Overlapping but Separable Determinants of DNA Binding and Nuclear Localization Map to the C-terminal End of the
Caenorhabditis elegans DAF-12 DNA Binding Domain*

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Proteins are commonly viewed as modular assemblies of functional domains. We analyzed a loss-of-function mutation in the Caenorhabditis elegans intracellular receptor DAF-12, a conservative substitution of an arginine to a lysine at position 197 (R197K). Arg197 resides in region similar to a nuclear localization signal, just downstream of the receptor minimal zinc finger DNA binding domain (DBD) core. We found that the R197K, but not mutations of neighboring arginine or lysine residues, dramatically reduced DAF-12 transcriptional regulatory activity in a yeast reporter assay. This reduction in regulatory activity correlated with greatly decreased DNA binding affinity in vitro, suggesting a role for the DAF-12 DBD C-terminal region (dbdC), and specifically for Arg197, in DNA binding. Remarkably, three basic residues immediately contiguous with Arg197 played little role in DNA binding and rather affected nuclear localization; in contrast, Arg197 itself was dispensable for nuclear localization. Thus, DAF-12 dbdC harbors overlapping but separable determinants of DNA binding and nuclear localization in a single small region.

Intracellular receptors (IRs), such as mammalian steroid, thyroid/retinoid, and orphan members of the superfamily, typically regulate target gene expression through direct interaction of the receptors' highly conserved DNA binding domain (DBD) with the cognate DNA response elements. The IR minimal "core" DBD is a centrally or N-terminally located 66-amino acid region in which the overall fold is determined by two zinc ions coordinating eight cysteine residues to form two "zinc finger"-like structures with two perpendicular amphipathic α-helices. The first zinc finger α-helix hydrophilic surface serves as a DNA response element recognition element by interactions with the DNA major groove; other DNA binding and receptor dimerization determinants are distributed throughout the zinc finger structure (1–8). IR DNA binding sequences commonly consist of two variously spaced imperfect hexameric half-sites arranged as direct or inverted repeats or a single hexamer, depending on the specific receptor hetero-, homo-, or monomeric mode of action (3).

In addition to the core DBD, the DBD C-terminal extension (CTE), a variable region just downstream of the core, has been implicated in DNA response element recognition and discrimination. The orphan IR NGFI-B CTE is required for the receptor's DNA binding; the CTE forms an α-helix that makes extensive contacts with the DNA phosphate backbone and bases in the minor groove, allowing NGFI-B to bind its response elements as a monomer (4, 9). The crystal structure of the retinoid X receptor-thyroid hormone receptor (TR) DBD heterodimer on its DNA response element was the first to reveal the α-helical structure of TR CTE, which engages in extensive, mostly phosphate backbone contacts in the minor groove and also contains a retinoid X receptor dimerization determinant (2, 3). Similarly, the crystal structure of the orphan receptor RevErb DBD homodimer on its response element revealed the CTE function in DNA binding and subunit interaction (5). Furthermore, the CTE has been proposed to serve as a "molecular ruler," measuring the spacing between the IR subunit binding half-sites (2, 3, 5). Although structural analyses did not reveal a particular conformation or function for the CTE in DNA binding by steroid receptors, such as the glucocorticoid receptor (GR) (1, 10), recent reports implicate steroid receptor CTEs in binding site discrimination and functional interaction with HMGB1/2 architectural proteins (11–13).

For several IRs, including GR, estrogen receptor, progesterone receptor, TR, androgen receptor, NGFI-B, and others (14–19), the DBD CTE has been demonstrated to contain a nuclear localization signal (NLS) determinant. Patches of the basic residues in the CTE (lysines and arginines) are responsible for NLS activity and interaction with nuclear import receptors (18, 20).Interestingly, some of the DNA binding surface determinants in the CTE map to lysine and arginine residues (2, 4). Mutation analysis of the vitamin D receptor CTE revealed selective influence of specific lysine and arginine residues on DNA binding and transcriptional regulatory activity (21). Furthermore, acetylation of lysine residues in androgen receptor and estrogen receptor CTEs has been reported to influence the receptors' transcriptional regulatory activities (22–24). Thus, the DBD CTE appears to function in several processes affecting mammalian IR action, as measured in cell-free and cell culture assays.

