. We then used a maximum likelihood analysis to test whether mortality patterns for carriers and noncarriers could be described by Gompertz models with the same parameters, or whether the slopes, intercepts, or both differed in carriers and noncarriers. Slopes represent rates of aging, while intercepts are the initial vulnerabilities to death. Only mice surviving past 500 days (130 mice in Experiment 1 and 100 mice in Experiment 2) were used for the analyses.

Results

Experiment 1 analyzed effects of carrying each of four alleles at each of 28 unlinked chromosome marker loci (detailed in Materials and Methods). Maximum life spans of randomly bred mice of the second segregating generation from the (LP × MOLD)F1 × (NZW × BALB)F1 four-way cross were significantly increased if they carried the MOLD allele at *D8Mit171*. The 18% longest-lived mice carrying this allele (10 mice) significantly ($P = 5.4 \times 10^{-5}$) outlived the 18% longest-lived noncarriers of the allele (18 mice) by 129 days (Table I and Fig. 1*a*). When we compared the longest-lived 40% of the carriers (18 mice) and noncarriers (32 mice), carriers outlived the noncarriers by 110 days ($P = 1.6 \times 10^{-4}$), indicating that our findings were not dependent on the fraction of the population we analyzed. Both the 18% and 40% fractions were chosen *ad hoc* based on simulations using the Gompertz model, which predicted that changes in aging rates in an animal population could be detected best in the oldest 18% to 40% of the population.

The life span curves for carriers and noncarriers were identical until the mice were about 900 days old (Fig. 1*b*). Thus, the MOLD allele seems to increase life span only in already long-lived animals. In short-lived animals, the allele's anti-aging effect might have been disguised by early-acting lethal genes. Only after these genes were gone from the population did carriers outlive noncarriers. We propose to name the effective gene at this locus *Legl* (longevity extending gene 1).

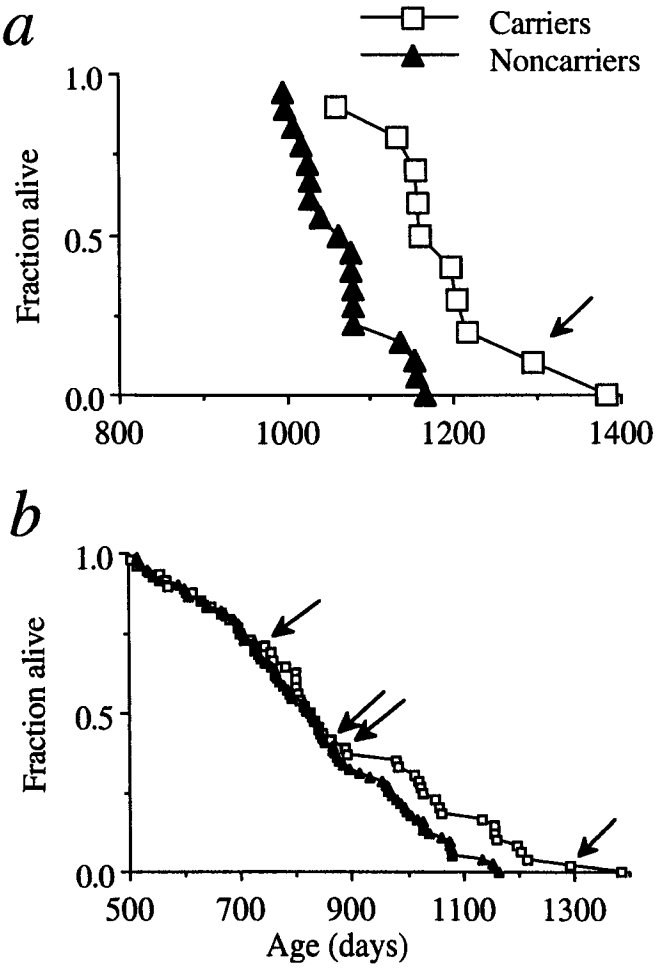

Figure 1. Survival curves for carriers and noncarriers of the MOLD allele of *D8Mit171* in Exp. 1. In both panels, the abscissa shows the fraction remaining alive, and the ordinate shows days of age. Each point represents an individual life span. Arrows denote homozygous carriers. (*a*) compares life spans of the longest-lived 18%, which included five male and five female carriers, shown with eight male and 10 female noncarriers; (*b*) compares life spans of all mice that lived more than 500 days.

