Positive Selection of *Caenorhabditis elegans* Mutants With Increased Stress Resistance and Longevity

Manuel J. Muñoz[1] and Donald L. Riddle[2]

Molecular Biology Program and Division of Biological Sciences, University of Missouri, Columbia, Missouri 65211-7400

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ABSTRACT

We developed selective conditions for long-lived mutants of the nematode *Caenorhabditis elegans* by subjecting the first larval stage (L1) to thermal stress at 30° for 7 days. The surviving larvae developed to fertile adults after the temperature was shifted to 15°. A total of one million F_2 progeny and a half million F_3 progeny of ethyl-methanesulfonate-mutagenized animals were treated in three separate experiments. Among the 81 putative mutants that recovered and matured to the reproductive adult, 63 retested as thermotolerant and 49 (80%) exhibited a >15% increase in mean life span. All the known classes of dauer formation (Daf) mutant that affect longevity were found, including six new alleles of *daf-2*, and a unique temperature-sensitive, dauer-constitutive allele of *age-1*. Alleles of *dyf-2* and *unc-13* were isolated, and mutants of *unc-18*, a gene that interacts with *unc-13*, were also found to be long lived. Thirteen additional mutations define at least four new genes.

RECENT work on *Caenorhabditis elegans*, Drosophila, and mammalian systems suggests that there is a conserved mechanism for regulation of life span (Kenyon 2001). Genetic studies on *C. elegans* have suggested an important role for protection from oxidative damage (Larsen 1993) as regulated by insulin-like signaling (Honda and Honda 1999). Metabolic rate (Vanfleteren and De Vreese 1995) and caloric intake (Lakowski and Hekimi 1998) also play a role, but the overall genetic complexity of life span regulation is not known. Until 1993, the only *C. elegans* mutant known to have increased adult longevity was *age-1(hx546)*, which encodes a phosphatidylinositol-3-kinase (PI3 kinase) catalytic subunit involved in insulin-like signaling (Morris *et al.* 1996; Kimura *et al.* 1997). Since then, nearly 50 genes have been implicated in the determination of life span, most of which were identified by testing the longevity of strains isolated for other reasons. Phenotypes associated with increased longevity include constitutive dauer larva formation (Kenyon *et al.* 1993), increased fat accumulation (Ogg *et al.* 1997), slow metabolism (Van Voorhies and Ward 1999), resistance to ultraviolet irradiation (Murakami and Johnson 1996), thermotolerance (Lithgow *et al.* 1995), and tolerance to oxidative damage (Larsen 1993; Martin *et al.* 1996; Finkel and Holbrook 2000).

The dauer diapause stage is normally triggered by food limitation and/or overcrowding (Cassada and Russell 1975). Dauer-constitutive (Daf-c) mutants arrest at this stage in the absence of the normally required environmental cues (reviewed by Riddle and Albert 1997). Pathways using transforming growth factor-β (TGF-β; Ren *et al.* 1996) and insulin-like ligands (Pierce *et al.* 2001) function via *daf-9* (Gerisch *et al.* 2001; Jia *et al.* 2002) to regulate the activity of the DAF-12 nuclear receptor (Antebi *et al.* 2000) to control the developmental switch. Genetic disruption of signaling by either pathway leads to a Daf-c phenotype. The Daf-c mutants that reduce insulin-like signaling activity are more pleiotropic (Gems *et al.* 1998) than mutants affecting TGF-β signaling; they extend adult life span by a factor of two or more (Kenyon *et al.* 1993; Gems *et al.* 1998).

The best-studied long-lived mutants are *age-1* and *daf-2*, the latter of which encodes an insulin-like growth factor (IGF) receptor (Kimura *et al.* 1997; Gems *et al.* 1998; Guarente and Kenyon 2000; Johnson *et al.* 2000; Pierce *et al.* 2001). Mutations in *daf-16* suppress the adult longevity (Age) and Daf-c phenotypes of both mutants, indicating that the DAF-16 Forkhead transcription factor (Lin *et al.* 1997; Ogg *et al.* 1997) is required for dauer formation and extension of adult longevity (Kenyon *et al.* 1993; Larsen *et al.* 1995). The AGE-1 PI3 kinase and DAF-16 transcription factor are both homologs of downstream regulators of insulin signaling in mammals (Morris *et al.* 1996; Lin *et al.* 1997; Ogg *et al.* 1997). Mutations that affect the insulin/IGF receptor in Drosophila (Tatar *et al.* 2001) and a mutation that impairs the development of the pituitary gland in mice increase life span. The latter mutation results in a reduction of several hormones, including IGF-1 (Brown-Borg *et al.* 1996; Flurkey *et al.* 2001).

Additional mutants with increased longevity should

[1]*Present address:* Laboratorio Andaluz de Biología, Universidad Pablo de Olavide, Carretera de Utrera, Km1, Seville 41013, Spain.

[2]*Corresponding author:* Molecular Biology Program and Division of Biological Sciences, University of Missouri, Columbia, MO 65211-7400. E-mail: RiddleD@missouri.edu

identify new elements in these pathways, but brute force screens for long-lived mutants are very laborious. Since long-lived animals are postreproductive, it is necessary to replicate populations of candidate mutant lines to recover a mutant once it has been identified. We examined stress-resistance phenotypes to determine whether they could be used as surrogate markers in a convenient positive selection to enrich for longevity mutants. Previous work used acute 40° or 35° heat-shock treatment of adults to enrich for thermotolerant mutants (Walker *et al.* 1998; Sampayo *et al.* 2000; Yang and Wilson 2000). We treated larval stage 1 (L1) larvae at 30° for 7 days and found that 80% of the surviving thermotolerant mutants were long lived. With this method we isolated Liv (long-*liv*ed and *vi*able after thermal stress) mutations affecting the dauer pathway, as well as mutations in new genes that do not produce a secondary Daf phenotype. We also found alleles of *unc-13* (uncoordinated) and *dyf-2* (neuronal dye-filling defective) and showed that they affect longevity as does *unc-18*, which together with *unc-13* plays a role in neurosecretion (Sassa *et al.* 1999).