Perhaps the most genetically characterized member of the Caenorhabditis elegans IR family is DAF-12, which mediates dauer formation, an alternative hibernation-like larval stage in response to unfavorable environmental conditions, regulates C. elegans developmental age and affects animal life span (25–32). Certain DAF-12 binding sites, response elements, and target genes have been recently identified (32). Multiple alleles
of daf-12 that partially uncouple its phenotypic effects as a dauer and developmental regulator were shown to have distinct protein sequence alterations (28). Loss-of-function, dauer-defective mutations in DAF-12 cluster predominantly in its core DNA binding domain. These lesions affect highly conserved core DBD residues, with the exception of R197K, an arginine to lysine substitution at position 197, 13 residues downstream of the core. We shall denote the segment encompassing the Arg197 as the DAF-12 DBD downstream region, dbdC. Interestingly, DAF-12 dbdC overlaps the position of the DBD CTE of the mammalian IRs; thus, characterization of the DAF-12 dbdC and specifically the R197K mutant might add to our understanding of CTE molecular and physiological roles in IR function. In this study, we examined in heterologous systems the molecular phenotypes of DAF-12 R197K as well as mutations in other nearby residues in DAF-12 dbdC.

MATERIALS AND METHODS

Yeast Transcriptional Reporter Assay—C. elegans DAF-12 response elements (5) containing the 4.2 and 4.3 genomic fragments (32) were subcloned upstream of a minimal CYC1 promoter driving LacZ expression in pdsS (2, URA-marked) vector (33) (plasmids pYSYR0001–3). Expression of C-terminally FLAG- or GFP-tagged (with or without SV40 NLS) wild type DAF-12 N500 (aa 1–500) and mutants (in order of appearance in Figs. 1, 2, and 4) was driven by the copper-inducible CUP1 promoter in pRS424 (2, TRP) (34) vector in the presence of 0.05 mM CuSO4 (plasmids pYSYR0002 and pYSYR0011, respectively). The plasmids were transformed (35) into Saccharomyces cerevisiae W303a strain. Expression assays and data analysis were performed as described (36), except after saturation, the cultures were diluted 1:20 and grown for 8 h with 0.05 mM CuSO4; lysis was performed for 15 min.

Immunoblotting—Yeast was boiled for 15 min in 2× SDS sample buffer, and the mixture was fractionated by SDS-PAGE (amounts loaded normalized to culture cell densities), transferred to Immobilon membrane (Millipore Corp.), and probed with a 1:500 dilution of anti-FLAG M2 antibody (Sigma). The primary antibody was detected with horseradish peroxidase-conjugated sheep anti-mouse antibody by developing with ECL substrate (Amersham Biosciences).

Protein Expression and Purification—For fluorescence anisotropy experiments, wild type DAF-12 DBD (aa 100–206) and mutants were expressed as GST fusions inserted into pET11b (Novagen). plasmids pYSYR0003–6 (in order of appearance in Fig. 3), in BL21-CodonPlus-RIL(DE3) cells. Expression was induced (1 liter of culture, 150 mM NaCl, 50 mM Tris, pH 7.4, 0.5 mM phenylmethylsulfonyl fluoride) and concentrated on Centricon-10 columns (Amicon). GST-DAF-12 DBD was then eluted with 10 mM reduced glutathione in TBS (150 mM NaCl, 50 mM Tris, pH 7.4, 100 mM KCl, 5 mM phenylmethylsulfonyl fluoride) and dialyzed against TBS buffer containing 1 mM isopropyl-1-thio-D-galactopyranoside at 30 °C for 2.5 h in the presence of 10 μM ZnCl2. After lysis by sonication in 20 mM Tris, pH 8.0, 2 mM EDTA, 1 mM NaCl, and 0.5 mM phenylmethylsulfonyl fluoride, the fusion proteins were purified on GST-bind resin (Novagen). After binding, the resin was washed with TBS buffer containing 1 mM NaCl (1 mM NaCl, 50 mM Tris, pH 7.4, 0.5 mM phenylmethylsulfonyl fluoride) followed by two washes with TBS containing 150 mM NaCl (150 mM NaCl, 50 mM Tris, pH 7.4, 0.5 mM phenylmethylsulfonyl fluoride). GST-DAF-12 DBD was then eluted with 10 mM reduced glutathione in TBS (150 mM NaCl, 50 mM Tris, pH 7.4, 0.5 mM phenylmethylsulfonyl fluoride) and concentrated on Centricon-10 columns (Amicon).