Experiment 2 analyzed effects of carrying each of four alleles at each of 30 unlinked chromosome marker loci (detailed in Materials and Methods). Maximum life spans of randomly bred mice of the second segregating generation from the (ST × B6)F1 × (CAST × D2)F1 four-way cross were significantly increased if they carried the CAST allele at *D10Mit267*. The 18% longest-lived mice carrying this allele (12 mice) significantly ($P = 1.6 \times 10^{-6}$) outlived the 18% longest-lived noncarriers of the allele (16 mice) by 125 days (Table I and Fig. 2*a*). As in Experiment 1, our results

Table I. Life Spans in Days of Longest-Lived 18% Mice

	Exp. 1: MOLD *D8Mit171*		Exp. 2: CAST *D10Mit267*	
	Mean, SD (*n*)	Maximum	Mean, SD (*n*)	Maximum
Carriers	1196, 90 (10)	1384	1089, 49 (12)	1214
Noncarriers	1067, 55 (18)	1164	964, 53 (16)	1088

Note. Mice were the S1 generation derived from randomly crossing the S0 population produced by mating (LP × MOLD)F1 × (NZW × D2)F1 in Exp. 1, or (ST × B6)F1 × (CAST × D2)F1 in Exp. 2.

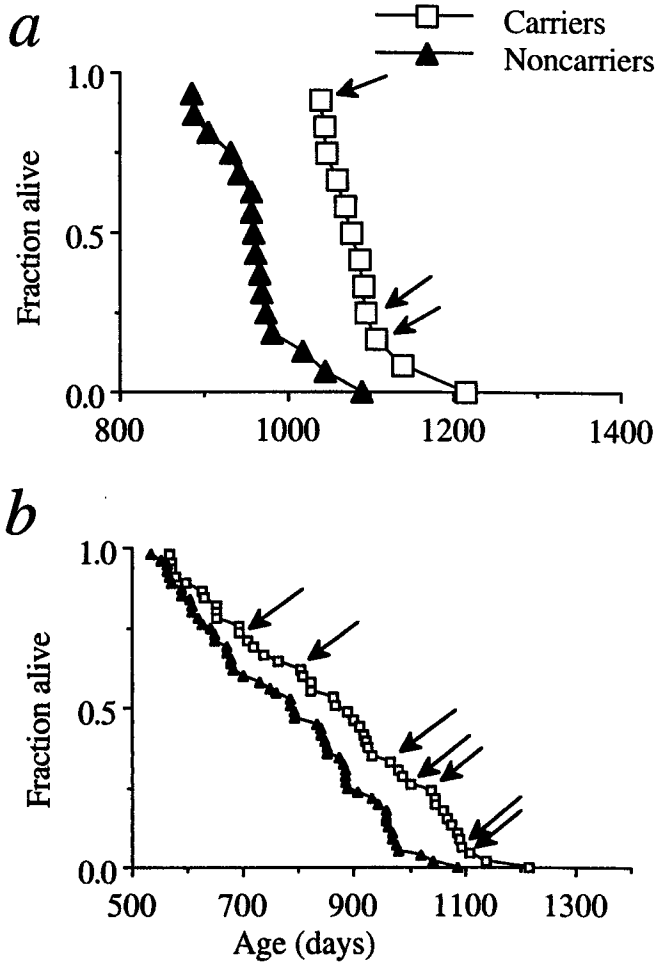

Figure 2. Like Figure 1, but showing survival curves for carriers and noncarriers of the CAST allele of *D10Mit267* in Exp. 2. (*a*) compares the longest-lived 18% (four male and eight female carriers shown with eight male and eight female noncarriers); (*b*) compares life spans of all mice that lived more than 500 days.

