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The genetics of caloric restriction in *Caenorhabditis elegans*

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ABSTRACT **Low caloric intake (caloric restriction) can lengthen the life span of a wide range of animals and possibly even of humans. To understand better how caloric restriction lengthens life span, we used genetic methods and criteria to investigate its mechanism of action in the nematode *Caenorhabditis elegans*. Mutations in many genes (*eat* genes) result in partial starvation of the worm by disrupting the function of the pharynx, the feeding organ. We found that most *eat* mutations significantly lengthen life span (by up to 50%). In *C. elegans,* mutations in a number of other genes that can extend life span have been found. Two genetically distinct mechanisms of life span extension are known: a mechanism involving genes that regulate dauer formation (*age-1, daf-2, daf-16*, and *daf-28*) and a mechanism involving genes that affect the rate of development and behavior (*clk-1, clk-2, clk-3*, and *gro-1*). We find that the long life of *eat-2* mutants does not require the activity of DAF-16 and that *eat-2; daf-2* double mutants live even longer than extremely long-lived *daf-2* mutants. These findings demonstrate that food restriction lengthens life span by a mechanism distinct from that of dauer-formation mutants. In contrast, we find that food restriction does not further increase the life span of long-lived *clk-1* mutants, suggesting that *clk-1* and caloric restriction affect similar processes.**

It was shown more than 50 years ago that reducing the caloric intake (caloric restriction) of rodents can significantly lengthen their mean and maximal life span (1). It subsequently has been shown that caloric restriction (CR) can lengthen the life span of a wide variety of animals (2). Trials have even begun with higher primates; based on preliminary evidence, calorically restricted rhesus monkeys show similar signs of delayed aging to those seen in the calorically restricted rodents (3–7). CR has been best studied in rodents, and it is known that rodents undergoing CR display many physiological changes, including reduced body weight, temperature, blood glucose, and insulin levels (reviewed in refs. 8 and 9). However, it is unclear which of these changes are required for an extended life span (8, 9). Several studies indicate that reducing caloric intake reduces the amount of damage attributable to free radicals (reviewed in refs. 8 and 9). One simple hypothesis to explain how CR extends life span is that CR may reduce basal metabolic rates. Rodents and primates undergoing CR have lowered body temperatures (10–12), an indication of lower metabolic rates. However, studies on the effect of CR on the metabolic rates of various mammals have given equivocal results, with some studies showing no change in oxygen consumption per unit of lean body mass (13, 14) and other studies showing a decrease of consumption under CR (15, 16). In spite of uncertainty about how CR affects life span, it remains the only experimental treatment that has been shown repeatedly to significantly prolong the life of vertebrates (8, 9, 17).

On the other hand, it is in *Caenorhabditis elegans* that genetic mechanisms that can extend life span have been best characterized, and the worm has become the foremost animal model system for the molecular analysis of life span (18–20). At least two different genetic mechanisms for extending life span have been identified in *C. elegans*. One mechanism involves the partial activation of the dauer pathway by mutations in the genes *age-1, daf-2*, and *daf-28* (reviewed in refs. 18–20). *age-1* and *daf-2* are involved in an insulin-like signaling cascade that regulates the activity of the forkhead-like transcription factor DAF-16 (21–24). Loss-of-function mutations in *daf-16* strongly suppress the extremely long life of the dauer mutants *age-1* and *daf-2*, as well as all other phenotypes of *daf-2* and *age-1* (23–28). The second mechanism involves the reduction of physiological rates by mutations in the Clock genes *clk-1*, *clk-2*, *clk-3*, and *gro-1* (12, 29, 30). So far, only one of these genes, *clk-1*, has been cloned. *clk-1* encodes a small protein of unknown biochemical function that may regulate metabolism (31).

Lowering food intake also lengthens *C. elegans* life span, indicating that the mechanism of CR may exist in the worm (32, 33). We wondered whether any of the known genetic factors extending life span involve the same mechanism as food restriction. To study food restriction directly, worms can be grown in liquid culture with different known concentrations of bacteria. However, most aging studies in the worm have been done under standard *C. elegans* culture conditions (i.e., worms are cultured on agar plates with lawns of *Escherichia coli* bacteria). Therefore, the response of many long-lived *C. elegans* strains to growth in liquid culture are unknown, and it is not even clear whether all of these strains can be cultured under such conditions. Doing aging studies in liquid culture also poses difficult technical problems such as accurately maintaining and reproducing certain bacterial concentrations. Consequently, we wanted to study food restriction under standard experimental conditions to compare our results directly to previously published work on the genetic basis of life span in *C. elegans*. To achieve this, we used *eat* mutations to reduce the food intake of worms.

eat mutants have defects in pharyngeal function leading to an insufficient food uptake and a starved appearance (34). In wild-type worms, the pharyngeal muscles contract to ingest bacteria more than 200 times per minute (35). Some *eat* mutations affect the strength or the proper sequence of contractions and relaxations of the various muscles of the pharynx, leading to inefficient feeding (34). Mutations in other genes, however, do not affect the coordination of the muscle contraction, but only drastically reduce the rate of pharyngeal pumping (34, 35). *eat* mutations were identified based on visible defects in feeding behavior that resemble those caused by experimental alterations in the muscles or nervous system of the pharynx (34). For some *eat* mutants, there is pharma-

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Abbreviations: CR, caloric restriction; Eat, eating defective; Unc, uncoordinated; ROS, reactive oxygen species.

