The hepatocyte nuclear factor 4 (HNF-4) is a member of the nuclear receptor superfamily and participates in the regulation of several genes involved in diverse metabolic pathways and developmental processes. To date, the functional domains of this nuclear receptor have not been identified, and it is not known whether its transcriptional activity is regulated by a ligand or other signals. In this report, we show that HNF-4 contains two transactivation domains, designated AF-1 and AF-2, which activate transcription in a cell type-independent manner. AF-1 consists of the extreme N-terminal 24 amino acids and functions as a constitutive autonomous activator of transcription. This short transactivator belongs to the class of acidic activators, and it is predicted to adopt an amphipathic $\alpha$-helical structure. In contrast, the AF-2 transactivator is complex, spanning the 128–366 region of HNF-4, and it cannot be further dissected without impairing activity. The 360–366 region of HNF-4 contains a motif that is highly conserved among transcriptionally active nuclear receptors, and it is essential for AF-2 activity, but it is not necessary for dimerization and DNA binding of HNF-4. Thus, HNF-4 deletion mutants lacking the 361–465 region bind efficiently to DNA as homo- and heterodimers and behave as dominant negative mutants. Remarkably, the full transactivation potential of AF-2 is inhibited by the region spanning residues 371–465 (region F). The inhibitory effect of region F on the HNF-4 AF-2 activity is a unique feature among members of the nuclear receptor superfamily, and we propose that it defines a distinct regulatory mechanism of transcriptional activation by HNF-4.

The nuclear receptor superfamily comprises a large set of ligand-regulated transcription factors. This superfamily includes receptors for steroid hormones, retinoids, thyroid hormone, and vitamin $D_3$, as well as a large number of structurally and functionally related transcription regulatory proteins whose natural ligands are not yet known, the so-called orphan receptors (reviewed in Refs. 1 and 2). Nuclear receptors exhibit a modular structure with six distinct regions (referred to as regions A–F), which correspond to functional domains. The N-terminal region A/B is highly variable among nuclear receptors and contains a ligand-independent transactivation function AF-1 (2). Region C contains a highly conserved DNA binding domain (DBD) composed of two zinc-coordinated modules and is responsible for specific binding to cognate response elements (Refs. 1 and 2 and references therein). The exact functions of regions D and F are not clear, although they appear to be well conserved for each receptor across species. Region D is postulated to function as a flexible hinge between the DBD and the ligand-binding domain (LBD), allowing rotational differences between these domains when dimeric receptors bind to direct, inverted, or palindromic repeats (2). Interestingly, the D regions of the thyroid hormone (TR) and retinoid acid receptors (RARs) interact with the co-repressor proteins N-CoR and SMRT, which mediate the ligand-independent transcriptional repression (3, 4).

Region E is functionally complex, since it contains the LBD, the dimerization interface, and the ligand-dependent transactivation function AF-2 (Ref. 2 and references therein). A short activating domain has been identified in the C-terminal part of AF-2 in many nuclear receptors, designated AF-2 AD, which is required for the ligand-dependent activity of AF-2 (5–8). Mutagenesis of the AF-2 AD can selectively abrogate AF-2 activity without affecting nuclear receptor dimerization and DNA binding (2, 5–8). The AF-2 AD contains the highly conserved motif $\phi_1\phi_2X\phi_3\phi_4$ (X being a hydrophobic amino acid, and $\phi$ a nonconserved amino acid), and it has been proposed to mediate interactions between the receptors and transcription coactivators (2, 5–8). Based on the importance of this conserved region in ligand-dependent transcriptional activation, it has been proposed that members of the nuclear receptor superfamily that contain this motif may stimulate transcription through a mechanism that involves a ligand (5). Recently, the crystal structures of the unliganded human RXRα LBD (9) and the liganded human RARγ (10) and rat TRα1 (11) LBDs revealed a novel fold, termed the antiparallel $\alpha$-helical sandwich, which consists of 12 $\alpha$-helices (H1–H12) packed in three layers and harboring...
an internal hydrophobic ligand-binding core. The dimerization interface of the RXR LBD is formed primarily by helix H10 and to a lesser degree H9 and a loop between H7 and H8 (9). Interestingly, the AF-2 AD corresponds to the amphipathic α-helix 12, which in the unliganded RXR LBD extends into the solvent but in the liganded RARα and TRα1 LBDs is packed onto the body of the receptor, contributing to the formation of the ligand-binding pocket and the surface that interacts with putative transactivation coactivators, such as TIF1, TRIP1, SRC-1, or RIP140 (12–15).