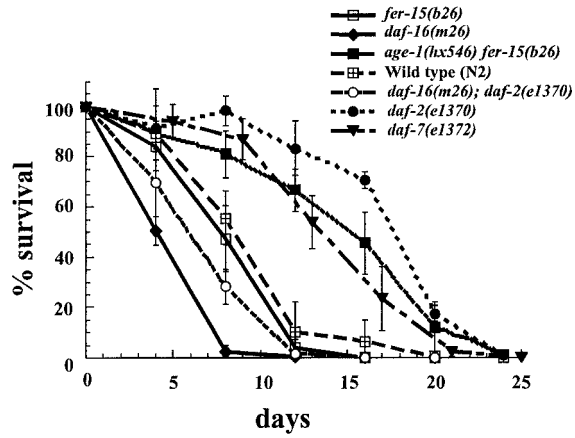


Figure 1.—Survival of L1 larvae in M9 buffer at 25.5° in the absence of food. Mean and SD of three independent trials are plotted. Daf-c *age-1(hx546)*, *daf-2(e1370)*, and *daf-7(e1372)* mutants survive starvation longer than wild type. The number of animals assayed for *fer-15(b26)* was 2775; for *daf-16(b26)*, 3861; for *age-1(hx546)*, 3090; for *daf-16(m26); daf-2(e1370)*, 4246; for *daf-2(e1370)*, 2851; for *daf-7(e1372)*, 3610; and for wild type, 4829.

MATERIALS AND METHODS

Phenotypic analyses: For L1 starvation assays, hypochlorite-treated eggs (Lewis and Fleming 1995) were incubated in 1 ml of M9 buffer (Brenner 1974) on a shaker at 20° for 16–24 hr, divided into three populations, and transferred to 25.5°. Three samples of each population were counted and used to calculate the mean and SD. The populations tested ranged from 2000 to 5000/milliliter. For thermal stress tests, L1 larvae synchronized as above were incubated on agar plates with *Escherichia coli* strain OP50 (Brenner 1974) at 30°. After treatment, plates were incubated at 15° and survivors were counted or harvested for mutant selection. Each set of assays included *daf-2(e1370)* and wild-type N2 controls and was repeated once.

To assay dauer formation, eggs were picked from populations grown at 20° and incubated at 25.5° or 27°. Dauer formation was scored visually after 3 or 2 days, respectively. To measure adult life span, L4 larvae grown at 15° were shifted to 25.5° to assay survival as described previously (Larsen *et al.* 1995). Day 1 is the first day of adulthood. Each assay used three populations of 33–45 animals and was repeated at least once. Figures represent one of the assays. To test neuronal dye filling, adults were exposed to 0.1 mg/ml fluorescein isothiocyanate (FITC) in the agar medium for 2 hr and then transferred to bacterial lawns without dye for ~30 min to flush unbound FITC from the intestines (Hedgecock *et al.* 1985). Neurons of anesthetized worms were observed with a Zeiss Axioscope equipped with fluorescence optics and FITC filters.

Mutant selection: The temperature-sensitive fertilization-defective mutant DH26, *fer-15(b26*ts*)*, was treated with 25 mm ethyl methanesulfonate (EMS) as described (Rosenbluth *et al.* 1985). Sets of 30 mutagenized L4 larvae were incubated for 8 days at 15° in tubes containing 6 ml S medium with *E. coli* (Sulston and Brenner 1974). F_2 eggs were purified by alkaline hypochlorite treatment to obtain synchronous L1 larvae (Lewis and Fleming 1995). Approximately 10,000 synchronous F_2 or F_3 L1 larvae were harvested from each tube and submitted to thermal stress at 30° for 7 days on agar plates spread with OP50. To ensure independence of the mutants, only one survivor per tube was saved after confirming L1 thermotolerance at 30°.

Genetics: All strains were derived from the wild-type N2 and were cultured as described (Brenner 1974). *dyf-2(m881* and *m886)*, *liv-2(m882)*, *liv-5(m884)*, and *aap-1(m889)* (AGE-1 adapter protein) were backcrossed three times with *fer-15* using the dye-filling-defective (Dyf) or Daf-c phenotype. *unc-13 (m873)* and *liv-4(m872)* were backcrossed six times using the uncoordinated (Unc) phenotype. Life span was measured after these crosses, showing that these traits were linked to longevity. *liv-2*, *liv-4*, *liv-5*, and *aap-1* were mapped by standard three-factor crosses, using markers *unc-38(e264)I*, *dpy-5(e61)I*, *dpy-10(e128)II*, *unc-4(e120)II*, *rol-1(e91)II*, *unc-42(e270)V*, and *sma-1(e30)V*. Populations homozygous for recombinant chromosomes were tested for Daf-c, Dyf, or Unc phenotypes. The following recombinants (in parentheses) were scored in the final mapping steps using flanking visible markers *in trans* to the Liv gene: *dpy-10* (15) *liv-2* (5) *unc-4*; *unc-42* (3) *liv-4* (15) *sma-1*; *unc-4* (3) *liv-5* (17) *rol-1*; *unc-38* (4) *aap-1* (3) *dpy-5*. For the *daf-16* double mutants, we confirmed the presence of the Liv mutation by the Unc phenotype or by noncomplementation of the Daf-c phenotype. *daf-16* was scored by the lack of dauer formation on three replicate starved plates.