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cological, electrophysiological, genetic, or molecular evidence that they affect the nervous system or muscles (34–38).

In this paper we show that many *eat* mutations prolong life span, almost certainly by restricting the caloric intake of the worm. We also demonstrate that food restriction lengthens the life span of *C. elegans* by a mechanism that is genetically distinct from that induced by mutations in the dauer genes, but which is similar to that induced by mutations in the gene *clk-1*.

MATERIALS AND METHODS

Aging Experiments. Aging experiments were performed as described (30) except that experiments were begun by allowing adult hermaphrodites to lay eggs for a limited time (4–6 hr). Animals were cultured at 20°C unless otherwise stated. Worms were examined every day until death and were scored as dead when they were no longer able to move even in response to prodding with a platinum pick. Each day, any dead worms were removed from plates and the deaths were recorded. Experiments were started with 50 experimental worms per genotype (10 per plate) and the wild type (N2) was always included as a control. A plate of approximately 30 spare worms was started at the same time as the experimental worms and was treated identically, except that deaths on this plate were not counted. Worms that died from matricidal hatching (the bag-of-worms phenotype) were replaced by spare worms when possible or were discarded from the analysis when spare worms were exhausted.

Strains Used. We used *eat* mutations to reduce the food intake of worms while they were maintained under standard experimental conditions. By maintaining such conditions, we can directly compare our results with previously published work on the genetic basis of life span in *C. elegans*. All *eat* mutants available from the Caenorhabditis Genetics Center were examined except those with mutations in *eat-9, -11, -14, -16* and *egl-19* (= *eat-12*). Three other alleles of *eat-2* and two alleles of *eat-18,* kindly provided by L. Avery and D. Raisen (University of Texas Southwestern Medical Center), also were examined. A selection of uncoordinated (*unc*) mutations also were examined. These mutations were chosen to represent a wide cross section of primary defects. However, *unc* mutations that lead to an egg laying-defective (Egl) phenotype were explicitly excluded, because most of these mutants die from matricidal hatching.

New Alleles of *unc-79* and *unc-80*. Mutations in *unc-79* and *unc-80* cause subtle movement defects. New alleles of *unc-79* and *unc-80* were recovered in a screen for maternal-effect viable mutations (39). These mutations subsequently were found to be fully zygotic mutations that fail to complement either *unc-79(e1030)* or *unc-80(e1272)*.

Backcrossing *eat* and *unc* Strains. In a preliminary study, we found that some strains containing *unc* mutations and some strains containing *eat* mutations could live longer than the wild type, whereas others did not (unpublished results). As the pattern of mutations that extended life span was difficult to interpret, we suspected that background effects could be affecting the life span of some strains. Many *C. elegans* strains available from the Caenorhabditis Genetics Center have been backcrossed only once or twice to the wild type and are likely to carry many background mutations. As a precaution against background effects, all of the *eat* and many of the *unc* mutations were backcrossed two times to the wild type before measuring life span. Because no significant differences in the effect of *eat* mutations on life span before and after backcrosses were noted (unpublished data), the strain DA465 *eat-2(ad465)* was used without backcrosses in experiments testing the interaction of *eat-2* with *daf-16*, *daf-2*, and *clk-1*.

Strain Construction. Double-mutant strains were constructed by using standard techniques (40, 41) selecting for easily identifiable phenotypes: slow growth (*clk-1*), Daf-c (*daf-2*), Eat (*eat-2*), and the ability to suppress the Daf-c phenotype of *daf-2* (*daf-16*).

Statistical Analysis. Statistics were calculated by using the Microsoft EXCEL 97 analysis ToolPak. Mean life spans were compared by using Student's *t* test assuming unequal variances.