Fluorescence Anisotropy—Fluorescein-labeled double-stranded DR5tt oligonucleotide (10 nm) in 2 ml of 20 mM Tris, pH 7.4, 100 mM KCl, 5 mM MgCl2, were used for fluorescence anisotropy measurements with wild type GST-DAF-12 DBD and mutants. Excitation was performed at 485-nm wavelength, and emission was monitored at 515 nm using a Photon Technologies International fluorometer. Anisotropy calculations and Kr determinations by curve fitting were performed as described previously (37, 38) using KaleidaGraph 3.51 (Synergy Software).

Subcellular Localization Determination in Yeast and CV-1 Cells—Subcellular localization of DAF-12 derivatives was determined by live GFP fluorescence visualization at either 25°C/11003 or 37°C/H9262 for mammalian CV-1 cells. For yeast experiments, DAF-12 derivatives were subcloned into a C. elegans vector generously provided by Joachim Li, University of California, San Francisco), plasmids pYSYR0021–212; the vector containing 3×GFP with SV40 NLS was also provided by J. Li.

Yeast W303a strain was transformed with plasmids as described above and grown in the presence of galactose similarly as for the yeast transcriptional reporter assay.

For mammalian subcellular localization studies, DAF-12 derivatives fused to 3×GFP were subcloned into pEGFP-C3 vector (Clontech), producing fusions with one N-terminal and three C-terminal GFPs (4×GFP), plasmids pYSYR0001–0019 (in order of appearance in Figs. 5 and 6). The plasmids (0.5 μg) were transiently transfected into 80–90% confluent CV-1 cells and grown in 24-well plates 20 h after seeding in serum-free medium with Lipofectamine-PLUS reagent (Invitrogen) using 2 μl/well Lipofectamine and 4 μl/well PLUS as directed by the manufacturer. Three hours after transfection, cells were incubated with phenol red-free medium with 5% fetal bovine serum. GFP fluorescence was visualized 18–20 h post-transfection.

RESULTS

DAF-12 DBD Mutants Fail to Activate Transcription—Genetic screens in C. elegans identified multiple DAF-12 alleles with distinct protein sequence alterations (27, 28, 31). Five of these alleles, each with a point mutation in or near the DAF-12 DBD, were transiently transfected into 80–90% confluent CV-1 cells and grown in 24-well plates 20 h after seeding in serum-free medium with Lipofectamine-PLUS reagent (Invitrogen) using 2 μl/well Lipofectamine and 4 μl/well PLUS as directed by the manufacturer. Three hours after transfection, cells were incubated with phenol red-free medium with 5% fetal bovine serum. GFP fluorescence was visualized 18–20 h post-transfection.
transcriptional regulatory activity.

Arg<sup>197</sup> Is Selectively Required for Transcriptional Activation—Four of the DAF-12 DBD mutations, C121Y, A125V, S137F, and R143K, affect highly conserved residues in the DBD core that function in zinc finger formation, DNA binding, or both (3). A fifth mutation, R197K, lies 13 residues downstream of the core DBD in a NLS-like region containing two arginines and a lysine, Arg<sup>195</sup>, Arg<sup>196</sup>, and Lys<sup>198</sup>. This conservative substitution creates a DAF-12 that is phenotypically defective in <i>C. elegans</i> and lacks transcriptional regulatory activity in yeast. In sharp contrast, we found that neighboring conservative substitutions, R195K, R196K, or K198R, retained the capacity to activate transcription at high levels in yeast from the 4.2 response element (Fig. 2K198R, or the R195K/R196K double mutant and the R195A/R196A/K198A triple mutant also had no major effect on transcriptional activity, thus resulting in a transcriptional activation-defective function phenotype of R197K in <i>C. elegans</i> appears to be explained by its inability to bind to DNA.

Arg<sup>197</sup> Has No Effect on DAF-12 Nuclear Localization—In many IRs, the DBD CTE contains an NLS activity (14–19). To examine a possible role of the R197K mutation in nuclear localization, we tested whether fusion of an exogenous NLS to the mutant protein could rescue its defect in transcriptional regulation. We found that fusion of the SV40 NLS to DAF-12 N500 R197K was not able to rescue the transcriptional activation defect from the 4.2 response element (Fig. 4A). In corroboration with the transcriptional reporter data, DAF-12 N500 R197K appeared to localize to the nucleus in yeast, similar to the wild type protein (Fig. 4B); fusion of the SV40 NLS either to wild type or to DAF-12 N500 R197K resulted in increased nuclear accumulation. These results indicate that the R197K mutation does not affect the nuclear localization properties of DAF-12, consistent with the finding that arginines and lysines typically function interchangeably within an NLS. We conclude that the R197K substitution leads to a defect in the DNA binding ability of DAF-12 and not in its subcellular localization, thus resulting in a transcriptional activation-defective protein.