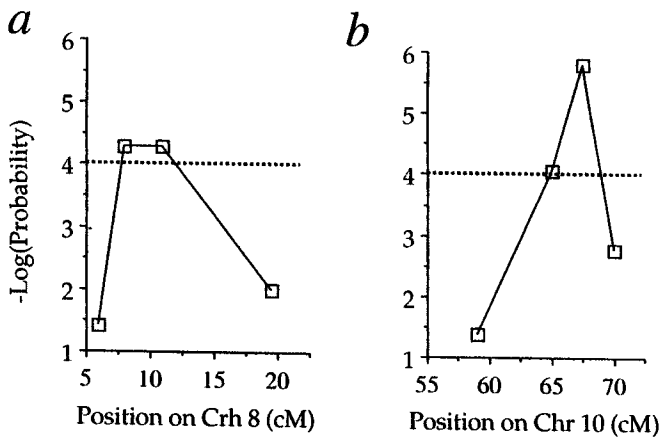

Figure 3. Single-point probabilities for markers of interest and adjacent markers. Negative logs of *P* values for several markers spanning the chromosomal regions from Exp. 1 and 2 are shown in (*a*) and (*b*), respectively. The markers are, from left to right, *D8Mit332*, *D8Mit171*, *D8Mit290*, and *D8Mit125* in (*a*) and *D10Mit134*, *D10Mit14*, *D10Mit267*, and *D10Mit103* in (*b*). The chromosome locations for these markers come from the MGD (see Materials and Methods). The dotted line is a single-point significance level $P = 1 \times 10^{-4}$ corresponding to experimental level of type I error, $P = 0.05$, for the backcross analysis (Ref. 20).

were virtually the same whether we analyzed the longest-lived 18% or 40% fractions of the population: in the 40% fraction, the 21 carriers significantly ($P = 2.6 \times 10^{-5}$) outlived the 27 noncarriers by 110 days.

The life span curves for mice in Experiment 2 separated gradually (Fig. 2*b*), suggesting that even the shorter-lived mice in that population may have benefited from the CAST allele. We propose to name the effective gene at this locus *Leg2* (longevity extending gene 2).

In addition to simply detecting these two QTL that increase longevity, we attempted to better delineate their chromosomal locations. Although no software is commercially available for the interval analysis of four-way crosses, single-point probabilities for markers adjacent to *Leg1* and *Leg2* indicated that *Leg1* was nearest the markers *D8Mit171* and *D8Mit290*, and that *Leg2* was nearest *D10Mit267* (Fig. 3).

It is difficult to estimate coverage of each marker. Assuming that each scanned ±10 cM, approximately one-half of the genome in each experiment was scanned. Because a substantial portion of the genome was not within 10 cM of a tested marker, we estimated how many additional "longevity" loci of the magnitude we detected might exist in our two experimental populations. For that we calculated how much variance in the life span of these populations was attributable to each of the *Leg1* and Leg 2 loci. A one factor (carrier versus noncarrier) analysis of variance showed that the Leg 1 and Leg 2 loci accounted for 46% and 61% of the variability in the life span of the longest-lived 18% of the mice in the Experiment 1 and the Experiment 2 populations, respectively. Because the remaining life span variability would be similar to that of genetically identical animals, it is unlikely that other QTLs of a magnitude similar to those of *Leg1* or *Leg2* exist in either of our two experimental populations.

To test whether the alleles that increased maximum life spans also reduced the slope of the Gompertz mortality curve, a measure of the rate of aging (22), we used the maximum likelihood analysis (21). In Experiment 1, the analysis showed that the most likely model was the one with different slopes, but equal intercepts for carriers and noncarriers of the MOLD allele at *Leg1* ($P = 0.006$). Allowing intercepts to change in addition to slopes did not significantly improve the model fit. This test provided additional evidence that the MOLD allele is associated with a decreased rate of aging. In Experiment 2, although the mortality rate was reduced by the CAST allele at *Leg2* ($P = 0.007$), there were insufficient data to determine whether the effect was caused by a change in the initial mortality in the Gompertzian slope.