RESULTS

Most *eat* Mutations Lengthen Life Span. We tested the life span of a large number of *eat* mutations to see whether these mutations extend life span. As expected, we found that mutations in many *eat* genes (*eat-1*, *eat-2*, *eat-3*, *eat-6*, *eat-13*, and *eat-18)* significantly extend mean and maximum life span (Table 1). All four tested alleles of *eat-2* and *eat-6,* as well as both alleles of *eat-1* and *eat-18,* significantly increase life span (Fig. 1, Table 1). Of all of the *eat* genes tested, the strongest effect was seen with *eat-2* mutants, which can live over 50% longer than the wild type (Table 1; Fig. 1). The life span extension of *eat-2* is comparable to other previously characterized long-lived mutants, such as *clk-1(e2519)* and *daf-2(e1370)* (ref. 30; Fig. 3), and is of similar magnitude to the effect of CR on the life span of mammals (42). The effect of *eat-2* on life span is also highly reproducible. The life span of

Table 1. Mean life span of a number of *eat* mutant strains

Strain	Genotype	*n*	Mean	Diff, %	Significance level
Experiment no. 1					
N2	+	50	21.6 ± 0.6	—	—
MQ581	***eat-1(ad427)***	50	28.8 ± 1.0	+33	<0.0005
MQ582*	***eat-1(e2343)***	50	23.9 ± 0.9	+11	0.03
MQ573	*eat-5(ad464)*	50	20.2 ± 0.5	−6	0.07
MQ574	*eat-7(ad450)*	50	14.0 ± 0.5	−35	<0.0005
Experiment no. 2					
N2	+	50	19.5 ± 0.5	—	—
MQ643	***eat-2(ad453)***	50	26.5 ± 0.7	+36	<0.0005
MQ594	***eat-2(ad465)***	50	25.1 ± 0.7	+29	<0.0005
MQ644	***eat-2(ad1113)***	50	28.4 ± 0.9	+46	<0.0005
MQ631	***eat-2(ad1116)***	36	30.6 ± 1.4	+57	<0.0005
MQ646	***eat-18(ad820sd)***	50	26.9 ± 1.0	+38	<0.0005
MQ649	***eat-18(ad1110)***	38	22.4 ± 0.9	+15	0.008
Experiment no. 3					
N2	+	50	19.9 ± 0.5	—	—
MQ676	***eat-3(ad426)***	50	22.0 ± 0.9	+11	0.04
MQ584	***eat-6(ad792)***	50	27.1 ± 0.6	+36	<0.0005
MQ583	***eat-6(ad467)***	50	27.2 ± 0.7	+37	<0.0005
MQ591	***eat-6(ad601)***	50	22.8 ± 0.7	+15	0.001
MQ645	***eat-6(ad997)***	39	23.6 ± 0.7	+19	<0.0005
MQ677	*eat-10(ad606)*	31	21.5 ± 0.8	+8	0.12
MQ596	***eat-13(ad522)***	50	26.0 ± 0.6	+31	<0.0005

Sample size (*n*), mean life span ± SEM (in days), and percent difference from the wild type are given. The final column displays the probability that the mean life span is the same as the wild type (Student's *t* test). Strains that lived significantly longer than the wild type ($P < 0.05$) are shown in bold, and short-lived strains are underlined. Three separate experiments were performed at different times, each with the wild type (N2) as a control.

*A high proportion of *eat-1(e2343)* worms died from a prolapsed gonad. If these worms are subtracted from the analysis, the mean life span increases significantly. We also tested the life span of *eat-8(ad599)*, *eat-15(ad602)*, and *eat-17(ad707)*; however, the sample sizes for these strains were too low to give reliable results because of high levels of matricidal hatching. *eat-4(ad572)*, *eat-4(ad819)*, and *eat-4(ky5)* also were tested, but most of the worms crawled off the plates and died from desiccation. The life span of DA531 *eat-1(ad427)*, DA631 *eat-3(ad426)*; *him-8(e1489)*, DA464 *eat-5(ad464)*, DA467 *eat-6(ad467)*, and DA521 *eat-7(ad450)* were determined twice, and that of DA465 *eat-2(ad465)* was determined six times. The life span of these strains was very similar to respective backcrossed strains presented above.

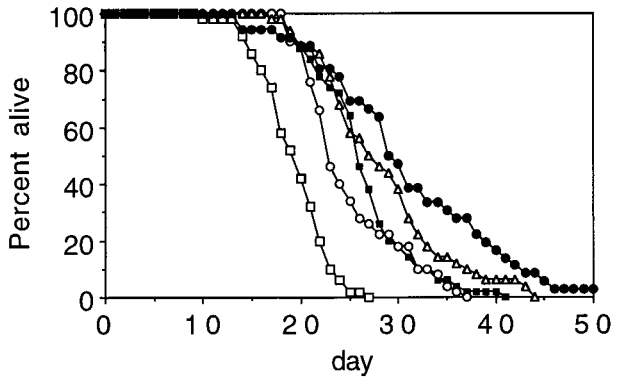

FIG. 1. Four alleles of *eat-2* lengthen life span. The percentage of worms alive on a given day after eggs being laid for a single experiment: N2 (□), *eat-2(ad465)* (○), *eat-2(ad453)* (■), *eat-2(ad1113)* (△), and *eat-2(ad1116)* (●). Mean life spans are given in Table 1. The death of the last surviving *eat-2(ad1116)* worm is not shown. This worm died on day 86.

eat-2(ad465) has been determined seven times, and in every trial mutants live substantially longer than the wild type, with an average lengthening of life span of 47% over the wild type (Table 1; Fig. 1 and 2*B*; and data not shown).

