The DNA Binding Domain of Hepatocyte Nuclear Factor 4 Mediates Cooperative, Specific Binding to DNA and Heterodimerization with the Retinoid X Receptor α*

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Guoqiang Jiang and Frances M. Sladek‡
From the Environmental Toxicology Graduate Program, University of California, Riverside, California 92521

We recently showed that hepatocyte nuclear factor 4 (HNF-4) defines a unique subclass of nuclear receptors that exist in solution and bind DNA elements as homodimers (Jiang, G., Nepomuceno, L., Hopkins, K., and Sladek, F. M. (1995) Mol. Cell. Biol. 15, 5131–5143). In this study, we show that the dimerization domains of HNF-4 map to both the DNA binding and the ligand binding domain. Whereas the latter is critical for dimerization in solution, the DNA binding domain mediates cooperative, specific binding to direct repeats of AGGTCA separated by one or two nucleotides. Whereas amino acid residues 117–125 (the T-box/third helix region) are insufficient for cooperative homodimerization and high affinity DNA binding, residues 126–142 (encompassing the A-box region) are required. Finally, in contrast to the full-length receptor, the DNA binding domain of HNF-4 is capable of heterodimerizing with that of the retinoid X receptor α but not with that of other receptors. These results indicate that the HNF-4 DNA binding domain is distinct from that of other receptors and that the determinants that prevent HNF-4 from heterodimerizing with RXR lie outside the DNA binding domain, presumably in the ligand binding domain.

Dimerization plays an important role in the function of many different types of proteins. From extracellular receptors to nuclear proteins, the ability to dimerize adds diversity as well as specificity to a variety of biological pathways. This is particularly true for the superfamily of ligand-dependent transcription factors. This family of soluble, nuclear receptors contains over 150 members, including the steroid, thyroid hormone, vitamin A, and vitamin D receptors, and an even larger number of orphan receptors for which ligands have not yet been identified (23).

The nuclear receptor superfamily is characterized by two highly conserved domains. The DNA binding domain (DBD), in the N-terminal half of the protein, consists of approximately 60–90 amino acids that form two zinc finger modules followed by a C-terminal extension. The so-called ligand binding domain (LBD) in the C-terminal half of the protein consists of approximately 200 amino acids. This region performs a variety of functions including transactivation, ligand binding, and protein dimerization via heptad repeats of hydrophobic residues (7, 8, 23).

With a few exceptions, the nuclear receptors all have the capacity to bind DNA as dimers. Several, particularly those related to the retinoid receptors, also bind DNA preferentially as heterodimers. Retinoid X receptor (RXR) is the most promiscuous of the receptors, dimerizing with at least 10 different receptors on a variety of DNA elements (23). Full-length RXR, in fact, binds DNA only poorly as a homodimer and not at all as a monomer (42).

Dimerization domains in the receptors have been localized to both the DBD and the LBD using mutagenesis studies (5, 7, 8, 23). Dimerization interfaces have also been visualized in three-dimensional structures of homo- and heterodimeric complexes of DBDs of various receptors bound to DNA: a homodimer of the estrogen receptor (30), a homodimer of the glucocorticoid receptor (21), and a heterodimer of RXRα and the thyroid hormone receptor β (TRβ) (28). The structure of a homodimer of the LBD of RXRα, in the absence of ligand, has also been solved and shown to contain a dimerization interface (3). Nevertheless, the precise role of the different dimerization motifs in DNA binding and other receptor functions has not been clearly defined.

Hepatocyte nuclear factor 4 (HNF-4) is a highly conserved receptor essential for development in organisms ranging from insect to man (4, 34, 44). Found predominantly in the liver, kidney, and intestine, HNF-4 transcriptionally activates a wide variety of genes including those involved in fatty acid and cholesterol metabolism, glucose metabolism, urea biosynthesis, blood coagulation, hepatitis B infections, and liver differentiation (reviewed in Refs. 32, 33). A ligand, however, has not yet been identified for HNF-4. (The term ligand binding domain (LBD), however, will be used for the sake of consistency with other receptors.)

We recently showed that HNF-4, while very similar in DNA binding specificity and amino acid sequence to RXRα, does not heterodimerize with full-length RXR or any other receptor analyzed. In fact, full-length HNF-4 bound DNA only as a homodimer and sedimented as a homodimer during glycerol gradient centrifugation. The strong homodimerization activity, as well as the exclusively nuclear localization of HNF-4, led us to conclude that HNF-4 defines a new subclass of receptors (12).