The hepatocyte nuclear factor 4 (HNF-4) belongs to the nuclear receptor superfamily, and it is expressed primarily in the adult liver, intestine, and kidney (16). The human and rat HNF-4 proteins are highly conserved, with an overall similarity of 96% (17). There is also a Drosophila homolog of HNF-4, which has lower similarity with the mammalian proteins, and it is expressed very early as maternal mRNA and during organogenesis (18). The mammalian HNF-4 has a distinctive F region among nuclear receptors whose function is unknown (16). Adult human and rat liver and kidney contain two isoforms of HNF-4, which differ by the presence or absence of a 10-amino acid segment in the middle of region F (17, 19). The longest of the isoforms, referred to as HNF-4A (17), is by far dominant in the liver, kidney, and the hepatoma cell line HepG2, whereas the shortest isoform (HNF-4B), which was cloned first, represents only a minor species in these tissues (17, 19). These isoforms are generated by differential splicing, but it is not known whether they have identical or different transactivation functions (20).

No ligand has been identified to date for HNF-4, which is classified as an orphan receptor. Nevertheless, it has been shown to play an important role in the regulation of several genes involved in diverse metabolic pathways. HNF-4 binds to its cognate elements as a dimer and is a positive regulator of several target genes, including the genes for apolipoprotein (apo) AI (21), apoB (22), apoCIII (22, 23), apoAI (22), apoAIV (24), medium chain acyl-CoA dehydrogenase (25), cellular retinol-binding protein II (26), the long terminal repeat of human immunodeficiency virus-1 (27), and the coagulation protease factor VII (28). Moreover, HNF-4 plays a key role in the transcriptional regulatory hierarchy of liver-specific gene expression, because it regulates the expression of HNF-1, a transcription factor important for the expression of several hepatic genes (29). Disruption of the murine HNF-4 gene, which is expressed in the visceral endoderm, leads to cell death in embryonic ectoderm and impaired gastrulation of mouse embryos, indicating that HNF-4 is critical for early development (30). However, despite the recognition of the importance of HNF-4 in developmental processes and the progress in identification of downstream targets of HNF-4, the functional domains of this nuclear receptor and the molecular mechanisms by which it regulates transcription are largely unknown.

