daf-16: An HNF-3/forkhead Family Member That Can Function to Double the Life-Span of Caenorhabditis elegans

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The wild-type Caenorhabditis elegans nematode ages rapidly, undergoing development, senescence, and death in less than 3 weeks. In contrast, mutants with reduced activity of the gene daf-2, a homolog of the insulin and insulin-like growth factor receptors, age more slowly than normal and live more than twice as long. These mutants are active and fully fertile and have normal metabolic rates. The life-span extension caused by daf-2 mutations requires the activity of the gene daf-16. daf-16 appears to play a unique role in life-span regulation and encodes a member of the hepatocyte nuclear factor 3 (HNF-3)/forkhead family of transcriptional regulators. In humans, insulin down-regulates the expression of certain genes by antagonizing the activity of HNF-3, raising the possibility that aspects of this regulatory system have been conserved.

The identification of genes that regulate aging (1) is an important breakthrough because it provides a means of investigating this fundamental but poorly understood process. The nematode Caenorhabditis elegans has a very rapid rate of aging (2), which is due in part to the activity of the gene daf-2. Mutations that reduce the activity of daf-2, a homolog of the insulin and insulin-like growth factor (IGF) receptors (3), can slow the rate of aging and more than double the life-span of the animal (4) without substantially affecting its activity or fertility (4–6). The life-span extension caused by daf-2 mutations requires the gene daf-16 (4).

In addition to regulating the rate of aging, daf-2 and daf-16 also regulate the decision to enter diapause (the dauer phase) (7–10). When food is limited, young animals become dauer larvae instead of developing to adulthood (9). The dauer is a resilient, long-lived, juvenile form that remains small and reproductively immature. Wild-type daf-2 activity promotes growth to adulthood and prevents dauer formation. Unlike partial loss of daf-2 function, which specifically affects life-span, more severe loss of daf-2 function causes the animals to become dauer larvae even in the presence of food (7–11). Thus, daf-2(-) has two functions: It promotes growth to adulthood, and it shortens the life-spans of adult animals (12). In addition to its role in life-span extension, the wild-type daf-16 gene is also required for dauer formation in both wild-type and daf-2(-) animals (7–11). Thus, daf-16 also has two functions: Under dauer-inducing conditions, it promotes dauer formation, and, under conditions that do not induce dauer formation, it allows fertile adults carrying weak daf-2 mutations to remain active for a much longer period and to live twice as long as normal (4).

Because so few genes are known to regulate aging in any organism, we asked how many other genes were likely to have functions similar to that of daf-16. To do this, we carried out a genetic screen for additional daf-16-like mutants. daf-16 mutations, as well as mutations in functionally related genes required for both dauer formation and life-span, can be isolated easily as suppressors of daf-2 mutations (7–9). We mutated daf-16 (–).

We cloned daf-16 by transposon tagging (16, 17). We isolated a daf-16::Tc1 insertion mutation, mut147, by looking for daf-2 suppressors in a daf-2; mut-6 strain, in which the transposon Tc1 is active (Fig. 1, A and B) (16). We then cloned the genomic DNA containing this Tc1 element and used sequences flanking Tc1 as a probe in Southern blot analysis of DNA isolated from our trimethylpsoralen-induced daf-16 mutants. In five mutants, we found changes in the mobility of the restriction fragments that hybridized to the probe (Fig. 1C).

The Tc1-tagged DNA was sequenced and found to be present on the cosmids R13H8, which was sequenced by the C. elegans sequencing project (18). We obtained the corresponding cDNA sequences by performing
The sequence of this gene was found to be homologous to members of the HNF-3/ forkhead family, a large class of transcription factors characterized by the presence of a forkhead domain, an ~110-amino acid domain that forms a winged helix structure and mediates DNA binding (20). Members of this family have many different roles in embryogenesis, tumorigenesis, and differentiation and have been found to function at downstream positions in several types of signaling pathways (20), including insulin pathways (described below). The daf-16 forkhead domain was most similar to those of human FKHR and AFX proteins (67% and 64% identity, respectively), both of which were identified as human oncogenic fusion proteins (20) (Fig. 3). As with other HNF-3/forkhead family members, little similarity was present outside of this region.