In this study, we analyze in greater detail the mechanism of dimerization of HNF-4 with the goal of deciphering the role and the determinants of homo- versus heterodimerization among the nuclear receptors. The dimerization domains of HNF-4 are localized to both the DBD and the LBD, and the DBD is shown...
to mediate cooperative, high affinity, specific binding to DNA. The LBD, in contrast, appears to be important for dimerization in solution. It is also shown that, in contrast, to the full-length receptor, the HNF-4 DBD is capable of heterodimerizing with the DBD of RXRa, although not with the DBDs of the retinoid acid receptor a (RARa) or thyroid hormone receptor b (TRb).

These results confirm the unique nature of HNF-4 and indicate the LBD as the major determinant of homo- versus heterodimerization of HNF-4.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—Expression vector pMT7 was constructed by inserting double-stranded oligonucleotide MT7 (top strand: 5'-GTAATACGACAACTATAGGGCCCCTCGAGGCG-3'; bottom strand: 5'-AATTCGCCCTCAGGGGGCCCTATAGGTCTCTGATATCTGAC-3') containing the T7 promoter and unique XhoI sites (underlined) into the parental vector pMT2 (13) and verified by dideoxy sequencing. pMT7 was used for in vitro expression of proteins in COS-7 cells and in vitro expression of proteins using reticulocyte lysate.

cDNAs encoding full-length and truncated rat HNF-4 proteins were prepared by the polymerase chain reaction (PCR) using as template the original HNF-4 clone pF7 (34), appropriate kinased oligonucleotides as primers, and Vent DNA Polymerase (New England Biolabs). The PCR products were gel-purified and subcloned into the pMT7 vector cut with EcoRI, filled-in with Klenow, and dephosphorylated. The sequence of the inserts were verified by dideoxy sequencing. The N-terminal primers, N245, N143, N145, N147, N149, N151, N153, N155, N157, N159, N161 contain start codons, and the C-terminal primers, C455, C374, C142, C125, C125N123E, contain stop codons. The relative position of the primers are diagrammed in Fig. 1 and defined by the N- or C-terminal-most residue of the region to which they anneal. N1 and C455 have been previously defined as Npf7 and Cpf7, respectively (12).

The sequence of the other primers are as follows: N45, 5'-GCCGACA-TTGATCTTGACTTGCTGATGCCC-3'; C374, 5'-GCAGGCCTCTACGGC-3'; C142, 5'-GGGAAGCTTCTACCAGGTATG-GCTGCTG-3'; C125, 5'-CGAAGCCTTACCCGCTTATTTTTGAGCCG-3'; C125N123E, 5'-TGGATACCCGCTTATTTTGAGCCG-3'. Restriction sites used for cloning purpose are underlined.

Protein Expression, Purification, and Electrophoretic Mobility Shift Analysis (EMSA)—Wild-type and truncated HNF-4 proteins were over-expressed in COS-7 cells, and nuclear extracts were prepared as described previously (12). RAR.BD, RAR.BD, and TR.BD were over-expressed in Escherichia coli as glutathione S-transferase fusion proteins using vectors generously provided by T. Perlmann (Karolinska Institute, Stockholm, Sweden). The fusion proteins were purified by glutathione-Sepharose columns (Sigma), and the glutathione S-transferase moiety was removed by cleavage with thrombin as described previously (26). Electrophoretic mobility shift analysis (EMSA) was performed as described previously (12, 26) and as indicated in the figure legends. The double-stranded oligonucleotides used for probes in EMSA are shown in Table I.

RESULTS

HNF-4 Contains Dimerization Domains in Both the DBD and the LBD—In order to locate the structural domain(s) responsible for the strong dimerization activity of HNF-4, several constructs were made and are diagrammed in Fig. 1. The constructs were overexpressed in COS cells and examined for DNA binding activity by electrophoretic mobility shift analysis (EMSA) using a variety of probes (see Table I). Since dimerization motifs had been previously mapped to both the DNA binding domain (DBD) and the ligand binding domain (LBD) of other receptors, those two domains were compared in HNF-4.