Three of the *eat* mutations tested did not lengthen life span. The mutations *eat-5(ad464)* and *eat-10(ad606)* do not affect life span (Table 1). It is not clear why these mutations do not lengthen life span, but it is possible that mutations in these genes may lead to a feeding defect that is too weak to affect life span. Alternatively, these mutations may produce deleterious pleiotropic effects that mask any positive effects of food restriction on life span. Surprisingly, the only known allele of another gene, *eat-7,* actually shortens life span (Table 1). However, the significance of this result is unclear, because *eat-7* has a very unusual phenotype (34). *eat-7(ad450)* is a poorly studied semidominant mutation that causes worms to "fall asleep" when left undisturbed. In this state, worms do not feed, move, or defecate.

Life Span Correlates with the Severity of the Feeding Defect. Thus, 14 of 17 *eat* mutations tested significantly lengthen life span, strongly suggesting that most *eat* mutants live long as a result of food restriction. This hypothesis is strengthened by the observation that in both *eat-2* and *eat-6* mutants, the severity of the eating defect appears to correlate with life span. Indeed, *eat-6(ad792)* and *eat-6(ad467)* have more severe feeding defects (36) and also live longer than *eat-6(ad601)* and *eat-6(ad997)*. The case is even clearer for *eat-2* mutants. Mutations in *eat-2* appear to affect only the rate of pharyngeal pumping (35). As Klass (32) has shown that food intake is linearly related to pumping rate, pumping rate is a reliable measure of nutritional status. We find that the *eat-2* alleles with the slowest pumping rates, *eat-2(ad1113)* and *eat-2(ad1116)* (35), live longer than one of the weakest *eat-2* alleles, *ad453* (Fig. 1; Table 1). In this experiment, another strong allele, *ad465*, did not lengthen life span as much as *ad453* (29% versus 36% increase over the wild type). However, the life span of *ad465* in this trial was much shorter than its average life span for seven trials (a 47% increase in life span over the wild type).

Most *unc* Mutations Do Not Lengthen Life Span. The fact that long-lived *eat* mutants display a range of defects with different underlying molecular or anatomical causes strongly suggests that these mutants live long because of the only phenotype they share: restricted food intake. It remains possible, however, that the *eat* mutants live long not because of food restriction, but rather as a consequence of some pleiotropic effect on the nervous system and/or muscles. To test this possibility, we examined the life span of a number of *unc* mutants. *unc* mutants display movement defects caused by

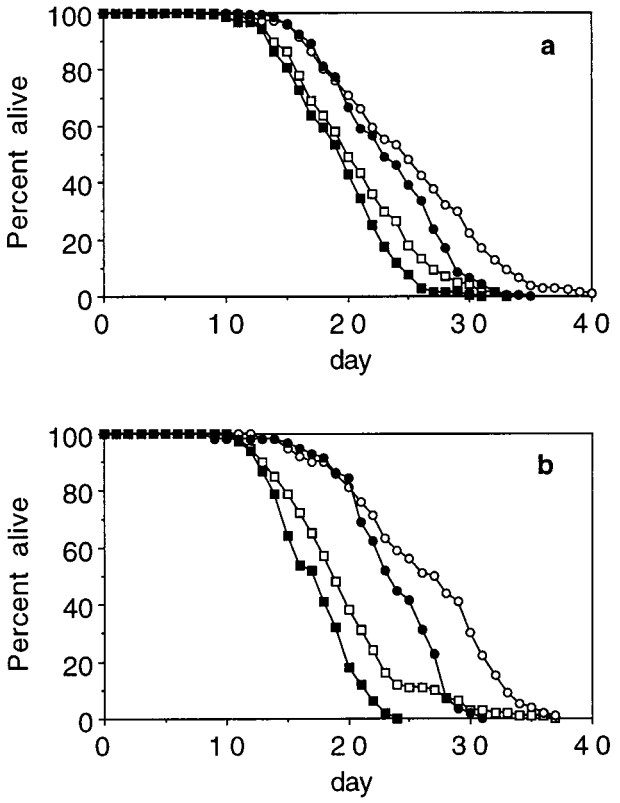

FIG. 2. The interaction of *daf-16* with *clk-1* and *eat-2.* (*a*) The percentage of worms alive on a given day after eggs being laid (day 0) for three pooled experiments at 18°C: N2(□), *daf-16(m26)*(■), *clk-1(e2519)* (○), and *daf-16(m26); clk-1(e2519)* (●). Mean life span ± SEM, with sample size in parentheses, are 20.8 ± 0.4 (145), 19.0 ± 0.6 (145), 25.0 ± 0.5 (141) and 23.5 ± 0.4 (149), respectively. (*b*) The percentage of worms alive on a given day after eggs being laid (day 0) for two pooled experiments at 20°C: N2 (□), *daf-16(m26)* (■), *eat-2(ad465)* (○), and *daf-16(m26); eat-2(ad465)* (●). Mean life spans are 19.7 ± 0.5 (100), 17.4 ± 0.3 (100), 26.3 ± 0.6 (100), and 23.6 ± 0.6 (57), respectively. In one of the two trials pooled for this figure, and in Fig. 3, the last few N2 worms lived much longer than normal. In this trial, the maximum life span *of eat-2(ad465)* and N2 were comparable. However, in the six other trials *eat-2(ad465)* had a maximum life span that was clearly greater than that of N2. All other *eat* mutations that lengthen mean life span also clearly lengthen maximum life span.