We verified that this gene was daf-16 by identifying sequence changes in the Tc1 insertion mutant, two previously identified daf-16 mutants, m26 and m27, and eight trimethylpsoralen-induced mutations that form was detected in the m26 mRNA. Also not shown is the mu100 mRNA analysis, which resulted in an abnormally spliced mRNA bearing an early stop codon, which predicted to remove most of the forkhead domain and all COOH-terminal regions. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
we analyzed (Fig. 2, A and B). Several mutations affected the conserved forkhead domain and were predicted to prevent DNA binding. The mu147 Tc1 insertion was located within the forkhead domain, m26 altered a splice donor sequence up-stream of the forkhead domain, mu84 deleted part of the forkhead domain and some of the COOH-terminal region, and mu100 and mu92 both affected splice junctions of exon 3, which encodes part of the forkhead domain. In contrast, one mutation, m27, created a stop codon −100 amino acids COOH-terminal to the forkhead domain. Semiquantitative RT-PCR analysis indicated that this mutation did not affect daf-16 mRNA levels, implying that it may encode a truncated DAF-16 protein containing the DNA binding domain but lacking the COOH-terminal region (21). This finding suggests that sequences downstream of the forkhead domain may be required for DAF-16 function. The COOH-terminal region of the forkhead family member FAST-1 has been found to mediate protein-protein interactions (22); whether a similar function exists for this region of DAF-16 awaits further analysis.

The dauer-defective phenotype of daf-16 is semidominant (8); therefore, it was particularly important to confirm that the Daf-16 mutant phenotype resulted from reduced rather than altered or novel gene activity. Our findings indicated that this is the case, because most of these mutations would be predicted to reduce or eliminate daf-16 activity. To determine whether any of these mutations were null alleles, we analyzed mRNA expression in the mutants. For the majority of alleles, it was not possible to completely rule out the possibility that residual daf-16 function might still exist (Fig. 2B). However, one mutation, mu86, was likely a null allele. This mutation was a large deletion that removed most of the coding sequence, including all of the forkhead domain. This mutant, like all other known daf-16 alleles, grew to become an active, fertile adult. This finding suggests that daf-16 functions primarily to regulate life-span and dauer formation and does not have essential activities.

The finding that daf-16 encodes an HNF-3/forkhead family member is important because it implies that mutations in the daf-2 insulin/IGF receptor homolog exert their effects not simply by changing the activities of preexisting enzymes (23) but instead by initiating a new genetic regulatory program that extends youthfulness and postpones death. There are several intriguing parallels between this C. elegans pathway and the human insulin/IGF pathways (3). In humans, insulin and IGF regulate food utilization pathways and promote growth (23). Similarly, daf-2 activity promotes growth to adulthood when food is abundant; conversely, lack of daf-2 activity maintains the dauer state, in which the animals use stored food sources. In animals that lack daf-16 activity, daf-16 mutations have little or no effect, which raises the possibility that the primary role of daf-2 is to prevent daf-16 function (7–9). In humans, insulin appears to mediate some of its effects by blocking the activity of HNF-3 (24). Of four insulin-repressed genes that have been studied extensively, three, phosphoenolpyruvate carboxykinase, tyrosine aminotransferase, and IGF binding protein-1, appear to be up-regulated by HNF-3 in the absence of insulin. Each gene contains a similar insulin-response sequence (IRS) that can act as a binding site for HNF-3 and that is required for repression by insulin. It has been proposed that insulin signaling acts by preventing HNF-3 from binding to the IRS (24, 25).

So far, little is known about how daf-2 might affect daf-16 activity in C. elegans. daf-2 may exert its effects by activating the phosphatidylinositol 3-kinase age-1, because mutations in this gene also extend life-span in a daf-16–dependent fashion (5, 26–30).

In vertebrates, caloric restriction, which affects insulin levels, has an effect analogous to that of weak daf-2 mutations: It also extends life-span without decreasing the rate of metabolism (31). Thus, life-span in both C. elegans and vertebrates may be regulated by an evolutionarily conserved mechanism involving a forkhead homolog that promotes longevity when food is scarce and an insulin family member that counteracts it. In addition, it is possible that the different aging rates of different individuals within a species, as well as the markedly different aging rates exhibited by members of different species, are due in part to intrinsic differences in the resting levels of this signaling pathway.

Note added in proof: Working independently, Ogg et al. have also cloned and molecularly analyzed daf-16 (36).

