Identification of the differential distribution patterns of mRNAs and consensus binding sequences for mouse DAF-16 homologues

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daf-16 is a forkhead-type transcription factor, functioning downstream of insulin-like signals, and is known to be critical to the regulation of life span in *Caenorhabditis elegans*. Mammalian DAF-16 homologues include AFX, FKHR and FKHRL1, which contain a conserved forkhead domain and three putative phosphorylation sites for the Ser/Thr kinase Akt/protein kinase B (PKB), as well as for DAF-16. To assess the function of the homologues, we examined tissue distribution patterns of mRNAs for DAF-16 homologues in mice. In the embryos, expressions of AFX, FKHR and FKHRL1 mRNAs were complementary to each other and were highest in muscle, adipose tissue and embryonic liver. The characteristic expression pattern remained in the adult, except that signals of FKHRL1 became evident in more tissues, including the brain. In order to clarify whether each DAF-16 homologue had different target genes, we determined the consensus sequences for the binding of DAF-16 and the mouse homologues. The binding sequences for all four proteins shared a core sequence, TTGTTTAC, daf-16 family protein-binding element (DBE) binding protein. However, electrophoretic mobility shift assay showed that the binding affinity of DAF-16 homologues to the core sequence was stronger than that to the insulin-responsive element in the insulin-like growth factor binding protein-1 promoter region, which has been identified as a binding sequence for them. We identified one copy of the DBE upstream of the first exon of *sod-3* by searching the genomic database of *C. elegans*. Taken together, DAF-16 homologues can fundamentally regulate the common target genes in insulin-responsive tissues and the specificity to target genes of each protein is partially determined by the differences in their expression patterns.

Key words: forkhead-type transcription factors (AFX, FKHR, FKHRL1), gene regulation, longevity, *sod-3*.

INTRODUCTION

It has been reported that the life span is genetically controlled [1]. The mechanisms of genetic control of aging have been examined in several models, such as *Caenorhabditis elegans*. Friedman and Johnson [2] found a mutant, *age-1*, with a 60% extension of life span in a screening programme for long-lived mutants. Kenyon et al. [3] exploited a temperature-sensitive dauer mutant, *daf-2*, which caused constitutive entry into diapause, even under conditions of plentiful food. They found that, by moving *daf-2* mutant organisms to a non-permissive temperature, they lived, as L4 larvae, for more than twice the normal life span. Moreover, *daf-2*/*age-1* double mutants lived no longer than *daf-2* mutants, suggesting that the two genes operate in the same signalling pathway. Life span extension resulting from either *age-1* or *daf-2* mutations is prevented by loss of functional mutations in the *daf-16* gene [3]. Therefore *daf-16* function is a prerequisite for life-span extension through the *daf-2* and *age-1* related signalling pathway. Recent molecular cloning analysis revealed that *daf-2*, *age-1* and *daf-16* encode the homologue of the insulin receptor, phosphatidylinositol 3-kinase and forkhead-transcription factor respectively [4–7]. These results suggest that an insulin-like signalling pathway controls life-span extension in *C. elegans*. More recently, Apfeld and Kenyon [8] revealed, by genetic mosaic analysis, that the daf-2 signalling pathway acts not as a cell-autonomous control system but as a systemic one. Thus, in mammals, DAF-16 homologues may control the transcription of certain intercellular signalling molecules, such as hormones, which are sent to systemic cells. The apparent similarity of the mammalian insulin-signalling pathway to that of the *C. elegans* *daf-2*/*age-1*/*daf-16* pathway raises the possibility that the systemic aging control mechanism may be conserved in evolution. If so, mammalian homologues of daf-16 may regulate an aging-related molecule.

In mammals, three members of the forkhead family of transcription factors, termed AFX, FKHR and FKHRL1, are most similar in amino-acid sequence to DAF-16 and represent the mammalian counterparts of daf-16. The human DAF-16 homologues were first identified at chromosomal breakpoints in human tumours [9–11]. They were reported recently to be phosphorylated by a Ser/Thr protein kinase, Akt/PKB, following insulin or insulin-like growth factor (IGF)-1 stimulation; and to bind the insulin-responsive sequence (IRS) in the insulin-like growth factor binding protein-1 (IGFBP1) promoter (BP-1) [12–18]. The IRS-related sequences also exist in the promoter region of other genes which are known to be down-regulated by insulin, such as phosphoenolpyruvate carboxykinase [19]. However, it is not clear which of the DAF-16 homologues more effectively binds to the IRS or IRS-related sequences. Moreover, if all homologues effectively bind to the sequences, it remains to be determined whether all homologues regulate the same genes *in vivo*.