DAF-12 dDBD Contains a Determinant That Contributes to Nuclear Localization and Is Separable from Its DNA Binding Activity—We wished to investigate whether the dDBD region contains DAF-12 nuclear localization determinants in addition to its DNA binding activity. For these studies, we tested two exogenous but experimentally accessible systems, yeast and mammalian cells. In the yeast experiments, we constructed plasmids containing DAF-12 derivatives fused to three copies of GFP in a yeast expression vector. Fig. 5A shows that a DAF-12 N500 equivalent, containing amino acids 2–500 fused to three copies of GFP, denoted D12.2–500.GF3, was localized to the nucleus in yeast. DAF-12 N500 derivative that lacks the N terminus, D12.101–500.GF3, as well as a derivative that lacks the “hinge” region, D12.2–206.GF3, were also localized to the nucleus; likewise, a DAF-12 derivative lacking both the N terminus and the “hinge” regions, D12.101–206.GF3, was localized to the nucleus in yeast. Interestingly, the DAF-12 N terminus, D12.2–100.GF3, and the “hinge” D12.207–500.GF3, displayed a slight nuclear accumulation preference. However, DAF-12 derivatives D12.2–191.GF3 and D12.101–191.GF3, which lack a portion of the dDBD, lost their preferential nuclear localization (Fig. 5A), suggesting that the 191–206 region of the DAF-12 dDBD is required for preferential nuclear localization in yeast. Similar to the results in Fig. 4B, the R197K or R196K function of Arg197 is required for high affinity binding of the DAF-12 DBD to its DR5 response element. Binding of the GST-DAF-12 DBD to fluorescein-labeled double-stranded oligonucleotides carrying the DR5tt element (10 nM) was measured by fluorescence anisotropy. Protein concentrations shown assume GST-DAF-12 DBD homodimers. Apparent K<sub>d</sub> values were calculated from curve fits. The graph inset provides an expanded view at protein concentrations up to 60 nM. wt, wild type.
mutations did not affect nuclear localization of D12.2–206.GF3 or D12.101–206.GF3 (Fig. 5A). Surprisingly, the aa 191–206 fragment by itself, fused to three copies of GFP, D12.191–206.GF3, was insufficient for nuclear accumulation; the fusion protein was distributed throughout the cells. A similar distribution pattern was observed with a control protein in yeast, a fusion protein consisting of three copies of GFP, GF3.

We extended our examination to mammalian CV-1 cells, a cell line with several advantageous characteristics for these types of studies (16), including large cell and nucleus size, flat morphology, and high transfection efficiency. In CV-1 cells, transiently transfected full-length DAF-12 and DAF-12 N500 equivalents fused to four copies of GFP, D12.2–753.GF4 and D12.2–500.GF4, respectively, showed strong nuclear localization (Fig. 5B). As in yeast, D12.2–206.GF4, with or without the R197K or R196K mutations, localized primarily to the nucleus in CV-1 cells; deletion of amino acids 197–206, resulting in the D12.2–191.GF4 construct, led to predominantly diffuse subcellular localization, suggesting that in mammalian cells this portion of the DAF-12 dbdC is also required for preferential nuclear localization. In these cells, the control protein containing four copies of GFP, GF4, was excluded from the nuclei of many cells (Fig. 5B). These experiments suggest that in both yeast and mammalian cells, the DAF-12 dbdC participates in nuclear localization but that Arg197 is dispensable for this activity.