RESULTS

Starvation tolerance: We used starved L1 larvae to test whether increased fat accumulation and decreased metabolic rate described in longevity mutants (Ogg *et al.* 1997; Van Voorhies and Ward 1999) might allow such strains to survive starvation better than wild type. As expected, the mean survival of long-lived *daf-2* and *age-1* mutants was longer than that of wild type, and this was suppressed by *daf-16*. However, a *daf-7* mutant (which accumulates fat, but is not long lived in the adult) survived nearly as long (Figure 1). In a separate trial (not shown), survival of *daf-1(m40)* was similar to that shown for *daf-7* and *age-1*. Hence, using starvation

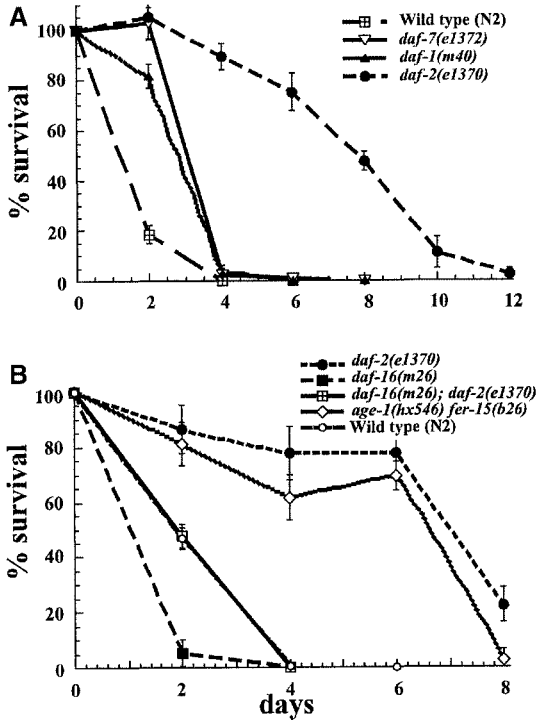


FIGURE 2.—Increased thermotolerance of *daf-2* and *age-1* mutants may share the same genetic pathway as increased adult longevity. (A) Survival of Daf-c and wild-type L1 larvae at 30°. The long-lived *daf-2(e1370)* mutant survived longer than the wild type (N2) and the Daf-c mutants *daf-7(el372)* and *daf-l (m40)*, which are not long lived as adults. (B) Survival of the long-lived mutant *age-1(hx456)* parallels that of *daf-2(e1370)*. The mutation *daf-16(m26)* suppressed the increased L1 thermotolerance of *daf-2(el370)*. The populations tested ranged from 1000 to 2000 worms per strain. A and B plot the mean and SD of three experiments.

to enrich for longevity mutants should also enrich for Daf-c mutants not associated with longevity.

Heat tolerance: *daf-2* adults survive an average of 800 min at 35° compared to ~500 min for wild-type survival (LITHGOW *et al.* 1995). We assayed larval thermotolerance to find convenient conditions favoring differential survival of long-lived *vs.* wild-type animals. When we incubated L1 animals at 27°, they developed to sterile adults, but at 30° they arrested development at the L1 stage. Temperature downshift from 30° to 15° before death permitted nearly all of them to develop to fertile adults. We treated L1 larvae of different strains to thermal stress at 30° and found that the mean survival of *daf-2* and *age-1* was three to seven times greater than the 2-day mean survival of wild type (Figure 2, A and B). To our knowledge, this is the largest difference in stress resistance thus far described between long-lived mutants and wild type.

At 30°, *daf-1(m40)* and *daf-7(e1372)* exhibited twice the mean wild-type survival, but this did not approach the increase observed in a *daf-2* mutant (Figure 2A). Mean survival of the wild-type strain differed among the three independent trials (Figure 2, A and B), possibly owing to small temperature fluctuations around 30° or to small differences in conditions prior to assay. Hence, each trial included all control strains and was repeated at least once. High thermotolerance is correlated with reduced *daf-2*/insulin-like pathway activity (LITHGOW *et al.* 1995). The *daf-16(m26)* mutation suppresses *daf-2(e1370)* thermotolerance (Figure 2B), as it suppresses the Daf-c and longevity traits (RIDDLE *et al.* 1981; KENYON *et al.* 1993). DAF-2 and AGE-1 negatively regulate DAF-16 activity, which in turn is necessary for increased longevity and thermotolerance (LITHGOW *et al.* 1995; GEMS *et al.* 1998).

Mutant selection and classification: Whereas longevity can be assayed only in the postreproductive adult, thermotolerance can also be detected in larvae. When used as a surrogate phenotype for increased longevity, thermotolerance allows one to recover stress-resistant animals that will be both fertile and long lived. To select thermotolerant mutants, we incubated synchronous L1 larvae at 30° on agar plates for 7 days. In preliminary tests, wild-type larvae were killed by this treatment, but ~50% of *daf-2* or *age-1* larvae survived (Figure 2, A and B) and resumed development at 15°. In aggregate, we treated ~1 million F_2 larvae, and 500,000 F_3 larvae derived from EMS-mutagenized *fer-15* hermaphrodites. The F_3 selection was performed to detect possible maternal-effect mutants. We recovered 57 F_2 and 24 F_3 survivors originating from independent F_1 animals. These 81 survivors were used to establish lines for retesting. Sixty-three of the lines were thermotolerant, and 49 of those were also long lived (>15% extension of mean life span). We named the latter class Liv mutants and only these were studied further.

All lines were tested for constitutive dauer formation at 25.5° and 27°. Of the 49 lines, 40 were Daf-c; of these, 6 failed to complement *daf-2(e1370)* and 5 failed to complement *age-1(hx546)*. The phenotypes of 5 of the new *daf-2* mutants were similar to the *e1370* reference allele (Daf-c at 25°), but one novel allele, *m883*, was Daf-c only at 27° (Table 1). The latter mutant exhibited a nearly doubled adult longevity (Figure 3A), but did not exhibit the impenetrant premature adult death at 25.5° reported for other *daf-2* alleles (LARSEN *et al.* 1995; GEMS *et al.* 1998). The *daf-2(m883)*, *age-1(hx546)*, *liv-5(m884)*, and *aap-1(m889)* mutants are Daf-c at 27°, and the adults are long lived at 25° and show a greater thermotolerance than other Liv mutants (Table 1, Figure 3J).