To clarify these issues, we made two suppositions: (1) that temporally and spatially different expressions of DAF-16 homologues confer the differences in functions *in vivo*, and (2) that one of these homologues has higher affinities for some IRS or IRS-related sequences than the others. In the present study, to

Abbreviations used: IRS, insulin responsive sequence; DBE, daf-16 family protein-binding element; IGF, insulin-like growth factor; IGFBP1, insulin-like growth factor binding protein-1; BP-1, insulin-responsive sequence in the IGFBP-1 promoter region; EMSA, electrophoretic mobility shift assay; Trx, thioredoxin; IRE, insulin-responsive element.

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investigate the first possibility, we determined the tissue distribution of the mRNAs for these homologues in embryonic and adult tissues. We then identified the binding sequences of the mouse DAF-16 homologues to test the latter.

MATERIALS AND METHODS

cDNA cloning

cDNA clones for mouse DAF-16 homologues, AFX, FKHR and FKHRL1, were obtained by screening a mouse neonatal brain cDNA library (Stratagene) using a PCR fragment corresponding to the region between two amino acid sequences (QIYEWM and WT/STFRP), which is conserved among DAF-16 homologues, as a probe. Cloned cDNAs were edited, and a cDNA containing a full open reading frame for each homologue resulted. Nucleotide sequences were determined on an ABI Prism 377 sequencer using the Big dye sequence kit.

Northern-blot analysis

ICR mice were purchased from SLC (Hamamatsu, Japan). Total RNAs were purified from each tissue using Trizol reagent (Gibco BRL), and 20 μg of total RNA was electrophoresed and blotted on to Gene Screen Plus membranes (NEN). A 660–1604 bp fragment for AFX (database accession number AB032770), a 1107–2257 bp fragment for FKHR (database accession number AF114258) and a 811–2556 bp fragment for FKHRL1 (database accession number AF114259), which locate in the 3′ region to the forkhead domain and have few similarities in their DNA sequences, were labelled with [α-^{32}P]dCTP using a Megaprime labelling kit (Amersham). 18 S ribosomal RNA was used as an internal control. ExpressHyb solution (Clontech) was used under standard conditions of hybridization at 65 °C and the blot was washed at 65 °C in 0.1 × SSC (1 × SSC = 0.15 M NaCl, 0.015 M sodium citrate) containing 0.1 % (w/v) SDS. The blot was exposed to an X-OMAT autoradiography film (Kodak) for 3–7 days at −80 °C.

In *situ* hybridization

ICR mice were mated and the day when a vaginal plug was discovered was recorded as embryonic day zero. Embryonic day 15.5 tissues or tissues of adult mice were frozen using solid CO_2, stored at −80 °C, and 15 μm-thick cryostat sections were prepared just before the *in situ* hybridization procedure described previously [20]. The fragments used in the Northern-blot analysis were inserted into pBluescriptSK− and used as a template for RNA probes. Antisense or sense RNA probes were synthesized with [^{35}S]UTP by T3 or T7 RNA polymerases. Sections were dipped in emulsion NTB2 (Kodak) and exposed for 14–21 days. Sections were observed with OptiPhoto2 (Nikon). Sense probes showed no significant signals.

Bacterial expression

The DNA fragment encoding the full sequence of the open reading frames of DAF-16, AFX, FKHR and FKHRL1 was amplified with specific primers with appropriate restriction enzyme sites. The PCR products were digested with *Eco*RI and *Xab*I for AFX, *Bam*H1 and *Xba*I for FKHR and *Bam*HI and *Xho*I for FKHRL1, and were cloned between the corresponding sites in pCDN3.1-myc-His-B (Invitrogen). The fragments excised from the expression vector with *Bam*HI and *Age*I were cloned between *Bam*HI and *Sma*I in the vector pET32a (Novagene), which is designed to produce thioredoxin (Trx)-fused proteins. Cultures of *Escherichia coli* BL21 (DE3), harbouring the respective plasmids, were induced with 0.1 mM isopropyl β-D-thiogalactoside and cultured for a further 3–5 h at 37 °C. The bacteria were collected by centrifugation and resuspended in ice-cold buffer. The suspension was sonicated five times at 50 % power for 1 min each time. The lysed bacteria were cleared by centrifugation at 15000 ***g*** for 30 min at 4 °C. The plasmid proteins were purified using Talon beads (Clontech) using the manufacturer's protocol.

Electrophoretic mobility shift assay (EMSA)

DNA-binding reactions were performed in binding buffer [20 mM Hepes (pH 7.9), 50 mM KCl, 2 mM $MgCl_2$, 0.5 mM EDTA, 10 % (v/v) glycerol, 0.1 mg/ml BSA, 2 mM dithiothreitol] containing 50 ng/ml poly(dI·dC), a probe 5′-end labelled with [γ-^{32}P]ATP and approx. 50 ng of Trx-fusion proteins. After incubation for 15 min at 25 °C, the reaction products were separated by PAGE (4 % polyacrylamide gel) in 0.25 × TBE (22.5 mM Tris/borate, 0.5 mM EDTA) containing 5 % glycerol. The gels were dried, exposed for 3–6 h to imaging plates and analysed on BAS2500 (Fuji Film).