**REFERENCES AND NOTES**

11. The process of dauer formation is facilitated by high temperature; therefore, many daf-2 mutations induce dauer formation at high but not low temperature. At low temperature or when shifted to high temperature as young adults, past the dauer decision point, these daf-2 mutants become long-lived adults (4).
12. Because dauers are long-lived, one explanation for these two roles is that weak daf-2 mutations allow adults to express only one feature of the dauer, namely its slow rate of aging.
14. daf-2(e1370ts); L4-stage animals were mutagenized with trimethylpsoralen followed by UV irradiation (13). P and F1 progeny were screened for their ability to grow to adulthood at 25°C. Twenty-four independent daf-2 suppressors, mu34 to mu707, were isolated, two of which proved to be daf-16 alleles. We performed complementation tests by crossing daf-16(mu26); daf-2(mu37); him-5(e1490) males with...
sup; daf-2(e1370) hermaphrodites at the non-permissive temperature and examining cross progeny for dauer formation. The descendants of the cross progeny were also examined to ensure that the mutations were not unlinked, noncomplementing mutations. We also mapped many of the mutations by testing for linkage with unc-29, which maps near daf-16, or by recombination fragment length polymorphisms using PCR (32).

15. One such gene may be daf-18. This gene has been identified by a single mutation that suppresses both dauer formation and the life-span extension of daf-2 mutants (7, 10, 28). However, many daf-18 individuals show severe morphological abnormalities, suggesting that this gene has other, possibly essential functions (29). The fact that we did not find any daf-18 alleles supports this hypothesis. In addition, we note that because we screened F2 progeny of mutated animals, we would have missed mutations that were maternally rescued.

16. We first attempted to clone daf-16 by positional mapping but found that the gene was located in a gap in the physical map between cosmid AET and ZK98. To isolate daf-16, Tc1 insertion mutants, we screened daf-2(sa189); mut-6 animals for spontaneous mutants that did not become dauer when cultured at 20°C. One mutant, mu147, also suppressed dauer formation at 25°C. This mutation failed to complement daf-16(m28) and was closely linked to unc-29, which maps near daf-16. mu147 was subsequently crossed to either unc-29(e1072); daf-2(e1370); him-5(e1490) or daf-2(e1370); him-5(e1490) mutants, and homozygous Daf-16(–) and Daf-16(+)- recombinants were obtained. Genomic DNA was prepared from these recombinants and analyzed by Southern blot hybridization with a 5.1-kb Tc1 sequence as probe. A 6.1-kb Tc1-hybridizing fragment was detected in the Xba I–digested genomic DNA, which was present in 20 of 20 daf-16(–) recombinants but absent in 15 of 15 daf-16(+) recombinants and also absent in the wild-type strain (N2). DNA from the corresponding region was then extracted from agarose gels and circularized by self-ligation. An inverse PCR strategy was used to identify a Tc1-containing fragment with the expected size of 5.1 kb. The Tc1-specific primers used for inverse PCR were 5′-CTCCTTGTCGAAGCACTACAATGTGCTGC-3′ and 5′-TGTGCACTCAGCACTCAGCCTG-TGTGT-C-3′. The 5.1-kb PCR product was cloned into the pGEM-T vector (Promega, Madison, WI), and the 0.6-kb flanking Tc1 sequence was removed by digestion with Eco RV. The remaining sequence was then used as a probe in subsequent experiments.


19. RT-PCR was performed with an SL1 primer to obtain the 3′ end of the gene and with Q1, Q2, and Q3 to obtain the 3′ end by rapid amplification of cDNA ends (RACE), as well as with several internal primers (positioned as shown in Fig. 2B). In addition, blast searches with sequences contained on R13H4 identified three cDNA clones (yk13111, yk31110, and yk3214) in a C. elegans EST database. Complete sequences of clones yk13111 and yk31110 were then obtained, and both contained the longer form of the transcripts, whereas the majority of the RT-PCR product was from a shorter RNA preparation contained in the shorter form (see Fig. 2). In addition, the longer spliced form was also detected by RT-PCR.


30. Neither gain of function (n1046) nor dominant negative (y100) mutations in the C. elegans Ras homolog let-60 affected C. elegans life-span (because these mutants cannot lay eggs, their gonads were ablated to prevent premature death from internal hatching). In addition, let-60(n1046) did not suppress the dauer-constitutive phenotype of daf-2(e1370) (J. Apfeld and C. Kenyon, unpublished data).


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