The *age-1(m895)* allele was Daf-c at 25.5° (Table 1), whereas the previously reported *hx546* allele (MALONE *et al.* 1996) and the other alleles reported here were Daf-c only at 27°. The maximum life span of *m895* was 35% ± 2.8 SD greater than that of *age-1*(+), but the mean life span was 29.6 ± 10.5 less than that of *age-1*(+) (Figure 3B). This premature adult death was similar to that observed for certain *daf-2* mutants (GEMS *et al.* 1998), but has not been reported previously for *age-1*. Null alleles of *age-1* (formerly *daf-23*) convey a noncondi-

TABLE 1

Daf-c phenotype of Liv mutants

Genotype	Other phenotypes	% dauer formation 25.5°	% dauer formation 27°	% dauer formation 27° with *daf-16(m26)*
Wild type (N2)		0	0	0
daf-2(m869)[a]		100	100	ND
daf-2(m883)		0	100	ND
age-1(m880)[b]		0	100	ND
age-1(m895)		90 ± 7	100	ND
liv-5(m884)		0	57 ± 1.2	14 ± 1.9
aap-1(m889)		0	100[c]	5 ± 2.6
dyf-2(m881)	Dyf	0	50 ± 4.3	0
dyf-2(m886)	Dyf	0	50 ± 6.6	ND
liv-2(m882)	Dyf	0	35 ± 2.3	2 ± 0.5
unc-31(m868)[d]	Unc	0	95 ± 3.2	ND
unc-13(m873)	Unc	0	5 ± 0.9	0
unc-13(e51)[e]	Unc	0	3 ± 0.4	ND
unc-13(e1091)[e]	Unc	0	0	ND
unc-13(e376)[e]	Unc	0	85 ± 5.7	ND
liv-4(m872)	Unc	0	10 ± 3.6	0

Mean and SD of three experiments are shown. Number of animals assayed was always >100. In addition, nine other Liv mutants that do not have visible phenotypes were isolated.

[a] Four other alleles with a similar phenotype were isolated.

[b] Three other alleles with a similar phenotype were isolated.

[c] All the animals that develop through L1 enter the dauer stage, but 40% of the eggs do not hatch or suffer L1 arrest.

[d] Twenty-one other alleles of similar phenotype were isolated.

[e] *unc-13* mutants not isolated in this selection.

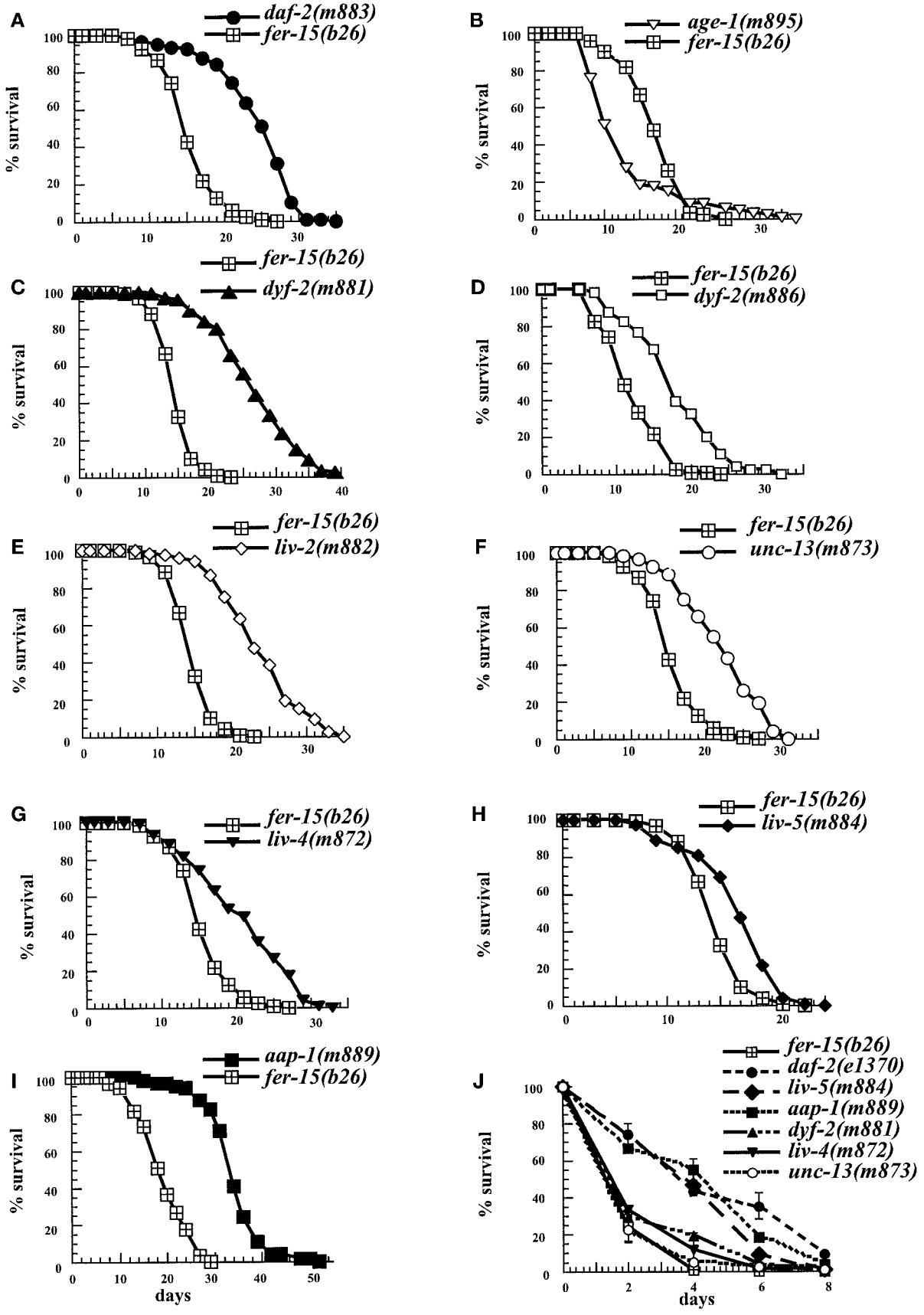
tional maternally rescued Daf-c phenotype (Larsen *et al.* 1995; Morris *et al.* 1996); mutants segregated from heterozygous parents do not form dauer larvae, but develop into long-lived adults.