As part of our ongoing studies on the mechanisms of transcriptional activation of apolipoprotein gene expression by HNF-4, we have performed a systematic analysis of the functional domains of HNF-4 that are involved in DNA binding, dimerization, and transactivation. We demonstrate here that HNF-4 contains two transactivation domains, designated AF-1 and AF-2, which are located in the A/B and D/E regions, respectively, and activate transcription in a cell type-independent manner. The AF-1 consists of the extreme N-terminal 24 amino acids and functions as an autonomous acidic transactivator. In contrast, the AF-2 transactivator is very complex, spanning the 128–366 region of HNF-4, and it cannot be further dissected without impairing activity. Unexpectedly, the full transactivation potential of the HNF-4 AF-2 is inhibited by sequences spanning region F. This is a unique feature among nuclear receptors, and it led us to propose that it may define a novel mechanism for regulation of the AF-2 activity.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructions**—All mutants of rat HNF-4 were generated by polymerase chain reaction (PCR). To construct the N- and C-terminal deletion mutants, the following oligonucleotides were designed as the N-terminal primers: HNF-N, 5′-GATAATACGTTGGCCGCCAT- GGACATGTTGACATCAGTGTGCT-3′; HNF-4S, 5′-TCTACGGAGAAACG- TGTCGCACCTGCGATCTTGGGATC-3′. The C-terminal primers were as follows: HNF-HF, 5′-GATGTCTGAG- ATCCCTATCATTAGTGTCGACATGTTGCT-3′; HNF-CD1, 5′-TCTAGAGATCCTTCAGGAGCAGACCTCAGGACCATCTC-3′; HNF-CD1b, 5′-GACTCGAGATCCTTCACATGTTGCTACTTGCGGCAA- T-3′; HNF-CD2, 5′-TCTAGAGATCCTTCAGTAAATGCTCTGTGATAG- TTGGGCAA-3′; HNF-CD3, 5′-TCTAGAGATCCTTCAGTACCTCGGATGCA- GAGATGATGCTG-3′; HNF-CD4, 5′-TCTAGAGATCCTTCAGGAGGCGAGGTGTA- GCGGCAA-3′; HNF-CD5, 5′-TCTAGAGATCCTTCAGGAGGCGAGGTG- GTAAGGA-3′. The underlined nucleotides indicate the cloning sites HindIII and BamHI, as well as the ATG and termination codon (TCA in reverse orientation) codons. The boldface letters indicate the Kozak sequence placed adjacent to the initiator ATG for optimal translation. The PCR products were digested with HindIII and BamHI and were cloned in the expression vector pcDNAII/amp (Invitrogen) to generate constructs HNF-4B, HNF-HF, CD1 to CD6, D1HNF-4, and DCD1.

The HNF-4A isoform was constructed as follows. HNF-4A fragment A was amplified by PCR using the forward primer H-HNF-4A (5′-GATGTCTGAG- GACGCGCCACAGCCACACA-3′) and the reverse primer H-3 (5′-GCTCTGCCCCCTGGGCGGGCCACTCATCAGCCTGGT-3′). Similarly, HNF-4B fragment B was generated using the forward primer H-2 (5′-GATGTGCGGCGGCGCCAGGGAGGACGCCAGCAGCAC- CACCTTGAAGCTCACAGCA-3′), which contains the reverse complement of the primer sequence of the alternative HNF-4B reverse primer GAL-455R (5′-TCTAGAGATCCCGGCGGCGGGTGATAGG- TCTC-3′). Aliquots containing 2% of each of the amplified fragments A and B were mixed and used for another round of PCR amplification in the presence of H-1 and GAL-455R primers. The resulting PCR fragment was digested with Hpal and BamHI, and it was used to replace the HpalBamHI fragment of HNF-4B cloned in the plasmid pBXG1, constructed by the GAL4 DBD(1–147)11. Following verification by sequencing, the resulting HNF-4A cDNA was subcloned as an EcoRI/BamHI fragment in the pCDNAI/amp vector.

Plasmids ND1–ND5, containing internal deletions of amino acids 129–174 (ND1), 175–239 (ND2), 175–289 (ND3), 175–336 (ND4), and 175–369 (ND5) were generated as follows. The HNF-4 C-terminal fragments spanning region D, containing the indicated deletions, and as reverse primer the oligonucleotide HNF-CD5 (5′-GACTGAGGATCCTCACAGGTTGTCAATCTTGGCCA-3′), were amplified using as forward primer the oligonucleotide HNF-HF (5′-GATCGAGCATCAGCATTTCGGAATGATGGCTGACTACAGTGCT3′) and the reverse primer HNF-CD5 (5′-GACTGAGGATCCTCACAGGTTGTCAATCTTGGCCA-3′). The PCR products were digested with Hpal and BamHI, and they were cloned in the expression vector pcDNAII/amp (Invitrogen) to generate constructs HNF-4B, HNF-HF, CD1 to CD6, D1HNF-4, and DCD1.