abnormalities in the function or development of the nervous system and/or the body-wall muscles (43). Thus, similar molecular defects may underlie the Eat and Unc phenotypes. This idea is borne out by the observation that *eat-8* and *eat-11* are also Unc, whereas *unc-2, -10, -11, -17, -18, -26, -32, -36, -37, -57, -75*, and *-104* mutants are also Eat (34). Thus, *unc* mutations that do not affect pharyngeal pumping are excellent controls for the effect of *eat* mutations on life span.

We tested the effect of mutations in 15 *unc* genes on life span and found that mutations in 14 of these genes (*unc-1, -4, -6, -7, -9, -24, -25, -29, -30, -46, -47, -49, -79*, and *-80*) do not lengthen life span (Table 2 and data not shown). However, some *unc* strains appear to contain background mutations that can extend life span. The strains CB138 *unc-24(e138)* and CB927 *unc-24(e927)* contain background mutations that are not strongly linked to *unc-24*; they could be removed by outcrossing the *unc-24* alleles to the wild type twice (Table 2). Three *unc* strains [containing the mutations *unc-25(e156), unc-79(e1069)*, and *unc-80(e1272)*] contain life-extending background mutations that appear to be linked to the *unc* mutation; they could not be removed by two backcrosses (Table 2). However, other alleles of these genes do not affect life span, indicating that these genes do not affect life span (Table 2).

Table 2. Mean life span of a number of *unc* mutant strains

Strain	Genotype	*n*	Mean	Diff, %	Significance level
Experiment no. 1*					
N2	+	50	19.0 ± 0.5	—	—
MQ545	*unc-24(e138)*	50	19.2 ± 0.6	+1	0.79
MQ546	*unc-24(e448)*	50	19.1 ± 0.4	+1	0.85
MQ547	*unc-24(e927)*	50	19.0 ± 0.5	0	0.95
MQ548	*unc-24(e1172)*	50	19.7 ± 0.4	+4	0.25
Experiment no. 2					
N2	+	50	21.7 ± 0.6	—	—
MQ613	*unc-25(e265)*	50	22.8 ± 1.1	+5	0.42
MQ612	*unc-25(e591)*	50	22.1 ± 0.9	+2	0.71
MQ611	*unc-25(e891)*	50	22.5 ± 1.0	+4	0.49
MQ633	*unc-49(e382)*	50	23.1 ± 0.6	+7	0.11
MQ603	***unc-79(e1068)***	50	24.1 ± 0.9	+11	0.03
MQ604	*unc-80(e1069)*	50	21.6 ± 0.6	0	0.95
Experiment no. 3					
N2	+	50	19.9 ± 0.5	—	—
MQ587	***unc-25(e156)***	50	25.1 ± 0.7	+26	<0.0005
MQ592	***unc-26(e205)***	50	28.7 ± 0.6	+44	<0.0005
MQ647	***unc-26(e345)***	34	29.2 ± 0.8	+47	<0.0005
MQ574	***unc-26(e1196)***	50	27.3 ± 0.7	+37	<0.0005
MQ648	***unc-26(m2)***	39	26.8 ± 0.7	+35	<0.0005
MQ632	*unc-30(e596)*	50	18.2 ± 0.5	−9	0.02
Experiment no. 4					
N2	+	50	21.6 ± 0.6	—	—
MQ575	*unc-30(e191)*	50	20.0 ± 0.6	−7	0.06
MQ588	*unc-30(e318)*	50	16.9 ± 0.5	−22	<0.0005
MQ578	*unc-46(e177)*	50	21.5 ± 1.0	0	0.96
MQ579	*unc-47(e367)*	50	20.5 ± 0.5	−5	0.16
Experiment no. 5					
N2	+	50	19.9 ± 0.7	—	—
MQ636	*unc-79(e1030)*	38	19.1 ± 0.7	−4	0.43
MQ635	*unc-79(qm12)*	50	19.7 ± 0.5	−1	0.89
MQ634	*unc-79(qm14)*	50	21.9 ± 0.8	+10	0.05
MQ638	***unc-80(e1272)***	50	22.0 ± 0.7	+11	0.03
MQ608	*unc-80(qm2)*	50	21.2 ± 0.7	+7	0.18
MQ609	*unc-80(qm3)*	50	20.1 ± 0.6	+1	0.83
MQ637	*unc-80(qm9)*	50	19.5 ± 0.6	−2	0.67

Columns are as described for Table 1. Before outcrossing all of the strains listed above, a number of other *unc* strains received from the Caenorhabditis Genetics Center or Medical Research Council (United Kingdom) also were tested. From these studies we found that mutations in the genes *unc-1, -4, -6, -7, -9*, and *-29* do not affect life span (unpublished data).
**unc-24* alleles have been tested four times with similar results.