The results, depicted in Fig. 2, indicate that, as seen previously, the full-length HNF-4 (HNF4.wt) yielded only a dimeric complex with the APF1 probe (Fig. 2, lanes 1–9). In contrast, HNF4.142, which contains the DBD but lacks the LBD, yielded both monomeric and dimeric binding species (lanes 4–5) (see Fig. 3 for verification of the dimer). HNF4.374, on the other hand, which contains both the DBD and the LBD but not the very N or C terminus, bound DNA only as a dimer (lanes 6–7). Furthermore, HNF4.374 dimerized with HNF4.wt, as evidenced by a shift in the intermediate mobility (lanes 9–11), whereas HNF4.142 did not dimerize with either HNF4.wt (lane 8) or HNF4.374 (lane 12). These results indicate that HNF-4 contains at least two dimerization domains, one located between amino acids 45 and 142, corresponding to the DBD, and an additional domain located between residues 143 and 374 (i.e. the LBD), which apparently precludes monomeric binding.

The HNF-4 DBD Is Responsible for DNA Binding Specificity Whereas the LBD Is Responsible for Dimerization in Solution—Our previous results from glycerol gradient sedimentation and order of dilution experiments indicated that the full-length HNF-4 is a homodimer even in the absence of DNA, i.e. in solution (12). In order to compare the dimerization activity of the HNF-4 DBD with that of the full-length protein, the following two experiments were performed. In the first, HNF4.wt and HNF4.142 were compared for cooperative binding to DNA. A saturation curve was calculated by performing EMSA with increasing amounts of COS cell nuclear extract containing ei-
ther HNF4.wt or HNF4.142 and a constant amount of $^{32}\text{P}$-APF1 as probe. The results, shown in Fig. 3, demonstrate that the saturation curve of the HNF4.142 dimer is sigmoidal, whereas that of the HNF4.wt dimer is hyperbolic. The sigmoidal shape indicates that the two subunits of the HNF4.142 dimer bind DNA in a cooperative, and thus two-step, fashion. This verifies the shift complex as a protein dimer bound to the probe, as opposed to two monomers that happen to bind to the same DNA molecule. It also indicates that HNF4.142 exists in solution as a monomer. The hyperbolic shape of the HNF4.wt curve, on the other hand, indicates that the two subunits of the dimer bind DNA in a noncooperative, and thus single-step, fashion, verifying that HNF4.wt exists in solution as a homodimer.

The second experiment to test for dimerization activity in solution also examined DNA binding specificity. The binding activity of HNF4.wt and HNF4.142 on DNA containing either a single half-site or direct repeats separated by an increasing number of nucleotides (DR0–5) was determined. The rationale was that a protein that exists as a dimer in solution might yield a DNA complex migrating as a dimer on a single half-site, whereas a protein that exists in solution only as a monomer would not.

The results, shown in Fig. 4, panel A, indicate that HNF4.wt binds DR1 (lanes 5–6), as expected, as well as DR2 (lanes 7–8). There was also a low level of binding activity on the probe containing a single half-site (lanes 1, 2, 1a, and 2a) but none on any of the other probes, DR3, DR4, or DR5 (lanes 9–14). The low binding activity on the half-site is presumably due to reduced protein-DNA contact and suggests that contacts to both half-sites are important for binding the consensus element (DR1). No monomeric binding of HNF4.wt to the half-site was detected even on very long exposures (not shown). In contrast to HNF4.wt, HNF4.142 bound a single half-site only as a monomer (panel B, lanes 1 and 2). These results, along with those of Fig. 3, confirm that HNF4.wt exists as a homodimer and HNF4.142 as a monomer in solution.

The results of Fig. 4 also show that HNF4.142 binds DNA with a specificity similar to that of HNF4.wt. Both HNF4.wt and the HNF4.142 dimer bind significantly only to direct repeats separated by one or two nucleotides (DR1 and DR2, respectively) with an apparent preference for DR1 (lanes 5–8 in Panels A and B). Similar results were obtained from a competition experiment in which HNF4.142 and HNF4.wt were subjected to EMSA using as probe $^{32}\text{P}$-APF1 and 40-fold molar excess of several different unlabeled oligonucleotides containing HNF-4 binding sites. The results indicated that the oligonucleotides competed the HNF4-APF1 complex in a similar fashion for both the HNF4.DBD dimer and HNF4.wt dimer. The only difference is that the extent of competition appeared to be somewhat less for the HNF4.142 dimer than for the HNF4.wt dimer (data not shown). Since the construct containing just the DBD and the LBD, HNF4.374, showed an identical binding specificity to that of HNF4.wt, these results, together with those of Figs. 2–4, indicate that the DBD of HNF-4 is primarily responsible for the DNA binding specificity and partially responsible for dimerization activity on DNA. The LBD, on the other hand, is apparently responsible for the strong