The HNF-4A isoform was constructed as follows. HNF-4A fragment A was amplified by PCR using the forward primer H-HNF-4A (5′-GATGTCTGAG- GACGCGCCACAGCCACACA-3′) and the reverse primer H-3 (5′-GCTCTGCCCCCTGGGCGGGCCACTCATCAGCCTGGT-3′). Similarly, HNF-4B fragment B was generated using the forward primer H-2 (5′-GATGTGCGGCGGCGCCAGGGAGGACGCCAGCAGCAC- CACCTTGAAGCTCACAGCA-3′), which contains the reverse complement of the primer sequence of the alternative HNF-4B reverse primer GAL-455R (5′-TCTAGAGATCCCGGCGGCGGGTGATAGG- TCTC-3′). Aliquots containing 2% of each of the amplified fragments A and B were mixed and used for another round of PCR amplification in the presence of H-1 and GAL-455R primers. The resulting PCR fragment was digested with Hpal and BamHI, and it was used to replace the HpalBamHI fragment of HNF-4B cloned in the plasmid pBXG1, constructed by the GAL4 DBD(1–147)11. Following verification by sequencing, the resulting HNF-4A cDNA was subcloned as an EcoRI/BamHI fragment in the pcDNAI/amp vector.

Plasmids ND1–ND5, containing internal deletions of amino acids 129–174 (ND1), 175–239 (ND2), 175–289 (ND3), 175–336 (ND4), and 175–369 (ND5) were generated as follows. The HNF-4 C-terminal fragments spanning region D, containing the indicated deletions, and as reverse primer the oligonucleotide PCHRNF-C. Similarly, the HNF-4 N-terminal fragments I–8 (for ND1) and 1–174 (for ND2 to ND5) were generated by PCR using as forward primer the oligonucleotide PCHRNF-N and as reverse primer the oligonucleotides PCR-IDS1 (5′-GATGGCGATCCTTGGCAGATC-3′) and PCR-IDS2 (5′-GCTCCAGATCCTTGGCAGATC-3′). The PCR-IDS1 is the reverse complement of the underlined sequence of oligonucleotide PCHRNF-N, and PCR-IDS2 is the reverse complement of the underlined sequence of oligonucleotides PCHRNF-N to PCHRNF-D5. Aliquots containing 2% of each of the amplified regions were mixed and used for another round of PCR amplification in the presence of PCHRNF-N and PCHRNF-D5 primers. The resulting PCR fragments were digested with HindIII and cloned in the pcDNAI/amp vector to generate constructs ND1–ND5.

The full-length and deletion mutants of GAL-HNF-4 chimeras were either obtained from pcDNAII/amp plasmids by PCR amplification or generated by PCR using appropriate primers and were cloned in frame into plasmid pBXG1 at the EcoRI and BamHI sites. Mutants GAL-L366K and GAL-E363K were generated by PCR-mediated site-directed
mutageneis, using appropriate primers. All constructs were verified by DNA sequencing analysis.

**In Vitro Transcription and Translation—pcDNAI/Amp constructs containing the wild type HNF-4 receptor and its deletion mutants were linearized with BanHI, transcribed in **in vitro** with T7 RNA polymerase, and translated with rabbit reticulocyte lysates (Promega) in the presence of [35S]methionine, as recommended by the manufacturer.

Electrophoretic Mobility Shift Assay (EMSA)—HNF-4 proteins produced by **in vitro** translation (2 μl) were incubated with 32P-labeled double-stranded oligonucleotide probes (10 fmol) for 15 min at 4 °C in the presence of 25 mM HEPES, pH 7.6, 40 mM KCl, 1 mM dithiothreitol, 5 mM MgCl2, and 0.6 μg of poly(dI-dC). Protein-DNA complexes were analyzed by electrophoresis in 5% nonnucleating gels, followed by autoradiography, as described previously (22, 27, 32).