Although the life span of *unc* mutants has not been systematically studied, some previous work has shown that at least five other *unc* genes (*unc-2, 15, 20, 54*, and *78*) do not lengthen life span (44).

Of the 15 *unc* genes that we examined, only mutations in *unc-26* clearly lengthen life span. After backcrossing four alleles of *unc-26* to the wild type twice, we determined that all four alleles significantly lengthen life span. However, because *unc-26* mutants are known to have a starved appearance and a feeding defect (34), the long life of these mutants is probably also the result of food restriction. Thus, mutations in 14 of 14 tested *unc* genes that do not affect pharyngeal pumping do not lengthen life span, whereas mutations in 7 of 10 genes that disrupt normal pharyngeal pumping do lengthen life span. This observation, along with the other evidence presented, indicates that *eat* genes indeed lengthen life span by reducing food intake and presumably caloric intake.

At Least Two Genetic Mechanisms Can Lengthen *C. elegans* Life Span. We wondered whether any of the known genetic factors extending life span in *C. elegans* involve the same mechanism as food restriction. Previously, we provided evidence for the existence of at least two distinct genetic mechanisms in *C. elegans* that can extend life span. Specifically, we showed that the dauer genes (*daf-2, age-1*, and *daf-16*) are involved in a mechanism that is distinct from that in which the Clock genes (*clk-1*, *clk-3*, and *gro-1*) are involved (30). Our main evidence was that mutations in *daf-16* that do suppress the long life of *daf-2* and *age-1* mutants do not suppress the long life of the Clock mutants (30). However, the result for *clk-1* has been disputed (45). To solve the question of the independence of the two pathways, we have retested the life span of *daf-16; clk-1* double mutants (Fig. 2*A*) by using the reference alleles of these genes. *daf-16(m26)* mutants live slightly shorter than the wild type (Fig. 2*A*), a result that we have seen repeatedly and that also has been noted by others (26, 27, 46). We find that *daf-16(m26)* has a similar, slightly deleterious effect on *clk-1* mutants; that is, *daf-16(m26); clk-1(e2519)* double mutants live slightly shorter than *clk-1(e2519)* mutants. However, *daf-16(m26); clk-1(e2519)* double mutants still live significantly longer than the wild type (Fig. 2*A*). This observation indicates that the shorter life span of *daf-16(m26); clk-1(e2519)* as compared with *clk-1(e2519)* is not caused by partial suppression of the long life of *clk-1(e2519)* by *daf-16(m26).* Rather, this suggests that the *daf-16(m26)* mutation, or a mutation strongly linked to it, has a weak deleterious effect on life span. That the dauer genes *age-1* and *daf-2* lengthen life span by a different mechanism than *clk-1* was further supported by our observation that *daf-2 clk-1* double mutants live much longer than either *clk-1* or *daf-2*, and can live up to five times longer than the wild type (30). This result contrasts with the result of combining *age-1* and *daf-2,* which, by genetic and molecular criteria, appear to function in the same pathway. In this case, the double mutants *age-1(hx546); daf-2(e1370)* do not live longer than *daf-2(e1370)* (28).

***daf-16* Does Not Suppress the Long Life of *eat-2*.** To test whether CR extends life span by the same mechanism as the dauer genes, we tested whether *daf-16* could suppress the long life of *ad465,* the reference allele of *eat-2*. The results of these experiments are very similar to those for *daf-16(m26); clk-1(e2519)* double mutants. *daf-16(m26); eat-2(ad465)* double mutants live slightly shorter than *eat-2(ad465)* (Fig. 2*B*). However, *eat-2(ad465)* mutants live 34% longer than the wild type and *daf-16(m26); eat-2(ad465)* double mutants live 36% longer than *daf-16(m26)*.

***eat-2; daf-2* Double Mutants Live Longer Than Either *daf-2* or *eat-2* Mutants.** The fact that *daf-16* does not suppress the long life of *eat-2* suggests that the dauer mutations and *eat* mutations lengthen life span by different mechanisms. One prediction of this observation is that, like *daf-2(e1370) clk-1(e2519)* double mutants, *eat-2(ad465); daf-2(e1370)* double mutants should live longer than animals carrying the individual mutations that compose these strains. This expectation is exactly what we find (Fig. 3*A*).