![Fig. 2. HNF-4 contains dimerization domains in both the DBD and the LBD.](image)

EMSA of COS cell nuclear extracts containing overexpressed HNF4.wt, HNF4.374, or HNF4.142 (0.5–1.0 μg of total protein each) and $^{32}\text{P}$-APF1 probe (1 ng per 15-μl reaction) was done at RT as described previously (12). Shown is a PhosphorImage (Molecular Dynamics) of the shift gel. α-445, antisera raised against the very C terminus of HNF-4 (34); Competition, 50 ng of unlabeled APF1, immune complex, supershift containing probe, receptor, and antisera. Dimers and monomers are identified by the proteins that comprised them: wt, HNF4.wt; 374, HNF4.374; 142, HNF4.142; Free probe, $^{32}\text{P}$-APF1 not bound by protein. For reactions containing two different HNF-4 proteins (lanes 8–12), extracts from single transfections were mixed and incubated at RT for 15 min prior to the addition of shift mix containing the probe. An endogenous complex from COS cells co-migrates with the HNF4.wt dimer in this gel, as seen in lanes 2–7, 10, and 12.

![Fig. 3. HNF4.142 binds DNA in a cooperative, two-step fashion while HNF4.wt binds in a noncooperative, one-step fashion.](image)

EMSA was performed as described in Fig. 2 using increasing amounts of COS nuclear extract containing overexpressed HNF4.wt (375 ng to 8.6 μg of total protein) or HNF4.142 (250 ng to 15 μg of total protein) and a constant amount of $^{32}\text{P}$-labeled APF1 oligonucleotide (0.5 ng) as probe. Total protein was kept constant at 15 μg per reaction by the addition of bovine serum albumin. Reactions were incubated at RT for 30 min to reach equilibrium and then subjected to electrophoresis. Shown are both the autoradiograms of the shift gels (A and B) and a graph of the corresponding densitometric readings (C) of the HNF4.wt dimer-DNA complex and HNF4.142 dimer-DNA complex. Each point in the graph represents the average of two separate experiments. The plots were fitted with either a hyperbolic (HNF4.wt) or sigmoidal curve (HNF4.142) using MicroCal Origin (MicroCal Software, Inc., MA).
homodimerization activity seen in the absence of DNA.

Amino Acids 126 to 142 (encompassing the A-box Region) Is Required for Cooperative Homodimerization and Specific and High Affinity Binding to DNA—In addition to the highly conserved zinc finger motifs, the DBD of many of the receptors also contains an A-box and a T-box (see Fig. 1). The A-box, which contacts the nucleotide bases flanking the core recognition sequence, is important for the high affinity binding of NGFI-B monomers and thyroid hormone receptor (TR) monomers and homodimers but not that of receptors such as RXR and retinoic acid receptor (RAR) (16, 28, 38, 40, 41). For these receptors, the T-box appears to be much more important for dimerization and high affinity binding (18, 28, 40, 41). The N-terminal portion of the T-box in RXR in particular, which forms an α-helix (the so-called third helix), is required for homodimerization of the RXRs DBD on DR1 (18). We therefore wished to determine which was more important for HNF-4 DBD binding, the A-box or the T-box/third helix.