Mutants other than *daf-2* and *age-1* were affected in sensory neurons (Dyf), exhibited uncoordinated movement (Unc), or were Liv, either with or without a Daf-c phenotype at 27°. We characterized the six Liv mutants with a Daf-c, Unc, or Dyf phenotype, which provided a surrogate marker for genetic analysis (Figure 3, C–J, Table 1). Each of the six mutants was mapped to a region of ~1 map unit (Figure 4), and complementation tests were then performed as described below.

Dyf mutants: Mutants with disrupted function of the amphid sensory neurons have increased longevity (Apfeld and Kenyon 1999). Such mutants are easily scored by the failure of their amphid neurons to stain with fluorescent dyes like FITC (Hedgecock *et al.* 1985; Starich *et al.* 1995). Three Liv mutants failed to stain with FITC; two were alleles of *dyf-2*, and one defined the *liv-2* gene. All three mutants are Daf-c at 27° (Table 1), a phenotype previously reported for some of the *dyf* mutants (Apfeld and Kenyon 1999).

Unc mutants: We tested whether any of the 24 Unc mutants we isolated were *unc-64* or *unc-31* alleles. *unc-64* sintaxin and *unc-31* Ca^{2+}-dependent activator protein for secretion homologs (Ann *et al.* 1997; Ogawa *et al.* 1998; Saifee *et al.* 1998) are *unc* genes reported to regulate the longevity of the hermaphrodite (Ailion *et al.* 1999). These genes are involved in the secretion of

Figure 3.—Longevity and resistance to thermal stress of different mutants isolated in a *fer-15* genetic background. (A–I) Adult life spans; data from one of two to three trials are shown. Day 1 is the first day of adulthood. *fer-15(b26)* is sterile at 25.5° and has a wild-type life span (Friedman and Johnson 1988). (A) Mean life span of *fer-15(b26)* is 14.2 ± 0.6 days and for *daf-2(m883)*, 24.8 ± 0.8. (B) *age-1(m895)* has a shorter mean life span (10.4 ± 0.8 days), but longer maximum life span (35 ± 2.8 days) than that of *fer-15(b26)* (27 ± 2 days). (C–I) The percentage of increase of life span (±SD) for *dyf-2(m881)* is 86 ± 9; for *dyf-2(m886)*, 50 ± 6; for *liv-2(m882)*, 66 ± 7; for *unc-13(m873)*, 52 + 4.5; for *liv-4(m872)*, 46 ± 15; for *liv-5(m884)*, 23 ± 1; and for *aap-1(m889)*, 85 ± 4. Each strain used three populations of 33–45 individuals. All the animals from the three populations are plotted. (J) Survival of L1 larvae at 30°. Mean and SD of three different experiments are plotted. Survival of *liv-5(m884)* and *aap-1(m889)* is similar to the control *daf-2(el370)*. The survival of *liv-2(m882)* was determined at a different time (data not shown) and was only slightly more thermotolerant than wild type. The populations tested ranged from 1000 to 2000 worms per strain. The three *unc-13* populations tested averaged 130.


A
daf-2(m883)
fer-15(b26)
% survival
B
age-1(m895)
fer-15(b26)
% survival
C
fer-15(b26)
dyf-2(m881)
% survival
D
fer-15(b26)
dyf-2(m886)
% survival
E
fer-15(b26)
liv-2(m882)
% survival
F
fer-15(b26)
unc-13(m873)
% survival
G
fer-15(b26)
liv-4(m872)
% survival
H
fer-15(b26)
liv-5(m884)
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aap-1(m889)
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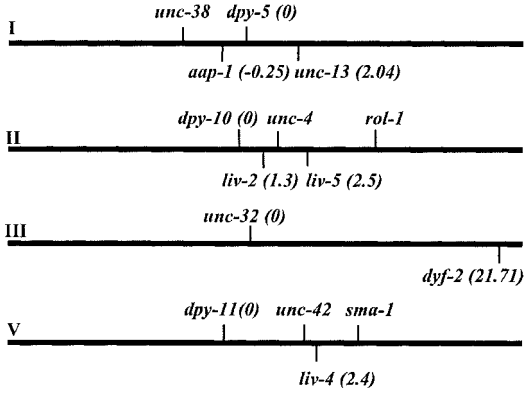

FIGURE 4.—Genetic map showing new mutations isolated in the selection for Liv mutants. Marker genes are shown above the lines. Chromosome positions (in parentheses) were obtained using three-factor mapping (see MATERIALS AND METHODS) and the Caenorhabditis Genetics Center database (http://biosci.umn.edu/CGC/CGChomepage.htm).

neurotransmitters and could be involved in the Ca^{2+}-regulated secretion of the ligand for the *daf-2* receptor. None of the Unc Liv mutants were *unc-64* alleles, but, surprisingly, 22 were independent *unc-31* mutants.

The *m873* Unc mutant was mapped on chromosome I, and it failed to complement *unc-13(e51)*. The UNC-13 protein has C1 and C2 homology domains. C1 binds diacylglycerol and phorbol esters, and C2 binds calcium and phospholipids (MARUYAMA and BRENNER 1991; AHMED *et al.* 1992). Mutants have impaired secretion via neuronal vesicles (RICHMOND *et al.* 1999). We found that the extant *unc-13* mutants *e376* and *e1091* exhibited increases in mean life span of 82.9 ± 12.5% and 60 ± 10.3%, respectively (Figure 5, B and C). The reference allele *e51* showed only a 28 ± 1.1% increase in mean life span, but a 54 ± 22% increase in maximum life span relative to the *fer-15* control (Figure 5A). Some *unc-13* strains were Daf-c at 27°, but this trait was variable (Table 1). Although these alleles conferred a similar increase in life span, they differed with respect to dauer formation at 27°.