**Transient Transfection Assays—**Plasmids were transfected into HepG2, Caco-2, and HeLa cells and were assayed for their ability to promote transcription of the chloramphenicol acetyltransferase (CAT) gene constructs. All transient transfactions were performed using the calcium phosphate DNA coprecipitation method, as described previously (22, 27, 32). The transfection mixture contained as a control the CAL-HNF-4 chimeras. Protein extracts corresponding to approximately 0.5–1×106 cells/ lane were combined with 5 × loading buffer (0.3 M Tris-HCl, pH 6.8, 10% SDS, 50% glycerol, 25% β-mercaptoethanol, and 0.05% bromophenol blue) in a total volume of 70 μl and separated on 10 or 13% SDS-polyacrylamide gels. Proteins were transferred to a nitrocellulose membrane by electroblotting. Membranes were pre-incubated in PBS containing 3% nonfat dry milk overnight at 4 °C. Subsequently, they were incubated with the primary anti-GAL rabbit polyclonal antibody (Upstate Biotechnology, Inc.) at a dilution of 1:500 in PBS containing 1% nonfat dry milk and 0.05% Tween 20 (PBS-T) for 1 h at 37 °C. Membranes were washed in the same buffer and incubated with the secondary antibody, goat anti-rabbit IgG conjugated to alkaline phosphatase (Kirkegaard & Perry Laboratories, Inc.) at a dilution 1:5,000 in PBS-T for 1 h at 37 °C. Membranes were washed in PBS-T, and proteins were visualized by developing the alkaline phosphatase color according to the manufacturer's specifications.

Immunofluorescence—For indirect immunofluorescence analysis, wild type HNF-4 and mutant proteins CD1 and CD1, carrying a FLAG peptide (MDYKDDDDK) (Kodak, IBi) at their N termini, were cloned in pcDNAI/amp vector and used to transfect COS-1 cells grown on coverslips (33). After fixation, cells on coverslips were fixed in 3% formaldehyde in PBS for 20 min at 20 °C, permeabilized with 0.1% Nonidet P-40 in PBS for 10 min at 20 °C, and incubated with a specific monoclonal antibody M2 to the FLAG peptide, at a concentration of 9 μg/ml in PBS for 40 min at 37 °C. Cells were stained, using as a secondary antibody a rhodamine-labeled anti-mouse IgG (Kirkegaard & Perry Laboratories) at a 1:10 dilution (50 μg/ml), and incubated at 37 °C for 40 min, followed by extensive washes in PBS.

**RESULTS**

**Domains of HNF-4 Involved in DNA Binding and Dimerization**—To investigate the functional properties of HNF-4 we cloned HNF-4A, HNF-4, and a number of N-terminal, C-terminal, and in-frame internal deletion mutants in the mammalian expression vector pcDNAI/Amp (Fig. 1A). In **in vitro** translated HNF-4A, HNF-4B, and various deletion mutants were tested for DNA binding in an EMSA, using the CIIB element of the apolipoprotein CIII promoter as a probe, which is highly specific for HNF-4 (22, 24). This analysis showed that HNF-4A and HNF-4B bound strongly to DNA (Fig. 1B, lanes 1 and 4), in agreement with previous studies (16, 17, 22). Mutants HNF-HF, CD1, and CD1b, lacking part or all of the F region also bound strongly to DNA, (Fig. 1B, lanes 2, 3, 5), indicating that region F is not required for DNA binding. In contrast, mutant CD2 lacking residues 340–465 lost its ability to bind to element CIIB (Fig. 1B, lane 6), indicating that sequences spanning the region 340–360 are required for efficient DNA binding. Similarly, mutants CD3 and CD4, harboring progressive C-terminal deletions within region E, lost their ability to bind to element CIIB (Fig. 1B, lanes 7 and 8). This inability to bind DNA was not due to inefficient synthesis of these proteins, because the integrity and amounts of the **in vitro** translated proteins were monitored by SDS-polyacrylamide gel electrophoresis and 35S-sensitive autoradiography prior to EMSA analysis (Fig. 1D). However, additional C-terminal deletions that removed either the entire region E (mutant CD5) or both regions E and D (mutant CD6) restored DNA binding, although with reduced affinity (Fig. 1B, lanes 9 and 10). Mutant DC1D, lacking both regions A/B and F, also bound strongly to DNA (Fig. 1B, lane 11), indicating that A/B is also not required for DNA binding. Taken together, these results map the HNF-4 DBD between residues 48–128, which corresponds to the well-studied DBD of the nuclear receptors that contains two zinc finger modules (2). In addition, these results indicate that, in the absence of the 340–465 region, DNA binding is inhibited by sequences carboxyterminal to residue 174, and at least a portion of these sequences is located in the 175–239 region.