Selection of binding sites

A 72-mer oligonucleotide in which the 32 central bases (N32) were completely degenerate, 5′-CGCTCGAGGGATCCGAATTC(N32)TCTAGAAAGCTTGTCGACGC-3′, was synthesized and purified with HPLC. A probe for use in the EMSA was generated using the 72-mer oligonucleotide as a template and primers 5′-GCGTCGACAAGCTTTCTAGA-3′ and 5′-GCGTCGACAAGCTTCTAGA-3′. Following binding to ∼ 50 ng of each fusion protein, EMSA was performed and the location of the DNA–protein complexes within the gels was approximated, based on the mobility of complexes generated with the BP-1 probe. The sequence of the BP-1 probe used was 5′-GAGTGCTAGCAAGCAAAACAAACTTATTTTGAACACGGGGATCCAA-3′. Gel slices were homogenized and incubated in gel elution buffer (0.5 M ammonium acetate, 10 mM $MgCl_2$, 1 mM EDTA and 0.1 % SDS) at 37 °C for 3 h. Recovered oligonucleotides were extracted with phenol/chloroform, precipitated with ethanol, reamplified and subjected to the next round of binding. Following completion of the fourth selection–amplification cycles, PCR products were digested with *Eco*RI and *Xba*I and cloned between the corresponding sites in pBluescriptSK−. Between 21 and 25 inserts were sequenced for each DAF-16 homologue.

Transfections and reporter assays

The reporters described here were derived from the pGL3-basic firefly luciferase vector with a minimal TATA box (Promega). Reporters containing the DAF-16 family protein-binding element (DBE) or the BP1 sequences were generated by ligation of concatemerized oligonucleotides to *Bgl*II-digested pGL3 or *Bgl*II–*Nhe*I-digested pGL3 respectively. They were designated p2xDBE-luc (with two copies of the DBE), p4xDBE-luc (with four copies of DBE), p6xDBE-luc (with six copies of the DBE) and pBP1-luc (with one copy of the BP1). The complementary oligonucleotides used for pDBE-luc constructions were 5′-GATCAAGTAAACAACTATGTAAACAA-3′ and 5′-GATCTTGTTTACATAGTTGTTTACTT-3′, whereas those used for pBP-1-luc construction were 5′-GAGTGCTAGCAAGCAAAACAAACTTATTTTGAACACGGGGATCCAA-3′ and 5′-TTGGATCCCCGTGTTCAAAATAAGTTTGTTTTGCTTGCTAGCACTC-3′. NIH3T3 cells grown in 24-well dishes were transfected with 200 ng of each reporter vector, 20 ng of pRL-

TK *Renilla* luciferase vector, as an internal control, and FKHR or *LacZ* expression vectors using LipofectAMINE plus reagents (GIBCO-BRL). For dose–response analysis, 10, 50, 100, 150, 200 or 300 ng of FKHR expression vector were used. *LacZ* expression vector was included in samples to adjust the total expression vector transfected to 300 ng. FKHR (150 ng) or *LacZ* expression vectors were used for examining the effects of multiple copies of DBEs on transcriptional activity. At 24 h after transfection, 50 ng/ml IGF-1 (GIBCO-BRL) or 20 % (v/v) fetal-bovine serum (GIBCO-BRL) was added. Then, 24 h later the relative luciferase activity was determined (the firefly luciferase activity divided by the *Renilla* luciferase activity) using the Dual Luciferase Reporter Assay System (Promega). Statistical significances were calculated using the Student's *t* test.

RESULTS

We first examined the tissue distribution of mRNAs for DAF-16 homologues in the adult by Northern-blot analysis (Figure 1). A single band was detected at approx. 3.4 kb for AFX, approx. 6.1 kb for FKHR and approx. 7.5 kb for FKHRL1. AFX mRNA was expressed at a higher level in the heart and skeletal muscle and at a moderate level in brown and white adipose tissues. FKHR mRNA was expressed at a higher level in brown adipose tissue, white adipose tissue and spleen, and a little was detected in liver and skeletal muscle. FKHRL1 mRNA was expressed at a higher level in heart, brain, spleen and kidney and at a moderate level in white adipose tissue and testis but, interestingly, little was detected in skeletal muscle and brown adipose tissue when compared with levels in heart and white adipose tissue. The expression of three genes were detected to a lesser extent in the liver.