OGAWA *et al.* (1998) reported that UNC-64 and UNC-18 proteins interact, and it has been suggested that UNC-13 is involved in the modulation of this interaction (SASSA *et al.* 1999). We found that the *unc-18* reference allele, *e81*, has a mean life span (35 + 0.5 days) 2.5 times that of *unc-18*(+) (13.3 + 0.2 days; Figure 5D). Hence, this gene is also involved in longevity, perhaps by the same mechanism as that of *unc-13*, *unc-64*, and *unc-31*. These gene products may be involved in the secretion of the ligand for DAF-2 (AILION *et al.* 1999). MUNC-18, a mammalian homolog of UNC-18, regulates exocytosis in pancreatic B-cells, which secrete insulin (ZHANG *et al.* 2000).

Finally, *liv-4(m872)* is a mild, slightly long, sluggish Unc that exhibits a 45.7 ± 14.6% increase in mean life span relative to the *fer-15* parent (Figure 3G). This gene maps to a region of chromosome V where no *Unc* mutant with similar phenotype has been previously identified, suggesting that *liv-4* may be a novel gene involved in neurosecretion and longevity.

Liv mutants with no visible phenotype: Nine mutants exhibited no obvious phenotype other than thermotolerance and increased longevity. Since these mutants can be scored only by using population-based assays, their analysis is beyond the scope of this report.

***daf-16* suppresses the Age and Daf-c phenotypes:** We constructed double mutants with *daf-16(m26)*, and in all six cases the *daf-16* mutation suppressed the Age and 27° Daf-c phenotype, indicating that all these genes function upstream of *daf-16* to negatively regulate its longevity-promoting function (Figure 6, Table 1). In three cases, *daf-16* epistasis was weak with respect to the 27° phenotype. The percentage of dauer formation at 27° was 57 ± 1% for *liv-5*, and it was 14 ± 2% for the *daf-16* double mutant (Table 1). With respect to Age, the *aap-1(m889) daf-16(m26)* double mutant had a slightly longer (38 ± 0.6%) mean and (21 ± 2%) maximum life span than that of *daf-16(m26)* itself (Figure 6F). These results suggest the presence of another mechanism independent of *daf-16* that regulates dauer formation and longevity. Alternatively, there may be some residual activity in the *daf-16(m26)* mutant (LIN *et al.* 1997; OGG *et al.* 1997) that makes epistasis incomplete.

Finally, the mean life span of *daf-16(m26); liv-4(m872)* (6.7 ± 0.2 days) was less than that of *daf-16(m26)* itself (12.2 ± 0.1 days; Figure 6D). It is possible that *liv-4* has a deleterious effect masked by its increased longevity. If so, blocking the longevity pathway with the *daf-16* mutation might unmask these effects.

DISCUSSION

We have isolated a total of 49 long-lived (Liv) mutants using a positive enrichment procedure based on the thermotolerance of L1 larvae. At least 36 of the long-lived mutations affect known genes, and at least four define new genes. We placed the mutants into four classes based on secondary phenotypes. Three of the classes exhibit visible traits (Dyf, Unc, and Daf-c) useful as surrogate markers for genetic analysis. Mutants with these visible phenotypes have been previously associated with longevity, suggesting that these traits result from the same genetic lesion. The Dyf, Unc, and Daf-c phenotype was used to resegregate the Liv mutants three to six times prior to retesting life span, and in each case the longevity trait cosegregated with the selected marker. Forty of the mutants are Daf-c at 25° or 27°. It is postulated that dauer larvae express a program for efficient life maintenance that increases their longevity (LARSEN *et al.* 1995; JONES *et al.* 2001) and that *daf-2* and *age-1* mutants express these functions in the adult stage (LARSEN *et al.* 1995; KENYON 2001). The longevity program appears to reduce oxidative damage and to in-