Amino acid sequence alignment, shown in Fig. 5A, indicates that the A-box of HNF-4, like that of the other receptors, is fairly distinct compared with the A-box of other receptors (e.g. RXR versus HNF-4) but relatively conserved among the different species of HNF-4 (i.e. rat versus Drosophila HNF-4). The T-box of HNF-4, on the other hand, is very similar to that of several other receptors, particularly in the third helix region. In fact, HNF-4 is closest to retinoid X receptor (RXR) (16, 28, 38, 40, 41). For these receptors, the A-box versus other members of the nuclear receptor superfamily. A, the T- and A-box regions as previously defined (38) are shown for HNF-4 and a variety of other receptors. The amino acid sequences (given in a single-letter code) start from the last conserved cysteine in the zinc finger region and end one amino acid after the A-box. Based residues correspond to the region in RXRα that forms an α-helical structure in solution (the 3rd helix) (18). rHNF-4 (34); hHNF-4 (44); hRXRα (22); mRXRβ (19); hRXRγ (19); hRARα (27); hRARβ (6); mRARγ (9); rRAR (36); hTRα (37); rNGFI-B (24); r, rat; d, Drosophila; h, human, m, mouse. B, schematic representation of the 3rd helix region (boxed) of hRXRα and the corresponding region in rHNF-4. Charge and polarity are indicated: +, positively charged; −, negatively charged; p, polar but uncharged; o, nonpolar and hydrophobic. Also indicated is the predicted secondary structure as determined by the Chou-Fasman method using the PEPTIDESTRUCTURE program (Genetic Computing Group, GCG): h, α-helical structure; −, no apparent structure.

The results of EMSA using HNF4.125 and HNF4.142 are shown in Fig. 6A. Under the conditions used, HNF4.125 binds AF1 as a monomer but not as a homodimer (lanes 1 and 2). In contrast, when HNF4.125 is mixed with extracts containing HNF4.142, a new shift complex appears which is dependent on the presence of both receptors. The identity of this complex was confirmed using specific antibodies against HNF-4.
upon the presence of both HNF4.125 and HNF4.142 (lanes 5–14). This indicates that a HNF4.125-HNF4.142 heterodimer is formed and suggests that residues 126 to 142, encompassing the A-box region, contain a dimerization motif that interacts with residues 1–125. Furthermore, the HNF4.142-HNF4.125 heterodimer appears to bind in a cooperative fashion, just as the HNF4.142 homodimer, since increasing amounts of either HNF4.142 (lanes 5–9) or HNF4.125 extract (lanes 10–14) lead to an increasing amount of heterodimer formation.

To determine whether residues 126–146 are also important in the specificity of binding of HNF-4 to DNA, a competition experiment was performed. The results, shown in Fig. 6B, indicate that the HNF4.142 homodimer and the HNF4.142-HNF4.125 heterodimer can both be efficiently competed by unlabeled competitor DNA, whereas neither the HNF4.142 nor the HNF4.125 monomer can be. This reinforces the notion that the dimerization mediated by residues 126–142 is important for the specific binding of the HNF-4 DBD to DNA.

To assess the role of the A-box in binding affinity, dissociation constants ($K_d$) for both HNF4.142 and HNF4.125 were determined on both a DR1 and a DR2 probe and compared with that of the RXRα DBD. Since the RXRα DBD did not homodimerize on any DR2 examined, RARα DBD and a native ApoAI site A probe, which can be considered a DR2 (see Table I), were used. The results, shown in Fig. 7, indicate that both the HNF4.142 and the RXR.DBD homodimer bind DR1 with high affinity ($K_d = 4.6$ and $2.2 \text{ nM}$ in panels A and C, respectively). The EMSA for Fig. 7 was done at $4^\circ \text{C}$ in order to detect the RXR and RAR homodimers. Under these conditions, a HNF4.125 homodimer shift complex was also detected, whereas it was not detected when the reaction was performed at RT (see Fig. 6). The HNF4.125 homodimer complex, however, had a very low binding affinity as evidenced by a nonsaturating curve (panel B). The binding of all the DBD monomers (HNF4.142, HNF4.125, RXR, and RAR) was also nonsaturating, yielding a straight line in each case (data not shown).

In comparison to the DR1, binding to ApoAI (a DR2) occurred at a lower affinity for the HNF4.142 homodimer but was comparable with that of the RARα DBD ($K_d = 32.3$ and $49.3 \text{ nM}$ in panels D and F, respectively). Interestingly, the affinity of the HNF4.125 homodimer for ApoAI site A was higher than that for the DR1 ($66.4 \text{ nM}$ in panel E) but still significantly lower than that of HNF4.142. These results show that, unlike the full-length HNF-4 which binds DNA much more efficiently than does either the full-length RXR or RAR homodimer (12), the HNF-4 DBD binds DNA as a dimer with a similar affinity as the RXR and the RAR DBD. The results also show that while amino acid residues 117–125 (the third helix region) are insufficient for high affinity binding of the HNF-4 DBD, the A-box region is required.