In a similar series of experiments, we also tested the DNA binding properties of the internal deletion mutants ND1–ND5 produced by **in vitro** translation. Mutant ND1 lacking most of D domain, bound strongly to CIIBB probe (Fig. 1B, lane 12), indicating that the 129–174 region is not required for DNA binding. Similarly, mutant ND2 lacking residues 175–240, also bound to DNA (Fig. 1B, lane 13). Interestingly, mutants ND3 and ND4 lacking the regions 175–289 and 175–336, respectively, lost their ability to interact efficiently with element CIIBB (Fig. 1B, lanes 14 and 15). In contrast, deletion of the entire E region in mutant ND5 restored DNA binding, and two weak protein-DNA complexes were observed reproducibly (Fig. 1B, lane 16). These complexes were not due to proteolytic degradation of ND5 protein, as shown in Fig. 1D. Comparison of the protein-DNA complexes formed by CD5 and ND5, suggests that the presence of region F in ND5 does not destroy DNA binding but leads to the formation of two complexes that have weaker intensities than the CD5-DNA complex. Taken together, these results indicate that the region between residues 175–240 is not required for DNA binding; nevertheless, it is necessary for the formation of a tight protein-DNA complex. Moreover, in the absence of this region, DNA binding is inhibited by sequences carboxy terminal to residue 241, and at least a portion of these sequences is located in the 337–369 region.

It was previously shown that HNF-4 binds to DNA as a dimer (16, 27, 35). To identify the dimerization domain(s) of HNF-4, we employed EMSA analysis to monitor the formation of heterodimers between wild type HNF-4B and its C-terminal deletion mutants CD1–CD6. When equal amounts of **in vitro** translated HNF-4B and CD1 were mixed and then tested for binding to CIIBB probe, in addition to complexes that corresponded to HNF-4B and CD1, a complex with intermediate electrophoretic mobility (heterodimer) was also formed (Fig. 1C, lanes 7–10), indicating that one molecule each of HNF-4B and CD1 bound concomitantly to element CIIB. In contrast, heterodimers were not observed between HNF-4B and any of the mutants CD2–CD6 (Fig. 1C, lanes 3–7), indicating that sequences within regions D and E are required for formation of dimers capable of DNA binding and that at least a portion of these sequences is located in the 340–360 region. Because mutants CD5 and CD6 retained their ability to bind to DNA but did not dimerize with HNF-4B, we conclude that they bind to DNA as monomers. Moreover, mutant DC1D also
formed dimers with HNF-4B that bound to DNA (Fig. 1C, lane 8), indicating that the A/B domain is not required for dimerization and that mutant DCD1 contains an intact dimerization interface. Similar results were obtained when HNF-4A and CD1b were used in a similar assay (Fig. 1C, lanes 9–11), indicating that sequences carboxyl to residue 360 are not required for efficient dimerization of HNF-4.