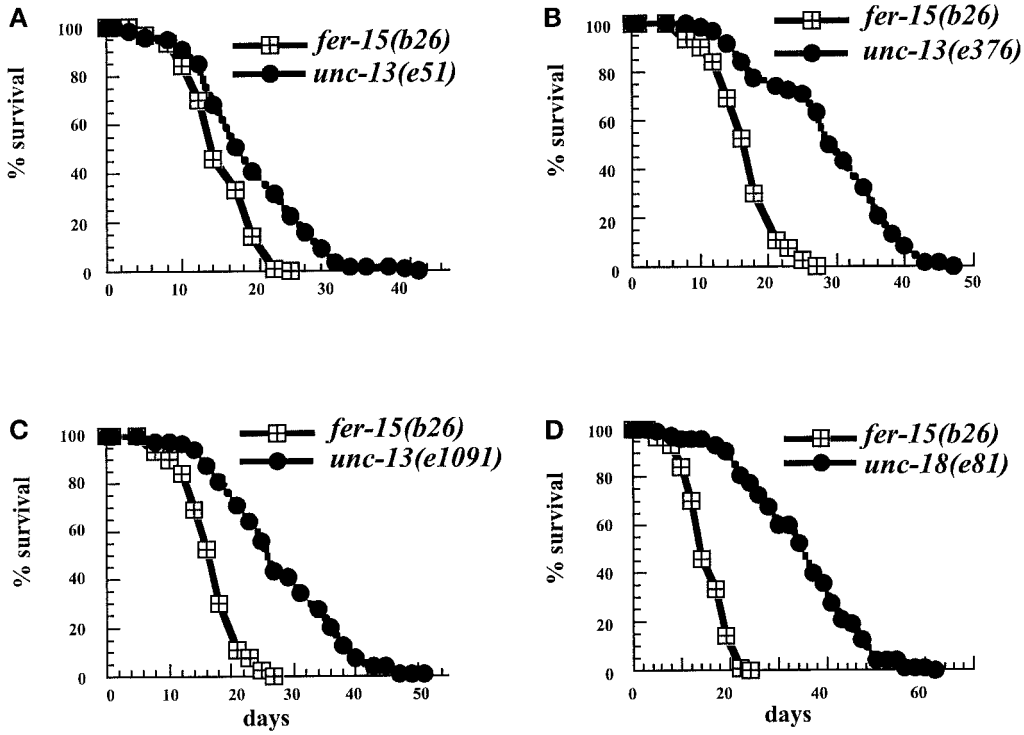

FIGURE 5.—Adult life spans of *unc-13* and *unc-18* mutants in a *fer-15* genetic background. (A–C) All the *unc-13* alleles showed increased longevity. The reference allele *unc-13(e51)* had a modestly increased mean life span, but an almost doubled maximum life span. (D) The mean life span of *unc-18(e81)* was increased 2.5-fold. Day 1 is the first day of adulthood.

crease resistance to stress (LARSEN 1993; LITHGOW *et al.* 1995; MELOV *et al.* 2000).

Food scarcity may be a cue modulating life span in divergent animal species (KENYON 2001). Caloric restriction increases longevity in *C. elegans* (KLASS 1977; LAKOWSKI and HEKIMI 1998) as well as in rodents (SOHAL and WEINDRUCH 1996). In *C. elegans*, chemosensory neurons could regulate the release of insulin from vesicles, involving *unc-31*, *unc-64*, *unc-13*, *unc-18*, and *liv-4*. The DAF-2 receptor and the AGE-1 PI3 kinase negatively regulate the DAF-16 transcription factor (LIN *et al.* 1997; OGG *et al.* 1997). Mutations in *daf-2* or *age-1* activate DAF-16 to promote longevity. Another Daf-c mutant, *daf-9*, encodes a cytochrome P450 acting downstream of *daf-16*, but upstream of *daf-12* (GERISCH *et al.* 2001; JIA *et al.* 2002). *daf-12* encodes the nuclear receptor likely to be the global switch for dauer *vs.* non-dauer development (ANTEBI *et al.* 2000). In all cases, a *daf-16* mutation suppressed the Daf-c phenotype of the Liv mutant, indicating that these mutations, like other mutations with similar phenotypes (KENYON *et al.* 1993; AILION *et al.* 1999; APFELD and KENYON 1999), define genes that are in the *daf-2* pathway or perhaps in another pathway negatively regulating DAF-16.

Longevity is highly correlated with resistance to thermal stress (LITHGOW and WALKER 2002), and our selection for L1 survival proved to be an efficient method to identify long-lived mutants. Nearly 80% of the 63 thermotolerant mutants we selected were long lived. This is by far the largest isolation of longevity mutants described to date. In fact, long-lived mutants of *C. elegans* (LITHGOW *et al.* 1995), Drosophila (LIN *et al.* 1998), mammals (MIGLIACCIO *et al.* 1999), and yeast (FABRIZIO *et al.* 2001) are resistant to a variety of stresses, including thermal stress (FINKEL and HOLBROOK 2000). Since many genes affect both stress sensitivity and longevity, it appears that the same molecular mechanisms defending cells against stress may also defend them from damage that causes aging. Thermal stress resistance provides an important tool to select mutants with increased longevity because previous attempts to isolate longevity mutants have had limited success (KLASS 1983; DUHON *et al.* 1996; YANG and WILSON 1999, 2000; SAMPAYO *et al.* 2000).

We also isolated 14 thermotolerant mutants that exhibited no obvious increase in longevity (*i.e.*, <15% increase in mean life span). These mutants could be (a) weak Liv alleles; (b) downstream of *daf-2*, past the point of divergence between the thermotolerance and longevity pathways; or (c) involved in unrelated pathways. Alternatively, these mutants could be temperature sensitive, with a phenotype detected at 30° (at which thermotolerance is assayed), but not at 25° (at which life spans were measured). Finally, the mutants may affect longevity pathways but have deleterious effects that mask the longevity phenotype, as may be the case with *age-1(m895)*.

The fact that we isolated 22 *unc-31*, 6 *daf-2*, and 5 *age-1* alleles suggests that the gene target for this selection is not large. However, we isolated only 1 allele of at least five other genes, indicating that the mutant spectrum is not saturated. In fact, we did not detect mutations in *unc-64* or *pdk-1*, genes that are known to be associated with dauer formation, longevity, and thermotolerance