In CV-1 cells, as in yeast, D12.191–206.GF4 or the complete dbdC, D12.192–206.GF4, was insufficient for nuclear localization; the fusions were excluded from the nucleus or in some cases were diffused throughout the cells (Fig. 6). However, we could show that the dbdC region is rate-limiting for nuclear localization; the addition of amino acids 191–206 to the fusion protein containing amino acids 2–206, D12.2–206 + 191–206.GF4, led to increased nuclear accumulation (Fig. 6). Further supporting the role of dbdC in nuclear localization, a dbdC-deleted DAF-12 DBD construct, D12.101–191.GF4, was now predominantly excluded from the nucleus. Finally, we noticed that in CV-1 cells, the DAF-12 N terminus, D12.2–100.GF4, displayed weak nuclear accumulation, whereas the fragment lacking the N terminus, D12.101–206.GF4, was predominantly diffused throughout the cell; these observations imply that the DAF-12 N terminus carries a weak NLS. Indeed, we found that the addition of an N-terminal stretch that includes amino acids 21–41, D12.101–206 + 21–41.GF4, displayed greatly increased nuclear localization (Fig. 6).

Further supporting the NLS activity of the aa 21–41 fragment, its fusion to cytoplasmically localized constructs D12.191–206.GF4 or D12.182–206.GF4, producing D12.191–206 + 21–41.GF4 or D12.182–206 + 21–41.GF4, respectively, resulted in nuclear accumulations of these proteins. In contrast, when we instead added aa 58–73, a region evolutionarily conserved in the Strongyloides stercoralis DAF-12 orthologue (28, 39), to D12.191–206.GF4 or D12.182–206.GF4, generating D12.191–206 + 58–73.GF4 and D12.182–206 + 58–73.GF4, respectively, the derivatives were excluded from the nucleus. Thus, the aa 21–41 segment appears to specifically confer the N-terminal NLS activity of DAF-12. Notably, however, nuclear localization was impaired when mutations in basic residues dispensable for DNA binding and transcriptional activation were introduced in these constructs (compare D12.191–206 + 21–41.GF4 and D12.182–206 + 21–41.GF4 with the respective constructs with R195A/R196A and K198A mutations (Fig. 6)), underscoring the critical role of the dbdC lysine and arginine residues 195, 196, and 198 in the dbdC nuclear localization function.

**DISCUSSION**

We have identified physically overlapping but functionally separable surfaces that serve as determinants of DAF-12 DNA binding and subcellular localization. We found that DAF-12 employs the dbdC region for at least two activities. First, arginine 197, the site of a loss-of-function mutation, serves as a key residue for high affinity DNA binding; second, the NLS1 element is involved in the receptor's nuclear localization (Fig. 7), but Arg197 is dispensable for the localization function. The sequence-specific DNA binding of IRs is conferred by a highly conserved C2–C3 zinc finger DNA binding domain. Here we show that DAF-12 loss-of-function mutants with alterations in conserved residues of the DNA binding domain are unable to activate transcription in a yeast reporter assay, most likely due to defects in DNA binding. We demonstrate that a conservative substitution mutation, arginine to lysine, at the 197-position, just 13 residues downstream of a core zinc finger DBD fold, leads to a dramatic loss in the ability of DAF-12 to activate transcription due to a great decrease in the protein's binding affinity for its specific DNA response element. Interestingly, mutations of the nearby residues, such as at the positions 195, 196, and 198, do not result in decreased transcriptional output or significant changes in DNA binding affinities of DAF-12. This suggests that arginine 197 is required for the high affinity DNA binding of DAF-12 and is probably a part of a DBD CTE.

Since mutations of the residues adjacent to arginine 197 do not exhibit transcriptional phenotypes, there must be other determinants, either in the dbdC or neighboring regions that lead to a precise positioning of this residue. One possibility is that, with TR and NGFI-B DBD CTE, DAF-12 dbdC forms a response element-induced α-helix, with the arginine 197 residue making a critical DNA backbone or base-specific contacts. Interestingly, the crystal structure of TR DBD (2) reveals that the residue at the same position as DAF-12 Arg197 makes DNA phosphate backbone as well as base-specific contacts; however, this residue is a lysine in TR. Although the precise cause of the DAF-12 R197K DNA binding defect is unknown, the combination of genetic data ascribing a dauer-defective phenotype to the R197K mutant DAF-12 (28) and our molecular characterization of the DAF-12 R197K mutation provide compelling evidence for a physiological role for the DAF-12 dbdC, a potential DBD CTE, as a required DNA binding interface.