***eat-2; clk-1* Double Mutants Live No Longer Than *eat-2* Mutants.** To examine whether food restriction and *clk-1* mutations lengthen life span by a similar mechanism, we examined the life span of *eat-2(ad465); clk-1(e2519)* double mutants. We find that *eat-2; clk-1* double mutants do not live longer than either of the single mutants (Fig. 3*B*). This result is similar to the interaction of *age-1* and *daf-2*, which function in the same pathway to determine life span. Thus, these results suggest that *eat-2* and *clk-1* mutations may regulate aging by affecting a common process.

DISCUSSION

***eat* Mutants Live Long Because of Food Restriction.** We find that most *eat* mutations significantly lengthen life span. Mutations in *eat-2* can lengthen life span by more that 50%. In the cases of *eat-2* and *eat-6*, life span appears to be correlated with the severity of the eating defect. These results are all consistent

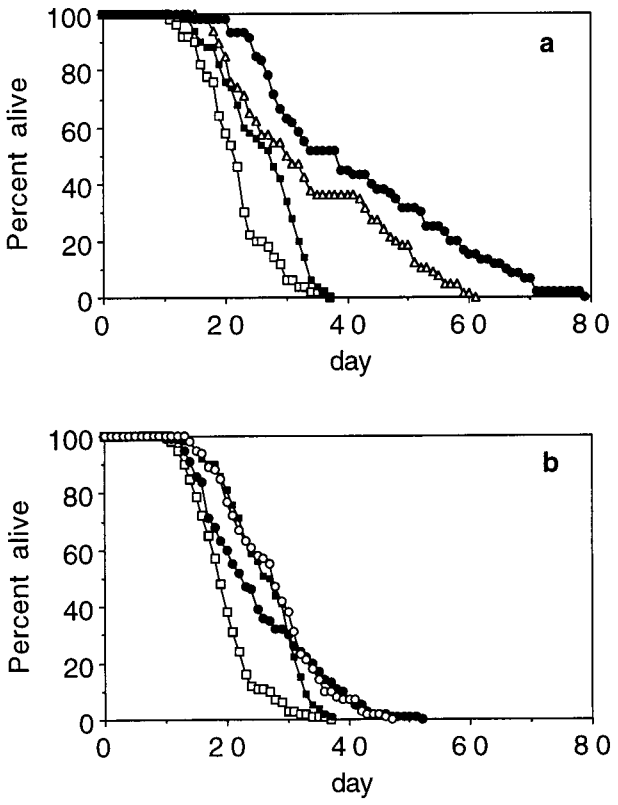

FIG. 3. The interaction of *eat-2* with *daf-2* and *clk-1.* (*a*) The percentage of worms alive on a given day after eggs being laid (day 0) at 20°C: N2(□), *eat-2(ad465)*(■), *daf-2(e1370)* (▲) and MQ413 *eat-2(ad465); daf-2(e1370)* (●). Mean life span ± SEM, with sample size in parentheses, are 21.9 ± 0.8 (50), 26.3 ± 0.9 (50), 34.1 ± 1.6 (66), and 41.6 ± 2.1 (60), respectively. *eat-2(ad465); daf-2(e1370)* worms live significantly longer than either *eat-2(ad465)* or *daf-2(e1370)* ($P < 0.0005$ and $P = 0.005$, respectively). A second trial with these strains gave very similar results. (*b*) The percentage of worms alive on a given day after eggs being laid (day 0) for two pooled experiments at 20°C: N2 (□), *eat-2(ad465)* (■), *clk-1(e2519)* (●) and *eat-2(ad465); clk-1(e2519)* (○). Mean life spans are 19.7 ± 0.5 (100), 26.3 ± 0.6 (100), 25.1 ± 0.9 (100), and 27.5 ± 0.8 (100), respectively. *eat-2(ad465); clk-1(e2519)* lives only marginally longer than *clk-1(e2519)* ($P = 0.05$) and no longer than *eat-2(ad465)* ($P = 0.23$). In one trial, *clk-1(qm30)* and *eat-2(ad465); clk-1(qm30)* were scored. The results for this experiment were: N2, 21.9 ± 0.8 (50); *eat-2,* 26.3 ± 0.9 (50); *clk-1,* 24.1 ± 1.4 (50), and *eat-2; clk-1,* 26.5 ± 1.5 (50).

with the long life of *eat* mutants being caused by CR. Because *eat* mutations are believed to affect feeding behavior by disrupting the pharyngeal muscle or the nervous system, it was possible that *eat* mutants would live long because of pleiotropic effects on muscles and/or the nervous system. However, we find that mutations in 14 *unc* genes that do not affect pharyngeal pumping do not lengthen life span. Of all of the *unc* mutations examined, only mutations in the gene *unc-26* lengthen life span. However *unc-26* mutations lead to feeding defects and they probably have increased life spans because of CR. The results with *unc* mutants indicate that defects in body-wall muscles or the extrapharyngeal nervous system do not affect life span. They also indicate that decreased movement *per se* does not affect life span.