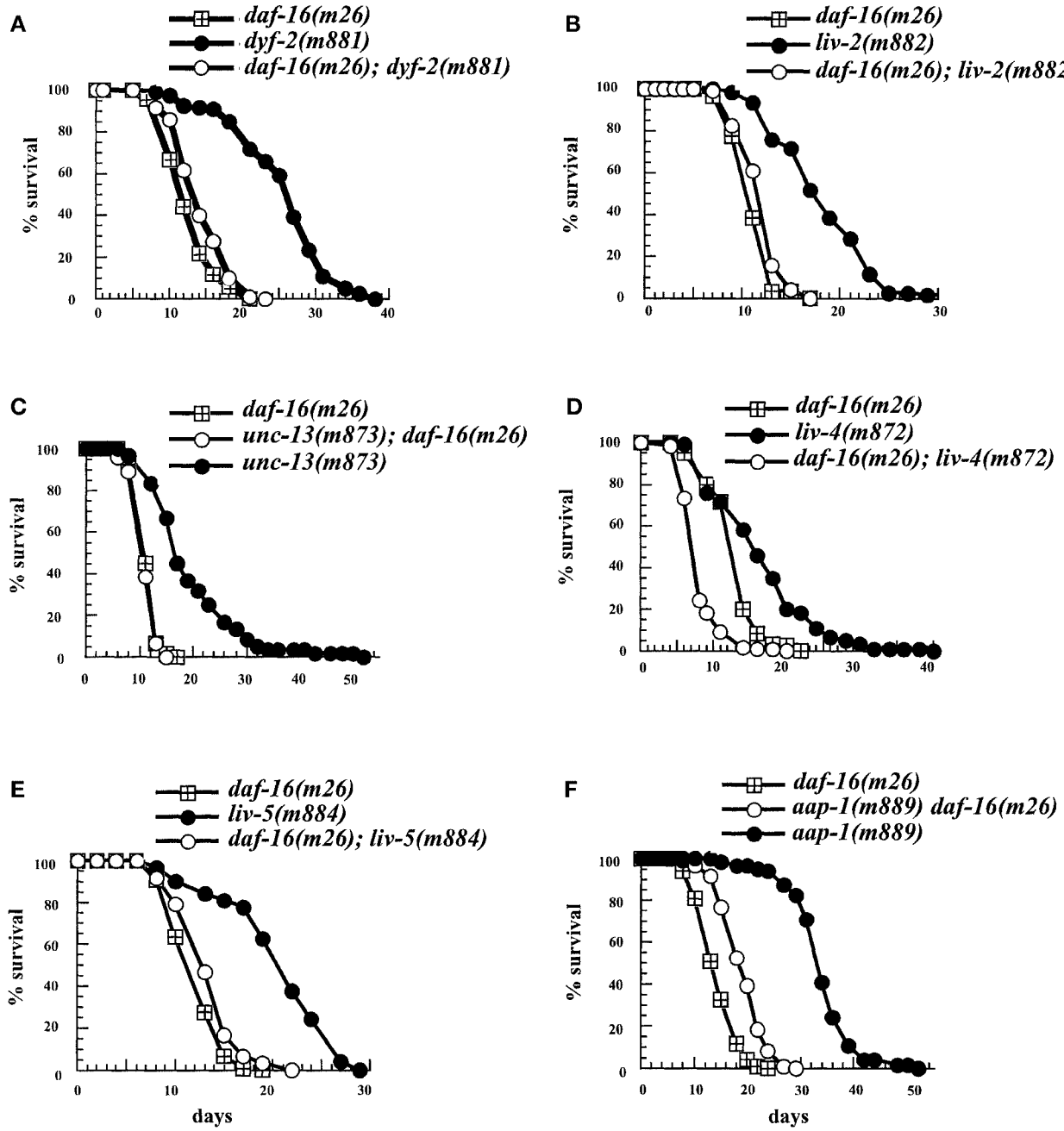

FIGURE 6.—*daf-16(m26)* suppressed the increased adult longevity of all the Liv mutants tested. All strains carry *fer-15(b26)*. Survival of a Liv mutant relative to *daf-16(m26)* and the corresponding double mutant is shown. Day 1 is the first day of adulthood.

(AILION *et al.* 1999; PARADIS *et al.* 1999). We did find a mutation in at least one such gene that had not been identified genetically. The *aap-1* mutant was mapped on chromosome I close to the location of a gene predicted to encode the *C. elegans* homolog of the p55 adapter subunit of PI3 kinase (WOLKOW *et al.* 2002). The *C. elegans* p55 transgene complements (rescues) *m889*, and a nonsense mutation was identified in the mutant strain. The *aap-1* mutation exhibits a Daf-c, longevity, and thermotolerance phenotype very similar to *age-1* mutations, which affect the catalytic subunit. *liv-5* also has a phenotype very similar to *age-1* and *aap-1*, and it may encode another component of the insulin-like signaling pathway.

The insulin-like pathway mutants (*daf-2* and *age-1*) appear to be the most thermotolerant. Survival of such mutants was favored in the selection, which was designed to kill about one-half of the *daf-2* or *age-1* larvae. Many chemosensory mutants are long lived (APFELD and KENYON 1999), and such genes constitute a large target (STARICH *et al.* 1995). However, we isolated only three of these mutants (identifying two genes), perhaps owing to relatively poor survival in the thermal stress assay. The ability to recover from L1 thermal stress may

also influence the mutant spectrum. For example, *unc-31* mutants are known to be constitutive feeders (Avery *et al.* 1993), possibly allowing better recovery than other mutants. Our largest target was *unc-31*. This gene encodes a 1214-amino-acid protein and may be a hot spot for mutagenesis. At least 14 alleles of *unc-31* have been found in previous screens for Unc mutants (http://biosci.umn.edu/CGC/CGChomepage.htm).

We performed a selection in the F_3 generation after EMS treatment to isolate maternal-effect mutants like *age-1* (Klass 1983). The *age-1* mutants were indeed isolated in this selection, as were *liv-2*, *liv-5*, and *aap-1*. *liv-2* and *liv-5* might also be detected in the F_2, since only *age-1* and *aap-1* exhibit a maternally rescued Daf-c phenotype. However, if the maternal effect influenced L1 thermotolerance and not the Daf-c phenotype, these mutants would not have been isolated in the F_2. In principle, the F_3 selection should yield the same mutants as the F_2, plus additional maternal-effect mutants. In fact, the frequency of *unc-31* mutations was similar in the F_2 and F_3 selections (26 and 29%, respectively), indicating that both selections have similar gene targets.

We did not construct *daf-16* double mutants with the nine Liv strains that lack a convenient phenotype. Our current work on these mutants involves development of a suitable surrogate marker to facilitate genetic analysis. This mutant set may simply consist of weak alleles of genes represented in the other three classes of mutants. Alternatively, they could define a different longevity pathway, or they could be downstream of insulin-like signaling past the point of divergence of the Daf and Liv pathways.

In summary, our thermotolerance-based strategy for selecting long-lived *C. elegans* mutants was remarkably successful. Nearly 80% of the isolated strains showed at least a 15% increase in mean life span. Among these are representatives of all known longevity mutants that affect dauer larva formation and a new class of long-lived, thermotolerant mutants with no other detectable phenotype. To facilitate molecular cloning of longevity genes, we are currently selecting for transposon insertions.

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