The DBD of HNF-4 Heterodimerizes with the DBD of RXRα but Not of Other Receptors—Since the HNF-4 DBD exists as a monomer in solution, we hypothesized that, unlike the full-length receptor, it might be able to heterodimerize with RARα. EMSA was performed as above using the HNF-4 and RXR DBD constructs (see Fig. 1) and DR1 and DR2 as probes. The results, shown in Fig. 8, indicate that whereas RXR.DBD did not heterodimerize with HNF4.142 on DR1 (panel A, lane 3), it did on DR2 (panel B, lane 3). HNF4.125, in contrast, heterodimerized with RXR.DBD on both DR1 and DR2 (lane 6 in panels A and B, respectively). None of the HNF-4 DBD constructs (HNF4.142, HNF4.125, and HNF4.N123E) heterodimerized with full-length RXRs (data not shown).

In order to determine whether the third helix/T-box region facilitates HNF-4 dimerization, EMSA was performed with RXR.DBD and extracts containing HNF4.N123E, which theoretically contains a third helix analogous to that of RXRα (see Fig. 5). The results show that HNF4.N123E does indeed form homo- and heterodimers somewhat more readily than does HNF4.125 on DR1 (Fig. 8A, compare lanes 10 and 11 to 5 and 6, respectively). Therefore, the third helix may facilitate but is evidently not required for homo- and heterodimerization. Interestingly, in contrast to the APF1 probe (Fig. 6), HNF4.125 and HNF4.N123E do not bind DR1 and DR2 as monomers, only as dimers (Fig. 8A, lanes 5 and 10; B, lanes 4 and data not shown). The significance of this difference is not known but could be due to different reaction conditions and/or nucleotide sequence of the probes.

Heterodimerization of the HNF-4 DBD with other receptors was also examined. However, in contrast to RXR.DBD, neither HNF4.142 nor HNF4.125 nor HNF4.N123E heterodimerized with either RAR.DBD (Fig. 8C) or TR.DBD (data not shown). Of potential interest is the observation that the amount of RAR DBD homodimer complex appears to be reduced by the presence of the HNF4.DBD extracts (lanes 6, 9, 12). Whereas the reason for this reduction is not known, it is doubtful that it is due to dimerization of the HNF4 and the RAR DBDs in solution since NMR studies of several different receptor DBDs, includ-
RXR (DBD) of HNF-4 readily heterodimerizes with the DBD of a synthetic DR2 element. This is the first published report of HNF-4 binding to DNA. Finally, this is the first report of direct evidence that modimerization and thus high affinity binding of HNF-4 DBD C-terminal end of the DBD is critical for the cooperative homodimerization of the HNF-4 DBD (Figs. 6 and 7), whereas the full-length receptors exist in solution as homodimers (12), the HNF-4 DBD exists in solution as a stable homodimer and binds DNA with the DBD of RXRα, which exists in solution as a monomer even at millimolar concentrations (11, 14, 18, 31).

**DISCUSSION**

The results of this study show that the DNA binding domain (DBD) of HNF-4 readily heterodimerizes with the DBD of RXRα. The results also show that the A-box region at the C-terminal end of the DBD is critical for the cooperative homodimerization and thus high affinity binding of HNF-4 DBD to DNA. Finally, this is the first report of direct evidence that HNF-4 binds DR2 elements as well as DR1 elements. Although HNF-4 has been previously shown to bind native elements comprised of nonconsensus half-sites separated by two nucleotides (2, 25), this is the first published report of HNF-4 binding to DNA. In contrast to the full-length HNF-4 also share response elements from at least six different genes as well as a consensus site of a direct repeat of AGGTCA separated by one nucleotide (DR1) (reviewed in Refs. 32, 33). These similarities suggested that the structural and functional properties of the HNF-4 DBD would be similar to that of the RXRα DBD. The results from this study show, however, that this is not the case.