DBD CTEs or CTE-like regions of several IRs have been shown to govern, independently of or in addition to DNA binding, nuclear localization of these proteins. For example, one of the GR NLSs, NL1, in the CTE, mediates the nuclear import of...
GR and does not appear to contribute significantly to its intrinsic DNA binding affinity (16, 40). In the case of the mammalian orphan IR NGFI-B, the CTE is required for DNA binding and is involved in nuclear localization (4, 18). Similar to its function in NGFI-B, our findings indicate that DAF-12 employs its CTE-like dbdC for high affinity DNA binding and nuclear localization. Interestingly, although these functions occupy a common region, we were able to uncouple a DNA binding determinant at Arg197 from the region’s nuclear localization function; whether these functions can be dissected in the case of NGFI-B has not been determined. In contrast to the NL1 NLS of GR, which can mediate nuclear import on its own, the DAF-12 dbdC participates in nuclear import, but the dbdC is not itself sufficient; dbdC NLS1, under the conditions of our assays in metazoan cells, functions only in conjunction with the N-terminal NLS1 (see Fig. 7). Since one of the goals of this study was to investigate the role of dbdC and its basic residues in nuclear localization, we have not addressed here the nuclear localization contributions of specific residues in the N-terminal NLS1. Similarly, we have not examined the subcellular localization contributions of dbdC NLS1, N-terminal NLS1, and other potential DAF-12 localization determinants in C. elegans. Cooperation between NLSs, also observed in other proteins such as estrogen receptor and progesterone receptor (14), might differentially regulate DAF-12 functions in certain contexts, resulting in distinct physiological outputs. Combinatorial utilization of NLSs in transcription factors might, furthermore, serve as “address codes” for subnuclear compartmentalization and gene-specific targeting and regulation; indeed, roles for nuclear localization machinery components in these processes have been suggested (41, 42).

Overlapping but separable determinants of distinct functions in a single small region are not unique to DAF-12, IRs, or transcription factors; an elegant study of CMP-Neu5Ac synthe-

**Fig. 5.** The DAF-12 dbdC participates in nuclear localization in yeast and mammalian cells, but Arg^{197} is dispensable for the localization function. **A**, subcellular localization of DAF-12 derivatives in yeast. Wild type or R197K-mutated DAF-12 derivatives were fused to three copies of GFP. The GF3 panel demonstrates subcellular localization of an empty vector containing three copies of GFP. The GF3-NLS panel shows nuclear localization of the described previously empty vector bearing SV40 NLS and served as a positive control. **B**, subcellular localization of DAF-12 derivatives in CV-1 cells. DAF-12 derivatives were fused to four copies of GFP and transiently transfected in CV-1 cells; the GF4 and GF4-NLS panels demonstrate cytoplasmic localization of an empty vector with four copies of GFP and nuclear localization of the vector bearing SV40 NLS, respectively. **C**, schematics of constructs in **A** and **B** as well as in Fig. 6 with the included DAF-12 domains and subregions, as indicated.
tase, an enzyme involved in synthesis of cell surface glycoconjugates, revealed overlapping but separable determinants for catalytic activity and nuclear localization resident in arginine and lysine residues within a small region (43). Although the rationale for this functional overlap is unclear, the co-evolution of overlapping nuclear localization and DNA binding determinants in the case of transcription factors is easier to envision. First, the site of transcription factor function is within the nucleus, on the DNA, and the determinants are used sequentially rather than simultaneously. Indeed, ~90% of transcription factors have proximal DNA binding and nuclear localization determinants (44). Second, similarity in charge polarities (DNA interacting with positively charged DBDs and import receptors binding positively charged NLSs) could contribute to DBD/NLS co-evolution. In DAF-12, a regulatory factor that functions in multiple physiological networks (25–32), it will be interesting to determine whether the selective use or interactions of the functions within dbdC contribute to the capacity of DAF-12 to differentially regulate its target genes.

DNA binding domains of IRs share strong sequence conservation across their core zinc finger folds; however, their DBD CTEs differ dramatically in sequence. In this context, it is particularly intriguing that the DAF-12 dbdC is 100% identical with the corresponding region in the *S. stercoralis* DAF-12 orthologue, compared with 35% identity over the entire protein and 95% identity in the core DBD. This striking evolutionary
conservation suggests that DAF-12 dbdCs are probably involved in multiple essential functions, including but perhaps not limited to the DNA binding and nuclear localization activities that we have determined here. It will be interesting to better understand the structure and evolution of this region, which may commonly acquire receptor-specific activities, thus resulting in stronger conservation across species for a given receptor than between receptors in a given species.

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