To map more precisely the dimerization domain of HNF-4, we tested mutants ND1–ND5 for their ability to dimerize with DCD1 in a similar series of EMSA experiments. The mutant DCD1 was used in these experiments, because its size (considerably smaller than HNF-4) facilitated the resolution of heterodimers in the dimerization assay. Mutant ND1 formed heterodimers with DCD1 that bound to DNA (Fig. 1C, lane 12), indicating that the region spanning residues 129–174 is not required for dimerization. In contrast, mutants ND2–ND5 failed to dimerize with DCD1 (Fig. 1C, lanes 13–16), indicating that sequences within E are required for formation of dimers capable of DNA binding and that at least a portion of these sequences is located in the 175–240 region. The inability of ND2 to dimerize with HNF-4 suggests that it binds to DNA as a monomer. Furthermore, since the region spanning residues 175–240 is required for efficient dimerization but not DNA binding, it is possible that the lack of this region in mutant ND2 may induce conformational changes in ND2 and explain, at least in part, the abnormal appearance of the ND2-DNA complex. Taken together, these data indicate that the dimerization domain of HNF-4 is located in the 175–360 region.
The Transactivation Potential of HNF-4 Is Negatively Regulated by Region F—

To study the transcription activation domain(s) of HNF-4 independently of its DNA binding, dimerization, and nuclear localization properties, we constructed a series of plasmids containing the full-length HNF-4 and its deletion mutants fused in-frame to the yeast GAL4 DBD-(1–147) (36). The GAL4 DBD-(1–147) in addition to specific DNA binding activity contains signals for dimerization and nuclear localization (36). The various GAL-HNF-4 chimeric constructs were cotransfected into HepG2 cells with the reporter plasmid pG\textsubscript{5}CAT, which contains five GAL4 DNA-binding sites upstream of the β-globin promoter and the CAT gene (37). The expression levels and integrity of the wild type HNF-4 and its deletion mutants fused to GAL4 DBD-(1–147) in transfected COS-1 cells was monitored by Western blot analysis using an anti-GAL4 antibody (Fig. 2). In general, the transfected cells were shown to express wild type and mutant proteins in comparable amounts, with insufficient variations to account for the observed differences in their transcriptional activation potential (Fig. 2). Cotransfection with GAL-HNF-4A and GAL-HNF-4B activated the transcription of reporter pG\textsubscript{5}CAT by approximately 10-fold as compared with the activation obtained by pBXG1, containing the GAL4 DBD-(1–147) alone (Fig. 3). This finding indicates that the full-length HNF-4A is a transcriptional activator, comparable with HNF-4B. Surprisingly, cotransfection with mutants GAL-HNF-HF and GAL-CD1 resulted in 45- and 65-fold increase of the pG\textsubscript{5}CAT activity, respectively (Fig. 3). This enhancement was not due to differences in expression or stability of the fusion proteins, as was demonstrated by Western blot analysis (Fig. 2). Therefore, we conclude that region F has an inhibitory function on the transcriptional activation potential of both HNF-4 isoforms, and it has been designated the negative regulatory domain.

The negative effect of region F was unexpected, because no similar function has been described to date for this region in other members of the nuclear receptor superfamily. In contrast, it was previously suggested that region F might be an activator of transcription because of its high content in proline.

**Fig. 2.** Western blot analysis of HNF-4 and its deletion mutants fused to GAL4 DBD-(1–147). COS-1 cells were transfected with the indicated GAL-HNF-4 chimeras. Protein extracts corresponding to approximately 0.5–1 × 10\textsuperscript{6} cells/lane were analyzed by SDS-polyacrylamide gel electrophoresis, followed by transfer to a nitrocellulose membrane. The expression of GAL-HNF-4 proteins were detected using an anti-GAL4 rabbit polyclonal antibody and, as secondary antibody, goat anti-rabbit IgG conjugated to alkaline phosphatase, as described under “Experimental Procedures.” Numbers indicate molecular mass protein markers in kDa.