Discussion

Because an organism dies when its first essential biological system fails, it can only live longer than normal if all

its biological systems function longer than normal. Hence, alleles that increase an organism's maximal life span will likely retard aging in all the organism's essential biological systems simultaneously (12). Thus, genetic models with extended maximal life spans are important for our understanding of basic mechanisms of aging.

Recent findings in *Caenorhabditis elegans* (3, 4) and *Drosophila* (5) indicate that single genes may substantially increase life span of a multicellular organism. These findings, while very important, may not be directly translated into mammalian biology. Nematodes and flies are evolutionarily distant from mammals and have very different life histories. For example, the life span of a nematode is extended by severalfold if the nematode goes through the dauer stage (23), a stage that does not exist in mammals. The life span of a fly is much more malleable than that of mammals: it may be extended in *Drosophila* by reducing ambient temperature (24), by reducing the density of food available (25), by reducing sexual activity (26, 27), and, in the housefly, by reducing physical activity (28). These differences in basic biology show the importance of mammalian models with extended maximal life spans.

We have apparently identified two novel alleles, designated as $Leg1^a$ and $Leg2^a$, each of which extend maximal murine life spans by about 10%. Increased life span has been associated with decreased body size in such conditions as caloric restriction (14, 15), pituitary dwarfism (29), and reduced activity of the somatotropic axis (30, 31). For both $Leg1^a$ and $Leg2^a$, mechanism of life extension is probably different because body weights and tail lengths of carriers and noncarriers were essentially identical from 1 to 7 months of age (data not shown). Therefore, our mice aged more slowly than normal not because they were food restricted (14, 15) or dwarfs (29), as growth is greatly delayed or prevented by these conditions. The only other gene reported to affect maximal life span, the $p66^{shc}$ adaptor protein (32), is also not involved as it is located on chromosome 18, not 8 or 10 (Mouse Genome Database, Mouse Genome Informatics website).

In prior genetic studies using recombinant inbred strains (6–8), loci identified as affecting life span are likely loci in which one of the two parental inbred strains had a normal allele, while the other strain had a deleterious allele. In contrast, *Leg1* and *Leg2* increased maximal life span and are unlikely to merely affect disease rates. It is also unlikely that $Leg1^a$ and $Leg2^a$, identified in four-way crosses, simply reflect an avoidance of deleterious alleles because it is unlikely that three unrelated strains carry deleterious alleles at the same locus. In genetically segregating four-way cross populations, alleles that increase life span can be detected because they are separated from alleles causing early death.

We suggest that we were able to isolate and identify the $Leg1^a$ and $Leg2^a$ QTLs in populations of fewer than 150 mice because we included in our crosses the MOLD and CAST strains, genetically very distant from most conventional mouse strains, and derived from wild, undomesticated stocks. Thus our populations contained sufficient genetic diversity to show heritabilities of 38% to 62% (33). Such strains were not used in other four-way cross studies (9). The MOLD and CAST inbred strains appear to have shorter life spans than the LP or B6 strains (data not shown). Presumably, deleterious recessive alleles kill the mice in these strains before they can benefit from the alleles that retard aging; in genetically segregating four-way crosses, such deleterious alleles can be separated from those that increase life span. Future attempts to identify other QTL, if they exist, that retard basic mechanisms of aging would do well to use four-way crosses and to include in those crosses highly diverse inbred strains derived from wild undomesticated stocks.

Currently, our mapping information is sufficient to localize *Leg1* and *Leg2* to within 10-cM regions. To narrow the regions further, progeny testing can be used. If enough progeny is tested to unequivocally determine whether a sire carries the longevity allele, 10 to 20 sires with independent recombinations in the interval of interest should be sufficient to narrow down *Leg1* and *Leg2* locations to within 1 cM.

If, as our study suggests, certain genes can retard basic mechanisms of aging in mammals, identifying them and understanding the biochemical pathways they control may tremendously improve the quality of human life.

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