The tissue distribution in the day 15.5 embryo was examined by *in situ* hybridization histochemistry (Figure 2). Interestingly, three members showed complementary distribution, and a high level of expression was detected in skeletal muscle for AFX (Figure 2A), in adipose tissue for FKHR (Figure 2B) and in the liver for FKHRL1 (Figure 2F).

We next examined the binding sequences of the three homologues. Each possesses a similar forkhead domain, although they

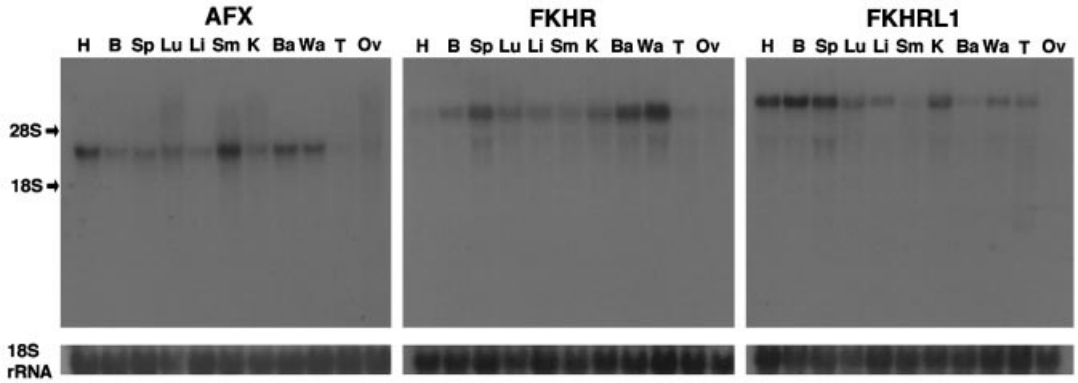


Figure 1 Northern-blot analysis of three DAF-16 homologues in adult mouse tissues

Top panel: total RNA (20 μg), isolated from ICR mouse tissues, was subjected to electrophoresis and blotted on to a nylon membrane as described in the Materials and methods section. The blots were hybridized with ^{32}P-labelled cDNA probes specific for mouse DAF-16 homologues. AFX (left panel), FKHR (middle panel) and FKHRL1 (right panel) showed a 3.4-kb, 6.5-kb and 7.1-kb transcripts respectively. H, heart; B, brain; Sp, spleen; Lu, lung; Li, liver; Sm, skeletal muscle; K, kidney; Wa, white adipose tissue; Ba, brown adipose tissue; T, testis; Ov, ovary. Bottom panel: 18 S ribosomal RNA served as an internal control.

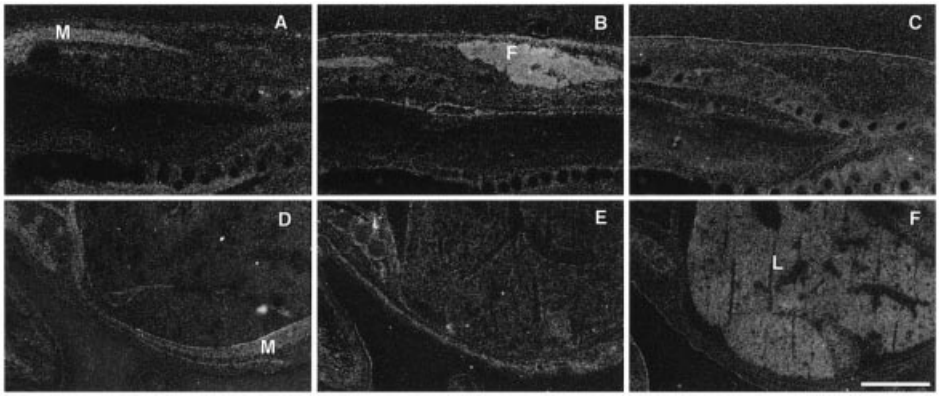


Figure 2 *In situ* hybridization histochemistry showing complementary tissue distribution among DAF-16 homologues in day 15.5 mouse embryos

(**A**,**B**) AFX, (**C**,**D**) FKHR, (**E**,**F**) FKHRL1. The highest expression levels of AFX, FKHR and FKHRL1 respectively were found in (**A**) skeletal muscles (M), (**B**) adipose tissue (F), and and (**F**) embryonic liver (L). Bar = 1 mm.