First, the A- and the T-boxes appear to play different roles in the homodimerization of the HNF-4 and the RXRα DBDs. For example, the A-box is required for the cooperative homodimerization of the HNF-4 DBD (Figs. 6 and 7), whereas the T-box is not necessary and sufficient for the cooperative homodimerization of the RXRα DBD (18, 40, 41). Second, the DBD of HNF-4 heterodimerizes with the RXRα DBD but not with the RAR or the TR DBD, suggesting that the HNF-4 DBD is more like the RAR or the TR DBD than the RXRα DBD (Fig. 8 and data not shown).
Since the HNF-4 DBD construct containing the A-box (HNF4.142) dimerizes cooperatively with the HNF-4 DBD construct lacking the A-box (HNF4.125), which by itself does not readily homodimerize (Fig. 6), it is concluded that the dimerization interface of the HNF-4 DBD homodimer is formed through the interaction between the A-box of one HNF-4 DBD and a more N-terminal region of the other HNF-4 DBD. This asymmetric nature of the HNF-4 DBD dimerization interface is consistent with arrangement of the two half-sites of the HNF-4 binding site as a direct repeat.

The finding that the A-box region is required for the cooperative homodimerization of the HNF-4 DBD on DNA is rather unique among nuclear receptors studied (see Table II). Although the A-box has shown to be important for the monomeric binding of NGFI-B and TR (16, 38), it has been shown to not be critical for the homo- and heterodimerization of RXR and RAR DBDs, for which the T-box is more important (28, 40, 41). The A-box, however, was shown to be important for the high affinity binding of the TR homodimer (16). It thus appears that HNF-4 DBD homodimer functions in a manner

| Requirement for the A-box in DNA binding |
|------------------------------------------|
| Information is from this study and a variety of other sources (10, 16, 18, 26, 40, 41). |

|            | Monomer | Homodimer | Heterodimer^a |
|------------|---------|-----------|---------------|
| RXR        | No      | No        | Not applicable |
| RAR        | No      | No        | No            |
| TR         | Yes     | Yes       | Yes           |
| HNF-4      | No      | Yes       | No            |

^a Heterodimerization with RXR.
similar to a TR DBD homodimer even though it is much more similar to the RXRa DBD in amino acid sequence, particularly in the T-box region. This similarity, however, is unexpected since HNF-4 and TR homodimers bind direct repeats with different spacings. Whereas HNF-4 binds both DR1 and DR2 elements, TR binds DR4 elements as well as inverted repeats. And binding of receptor dimers to direct repeats with different spacings are expected to involve dimerization interfaces formed between different regions of the receptors (28).

Despite the similar role of the A-box in HNF-4 and TR DBD homodimerization, other evidence suggests that the structure of HNF-4 DBD is different from that of the TR DBD. First, the A-box plays apparently different roles in the monomeric binding of the HNF-4 DBD and the TR DBD. Whereas the A-box is absolutely required for the monomeric binding of TR DBD (41), it appears to facilitate but is not necessary for the monomeric binding of HNF-4 DBD (Fig. 8). Second, the A-box of TR is required for heterodimerization with RXR but it is not required for heterodimerization of the HNF-4 DBD (Fig. 8). Third, structural modeling indicates that the A-box of the TR DBD will interfere with the second zinc module of RXR DBD on DR1 and DR2 elements and therefore prevent the two DBDs from heterodimerizing on those elements (28). In contrast, the HNF-4 DBD construct containing the A-box (HNF4.142) heterodimerizes with the RXR DBD construct containing the A-box (HNF4-RXR DBD Heterodimerization) even though it is much more similar to the RXRa DBD in amino acid sequence, particularly in the T-box region. This similarity, however, is unexpected since HNF-4 and TR homodimers bind direct repeats with different spacings. Whereas HNF-4 binds both DR1 and DR2 elements, TR binds DR4 elements as well as inverted repeats. And binding of receptor dimers to direct repeats with different spacings are expected to involve dimerization interfaces formed between different regions of the receptors (28).

In conclusion, the results of this study show not only that the determinants of homo- versus heterodimerization reside within the LBD of the receptors but also that much remains to be learned about the role of the A-box and the T-box in the nuclear receptors. Furthermore, the results indicate that the dimerization interface of HNF-4 DBD is different from that of other nuclear receptors, which could explain why HNF-4, which binds DR1 and DR2, has a different DNA binding specificity than that of other receptors, including RXR, TR, andRAR, which preferentially bind DR1, DR4, and DR5, respectively. Finally, this study provides yet another example of how broad functional diversity among proteins within a highly conserved superfamily can be achieved with a minimal amount of alteration in primary amino acid sequence.

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