Many Genes Affect Life Span. We have found that mutations in 7 of 10 genes that cause a feeding defect can lengthen life span. Because Avery (34) has estimated that there are at least 60 such genes that can mutate to give a feeding defect, mutations that extend life span by restricting food intake should be isolated at high frequency in genetic screens for long-lived mutants. We have also found that many *unc* strains harbor background mutations that extend life span. It is not clear whether these mutations affect feeding behavior; however, they do not affect developmental rates or cause dauer-formation defects (unpublished data).

Food Restriction and Dauer Mutations Lengthen Life Span by Distinct Mechanisms. It has been hypothesized that CR may lengthen life span by affecting metabolism (reviewed in refs. 8 and 9). Because *daf-2* encodes an insulin receptor-like molecule, and insulin regulates metabolism in mammals, it has been suggested that there may be parallels between life extension by CR and by *daf-2* mutations (22). However, by two different criteria, the results of our aging studies strongly suggest that *daf-2* mutations lengthen life span by a mechanism distinct from that of food restriction. This interpretation is supported by the phenotype of *eat-2; daf-2* double mutants. *eat-2* mutations appear to have no effect on the Daf-c phenotype of *daf-2* mutants, consistent with these genes acting in different pathways. *daf-2* mutants also have very different visible effects on metabolism than *eat* mutants. *daf-2* mutants accumulate fat in the intestine, giving these mutants a very dark appearance (22). *eat* mutants, on the other hand, appear very pale, probably indicating reduced intestinal stores of fat. *eat-2; daf-2* double mutants have an intermediate appearance: they are paler than *daf-2* and darker than *eat-2*, suggesting that these two genes affect metabolism in different and perhaps antagonistic ways.

CR and *clk-1* Mutations May Extend Life Span by a Similar Process. Our results suggest that *eat-2* and *clk-1* mutations may affect life span by affecting a common process. *eat-2* mutants and *clk-1* mutants, however, have very different phenotypes. *clk-1* mutations slow a range of timed phenomena, including the rate of embryonic and postembryonic development, as well as several behavioral rhythms (29). However, in spite of these wide-ranging defects, *clk-1* mutants superficially have a wild-type appearance. We have interpreted the long life of *clk-1* mutants to be the result of a slower rate of living, presumably concomitant with a reduced metabolic rate (20, 30). *clk-1* encodes a small protein with an unknown, but evolutionarily conserved, biochemical function (31). Consistent with our interpretation of the function of *clk-1*, the yeast homologue of CLK-1 is localized to the inner membrane of mitochondria, where it is necessary for respiration (47, 48). On the other hand, *eat-2* mutations have obvious deleterious effects. Mutations in the *eat-2* gene are thought to affect primarily pharyngeal pumping rates, slowing pumping to 10–20% of normal (35). *eat-2* mutants appear pale and small, two phenotypes that also are seen when wild-type worms are grown under low food concentrations (34). In spite of this, *eat-2* mutants develop at an almost-wild-type rate. *eat-2; clk-1* double mutants display the phenotypes of both mutations, developing at the same rate as *clk-1* mutants and appearing pale and starved like *eat-2* mutants, suggesting that only some aspects of their phenotype are relevant to the effect of these mutations on life span.

We hypothesize that mutations in both *clk-1* and *eat-2* genes may reduce energy production in the worm, and this is why they affect life span in a similar manner. *eat-2* mutants are starved, which may lead, through some physiological feedback mechanism, to decreased metabolic rates. As the yeast homologue of CLK-1 is a mitochondrial protein, *clk-1* mutations probably reduce metabolic rates more directly. However, if this is the case, *clk-1* mutations also must lead to decreased energy requirements because unlike *eat-2* mutants, *clk-1* mutants are not starved.

Evidence is mounting that damage caused by reactive oxygen species (ROS) produced in the mitochondria may be a major cause of aging (8, 9). In other systems, CR has been shown to reduce oxidative stress produced by ROS (8, 9). If mutations in *clk-1* and *eat-2* genes reduce metabolic rates, this could lead to reduced rates of production of ROS, which in

turn would lead to a slower accumulation of ROS-associated damage and slower aging.

The Relationship Between CR and Aging in Worms and Vertebrates. We show that reducing food intake by *eat* mutations lengthens the life span of *C. elegans*. It is not well understood in *C. elegans* how reduced food intake lengthens life span, but by analogy to mammals it is likely that it is reduced caloric intake that lengthens life span. However, it has not been shown that this effect is caused by reduced caloric intake, as opposed to reduced intake of protein or some other nutrient. It also is not clear how similar the physiological response to reduced food intake is between worms and mammals. However, the fact that food restriction in worms, mammals, and many other types of organisms lengthens life span suggests that there may be important conserved physiological responses to reduced food intake across divergent phyla. The *eat* mutants provide excellent tools to help study these responses in a genetically tractable system.

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