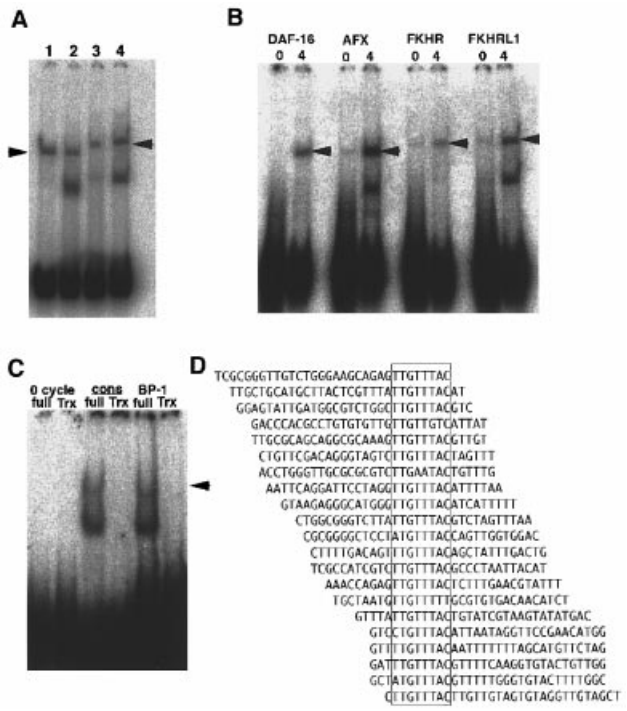
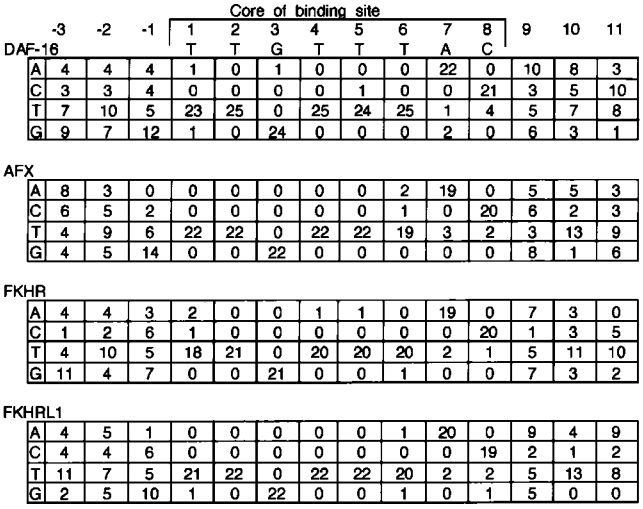


Figure 3 Selection of binding sites for random-sequence oligonucleotides with Trx–DAF16, Trx–AFX, Trx–FKHR and Trx–FKHRL1 fusion proteins

(**A**) Binding of the BP-1 to DAF-16 and DAF-16 homologues: 1, Trx–DAF16; 2, Trx–AFX; 3, Trx–FKHR; 4, Trx–FKHRL1. (**B**) Enrichment of high-affinity binding sites following four cycles of sequential selection and amplification. EMSA of the PCR products obtained after the fourth amplification (4) and the randomized oligonucleotides used as starting material for the selection (0). (**C**) Trx–FKHR fusion protein (cons) showed a shifted band as did the BP-1, but Trx alone did not bind to the consensus sequence. The arrowheads indicate the shifted bands which may be due to degraded products. (**D**) Summary of sequences selected with Trx–FKHR. The 32 nt, corresponding to the randomized segment of 21 selected PCR products, are shown. The core sequence of the FKHR binding site is boxed.



Core of binding site

DAF-16	-3	-2	-1	1 T	2 T	3 G	4 T	5 T	6 T	7 A	8 C	9	10	11
A	4	4	4	1	0	1	0	0	0	22	0	10	8	3
C	3	3	4	0	0	0	0	1	0	0	21	3	5	10
T	7	10	5	23	25	0	25	24	25	1	4	5	7	8
G	9	7	12	1	0	24	0	0	0	2	0	6	3	1

AFX														
A	8	3	0	0	0	0	0	0	2	19	0	5	5	3
C	6	5	2	0	0	0	0	0	1	0	20	6	2	3
T	4	9	6	22	22	0	22	22	19	3	2	3	13	9
G	4	5	14	0	0	22	0	0	0	0	0	8	1	6

FKHR														
A	4	4	3	2	0	0	1	1	0	19	0	7	3	0
C	1	2	6	1	0	0	0	0	0	0	20	1	3	5
T	4	10	5	18	21	0	20	20	20	2	1	5	11	10
G	11	4	7	0	0	21	0	0	1	0	0	7	3	2

FKHRL1														
A	4	5	1	0	0	0	0	0	1	20	0	9	4	9
C	4	4	6	0	0	0	0	0	0	0	19	2	1	2
T	11	7	5	21	22	0	22	22	20	2	2	5	13	8
G	2	5	10	1	0	22	0	0	1	0	1	5	0	0

Figure 4 Summary of selected binding sequences for DAF16, AFX, FKHR and FKHRL1 proteins

The numbers represent the frequency of each nucleotide at each position. The number of sequences on which this summary is based is: 25 for DAF16, 22 for AFX, 21 for FKHR and 22 for FKHRL1. The core sequence, TTGTTTAC, was shared and the sequence is named as DBE.

vary in their primary structure, suggesting that they may have different DNA binding specificities. First, we confirmed that DAF-16 and DAF-16 homologues could bind to the BP-1 (Figure 3A), as reported previously [12–18]. Binding was performed with a random pool of oligonucleotides, using a variety of PCR/selection strategies described previously [21]. After four rounds of selection and amplification, the enriched oligonucleotides were cloned and sequenced. For each DAF-16 homologue, 21–25 different sequences were compared and a consensus sequence was calculated. To rule out that the Trx moiety of the fusion proteins may have any affinity for the selected sequences, Trx protein alone and with the consensus sequence was also tested by EMSA, and no band shift was evident (Figure 3C). The sequencing results revealed that a related 8-bp sequence (5′-TTGTTTAC-3′) or its complement was present in each clone, for example sequences selected with FKHR (Figure 3D). DAF16, AFX and FKHRL1 also shared a consensus sequence identical to that obtained for FKHR (Figure 4). Moreover, DAF-16 and DAF-16 homologues exhibited different preferences in the 5′ flanking sequence of the consensus sequence, i.e. GNA for FKHR and TNG for FKHRL1. In the 3′ flanking region of the consensus sequence they shared a similar sequence, NTTT. This consensus sequence was most similar to that of FREAC-3, another forkhead family member described previously [21].

To test whether the 8-bp consensus sequence (DBE, for daf-16 family member binding element) was sufficient for binding to the DAF-16 homologues, complementary 20-bp oligonucleotide strands containing a single DBE and a single DBE with a single base-pair change of T or G to C (TCGTTTAC, TTCTTTAC, TTGCTTAC and TTGTCTAC) were synthesized and used in an EMSA. The DAF-16 homologues bound the probe containing native DBE, but the mutated probes bound to a much lesser extent or not at all (Figure 5A). The insulin-responsive element (IRE) in the BP-1 is 5′-TT(G/A)TTTTG-3′ and differs from the DBE in the last two bases. We examined for which of the two sequences DAF-16 homologues had a stronger affinity by EMSA with complementary oligonucleotides in which DBE was replaced by a single IRE. All the DAF-16 homologues bound more strongly to the DBE sequence than to the IRE, at least *in vitro* (Figure 5B).

To test whether the DBE recognized by DAF-16 homologues could function *in vivo*, we carried out a reporter assay with p2xDBE-luc, p4xDBE-luc, p6xDBE-luc and pBP1-luc using NIH3T3 cells and the FKHR expression vector. We first examined the dose response of the FKHR expression vector with p2xDBE-luc. The transcriptional activity of the FKHR expression vector increased in a dose-dependent manner and, with $\geqslant$ 100 ng of FKHR expression vector, it was three-fold that with the LacZ vector (Figure 6A). The effects of numbers of copies of the DBE in the promoter region on the transcriptional activity was examined and FKHR showed approximately twice as much reporter activity as LacZ in p2xDBE-luc (Figure 6B). In addition, upon treatment of the cells with IGF-1 or serum, the activity decreased by half. The levels of reporter activity achieved with p4xDBE-luc and p6xDBE-luc were significantly increased to $>$ 4- and 10-fold respectively when compared with that with the LacZ vector alone.

We searched for a candidate for target genes for DAF-16 using the BLASTN program in a genomic database of *C. elegans* (Sanger Center) and identified one copy of the DBE upstream of the first exon of *sod-3* (Figure 7).

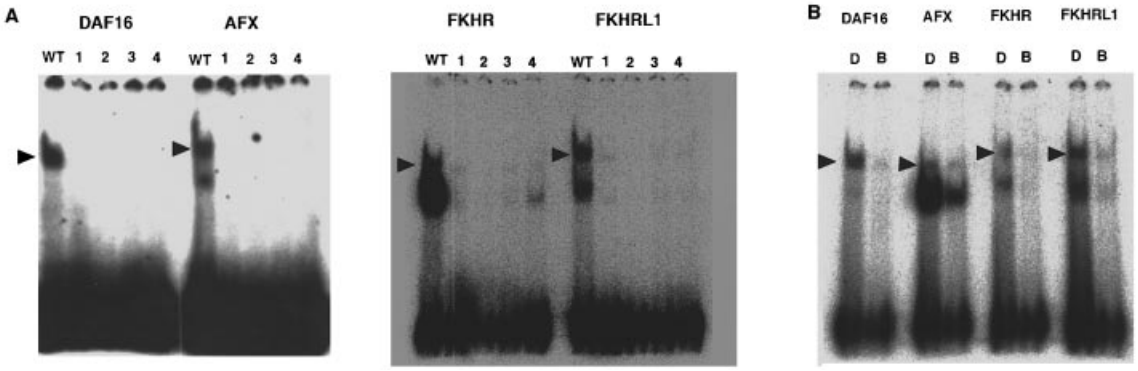


Figure 5 DBE oligonucleotides bind to DAF-16 homologues

(**A**) Examples of EMSA using the Trx-fused DAF-16 homologues and a ^{32}P-labelled DBE probe (WT) or similar probes in which each nucleotide, respectively, of TGTT in TTGTTTAC was changed to C (lanes 1, 2, 3 and 4 respectively). These mutations destroyed the complex of probes and DAF-16 homologues. (**B**) EMSA using ^{32}P-labelled DBE (D) or ^{32}P-labelled BP-1 (B) as a probe for examining the affinity for each DAF-16 homologue. DAF-16 homologues bound more strongly to the DBE than to the BP-1. Arrowheads indicate the shifted bands due to degraded products.

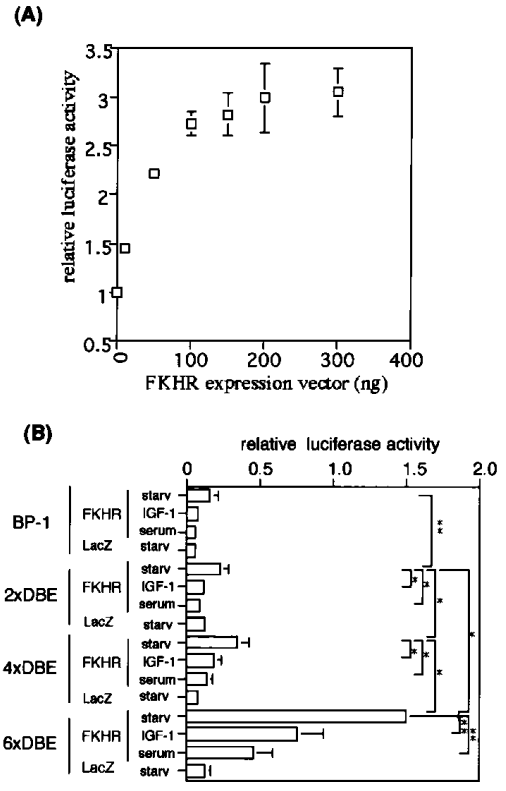


Figure 6 DBE can function *in vivo*

(**A**) The FKHR expression vector increased the relative luciferase activity of a p2xDBE-luc reporter gene in a dose-dependent manner. The activity saturated at $\geqslant$ 100 ng of the FKHR expression vector. NIH3T3 cells were transfected with p2xDBE-luc, pRL-TK and various amounts of FKHR expression vector and 48 h after transfection the cells were harvested and assayed as described in the Materials and methods section. The relative luciferase activity was determined as the firefly luciferase activity divided by the *Renilla* luciferase activity and the points shown represent the means $\pm$ S.E.M. of three experiments. (**B**) NIH3T3 cells were transfected with the indicated plasmids (pBP-1-luc, p2xDBE-luc, p4xDBE-luc and p6xDBE-luc), pRL-TK and FKHR expression vector, and 24 h after the transfection were treated with or without 50 ng/ml IGF-1 or 20% bovine-fetal serum. The transcriptional activities were determined as described in (**A**). The values are the means $\pm$ S.E.M. of three experiments. Asterisks show statistically significant differences calculated by Student's *t* test ($^*P < 0.05$ and $^{**}P < 0.01$).

sod-3:

…

tctctttctctcaacaacaatgtgctggccttgcatgtttg
ccagtgcgggttgtttacgcgttttcaagatttttggtctc
ctatctaacgtcccgaaatgcatttttttcctttcatttggt
ttttttctgttcgagaaaagTGACCGTTTGTCAAATCTTCT
AATTTTCAGTGAATAAAATGCTGCAATCTACTGCTCGCACT
GCTTCAAAGCTTGTTCAACCGGTTGCGGGgtaagtcaaaat
gaaattttcgtttaaaaattggtttttttttggtattataga
taaaacttataccaaaacaaaacatatttagaaaaacttta
atagagaataattgtttaataattaatttttgcaagctcct
ttt…

Figure 7 *C. elegans sod-3* is a candidate target gene of DAF-16

One copy of DBE is located upstream of the first exon of *C. elegans sod-3*. The DBE is underlined and capital letters indicate the exon region.

DISCUSSION

In ICR mice, the expression of DAF-16 homologues were detected at a high levels in the insulin/IGF-1 responsive tissues, such as muscle and adipose tissue in both adults and embryos. This was consistent with the regulation of the transcriptional activities of these proteins by insulin/IGF-1 ([12–18]; T. Furuyama, T. Nakazawa, I. Nakano and N. Mori, unpublished work). However, not all insulin responsive tissues expressed the DAF-16 homologues. The distribution pattern of the three DAF-16 homologues was similar in embryos, i.e. AFX, FKHR and FKHRL1 were expressed more strongly in muscle, adipose tissue and liver respectively. The characteristic distribution pattern remained in the adult and AFX mRNA was expressed at a higher level in muscle, and FKHR in brown and white adipose tissue. This suggested that each homologue plays an important role in the regulation of a target gene in a specific tissue. For example, FKHR may regulate a target gene in adipose tissues. However, the signal level of FKHRL1 was found to be higher in many adult tissues than that of embryonic tissues, so it may function in the mature tissues. Moreover, the distribution pattern in the embryos was also similar to that of Akt2 among Akt

family members, suggesting that Akt2 is critical in phosphorylating DAF-16 homologues following insulin/IGF-1 stimulation [22]. It remains to be clarified whether Akt2 can phosphorylate DAF-16 homologues more efficiently than Akt1.

We next examined the possibility that each DAF-16 homologue recognized a specific sequence for DNA binding. The DBE selected by each DAF-16 homologue, using binding-site selection, was identical, and was 5′-TTGTTTAC-3′. This sequence includes the sequence conserved among forkhead family members, TRTTTAY. A change of any one base in TGTT to C caused the DAF-16 homologue to dissociate from the DBE. Similarly, the change of G or T to C in TRTTTAY was reported previously to prevent binding to the forkhead domain [21]. Taken together, this suggests that DAF-16 homologues also bind to DNA via a forkhead domain, and a variation from the typical forkhead domain, conserved among the family members, may determine the base pairs flanking TGTT in the DBE. Moreover, the binding-site selection revealed that each DAF-16 homologue exhibits different preferences in the 5′-flanking sequence of the DBE. This difference may determine which target genes each DAF-16 homologue regulates.

The BP-1 has been reported to be recognized by DAF-16 homologues [12–18]. The IREs are TT(G/A)TTTTC and are different from the DBE in the last two bases. EMSA revealed that DAF-16 homologues bound more strongly to the DBE than to the IRE in the BP-1. This finding is consistent with the fact that we detected the DBE sequence exclusively by its binding site. However, it is possible that the common regions flanking the IRE or the DBE in the oligonucleotide probes confer a more rigid affinity for the DBE. Indeed, the regions flanking TRTTTAY were reported to be critical for binding between DNA and the protein in another forkhead family member, FREAC-3 [21].

The reporter gene assays confirmed that the DBE could also function *in vivo*. Since the transcriptional activity was dose-dependent on the FKHR expression vector and copy-number dependent on the DBE, it suggests that DAF-16 homologues could also bind to the DBE *in vivo* and transactivate the reporter genes through the DBE. Moreover, the finding that the transcriptional activity with p2xDBE-luc was higher than that with pBP-1-luc suggests that the DAF-16 homologues bind more strongly to the DBE than the BP-1. In addition, the reporter-gene assay showed that the DBE alone is sufficient for repression by IGF-1 or serum. Indeed, FKHRL1 is phosphorylated by Akt/PKB following stimulation of insulin or serum and translocates to the cytoplasm from the nuclei, which results in a decrease in the amount of FKHRL1 proteins in the nuclei and repression of a target gene ([17]; T. Furuyama, T. Nakazawa, I. Nakano and N. Mori, unpublished work). The translocation in the cells is critical for the determination of transcriptional activities by DAF-16 homologues. However, the DBE is only 8-bp and is similar to a consensus sequence recognized by other forkhead members, and so the specificity may be low. Therefore it is likely that the specificity is increased by interaction with particular molecules and by the different tissue distribution patterns.

In the process of searching for a candidate for the target genes of daf-16 in the genomic database of *C. elegans*, we found one copy of DBE upstream of the first exon of *sod-3*. The product of *sod-3* is a Mn-containing superoxide dismutase and is localized in the mitochondria. It is reported that the expression level of *sod-3* is up-regulated in the dauer larva and the *daf-2* mutant and it disappears in the double mutant *daf-2/daf-16* [23]. Our study supports these findings and strongly suggests that DAF-16 directly regulates the expression level of *sod-3* through the DBE.

Recent studies have revealed that mammalian DAF-16 homologues play an important role downstream of the insulin/IGF-1 signalling pathway, as in *C. elegans* [12–18]. However, there is no evidence for a direct relationship between the DAF-16 homologues and the regulation of aging or life span in mammals. To explore the issue further, we need to identify the target genes for DAF-16 homologues and their functions. The DBE may be valuable as a marker in the search for the target genes.

In summary, DAF-16 homologues can fundamentally regulate the same target genes through the DBE, and the specificity of each homologue to the target genes is partially determined by the differences in their expression patterns.

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