**Fig. 3.** Transcriptional activity of HNF-4 and its C-terminal deletion mutants fused to GAL4 DBD-(1–147). The reporter plasmid pG\textsubscript{5}CAT was cotransfected into HepG2 cells with pRSV-β-gal plasmid and effector plasmids expressing HNF-4 and the indicated deletion mutants fused in-frame to the yeast GAL4 DBD-(1–147). Cells were harvested 48 h later and assayed for CAT and β-galactosidase activities. The normalized CAT values (CAT/β-galactosidase) are shown as the percentages of the activity obtained with the pG\textsubscript{5}CAT reporter construct cotransfected with GAL-HNF-4A. Data represent the mean ± S.E. of at least three independent experiments.
residues (16). However, our results clearly indicate that the activation potential of HNF-4 resides outside region F, and in fact, its activity is inhibited by F.

A Stretch of 24 Amino Acids at the Extreme N-terminal Domain of HNF-4 Is an Autonomous Transactivator—To identify the activation domain(s) of mutant CD1, we tested a series of HNF-4 C-terminal deletion mutants fused to GAL4 DBD-(1–147) for their potential to transactivate the pG5CAT reporter construct in HepG2 cells. Cotransfection with mutant GAL-CD2 resulted in loss of transcriptional activation of pG5CAT (Fig. 3). The dramatic decrease in the transactivation potential of GAL-CD2 indicates that region 340–370 is necessary for high levels of transcriptional activation by GAL-CD1.

Low levels of pG5CAT activity were maintained upon cotransfection with expression plasmids GAL-CD3 and GAL-CD4, which lack regions 290–465 and 240–465, respectively (Fig. 3). However, deletion of the residues 175–239 in expression plasmid GAL-CD5 resulted in a dramatic increase of the pG5CAT transcriptional activity (Fig. 3). The results indicate that the region 340–370 is necessary for high levels of transcriptional activation by GAL-CD1.

Low levels of pG5CAT activity were maintained upon cotransfection with expression plasmids GAL-CD3 and GAL-CD4, which lack regions 290–465 and 240–465, respectively (Fig. 3). However, deletion of the residues 175–239 in expression plasmid GAL-CD5 resulted in a dramatic increase of the pG5CAT transcriptional activity (Fig. 3). These results indicate that an activation function(s) of HNF-4 is located in the region 1–174, which includes domains A/B, C, and D. To examine the influence of region F on this activation function(s), we tested the transactivation potential of GAL-ND5. As shown in Fig. 3, the activity of GAL-ND5 was only 2-fold lower than that of GAL-CD5, indicating that the negative regulatory domain only slightly affects the transactivation activity elicited by domains A/B, C, and D. Furthermore, to identify which of these domains was responsible for the observed transactivation potential of GAL-CD5, we tested constructs GAL-CD6 to GAL-CD10, which contain deletions of regions A/B, C, and D in various combinations. Transactivation was obtained with GAL-CD6 but not with GAL-CD7, GAL-CD8, or GAL-CD9, suggesting the presence of an activating domain in region A/B (Fig. 3). This was confirmed by the high transactivation elicited by GAL-CD10, demonstrating the presence of a potent activator in the region spanning residues 1–48. Interestingly, Chou-Fasman (38) and Garnier-Robson (39) algorithms predicted that the region spanning residues 1–24 has the potential to adopt an α-helical structure, whereas the region spanning residues 25–48 is unstructured. Based on these structural predictions, we asked whether the transactivation potential could be further sublocalized in either of these two regions, by testing mutants GAL-CD11 and GAL-CD12 for their potential to activate the reporter pG5CAT. The results showed that GAL-CD11 retained full transactivation potential, whereas GAL-CD12 had no activity above background levels (Fig. 3). Therefore, we conclude that the small region spanning residues 1–24 contains a potent transactivation domain, designated AF-1. This transactivator has an estimated isoelectric point of 3.04 (DNASTAR package), and it is therefore classified as an acidic activator (40). These results suggest that although AF-1 is a powerful autonomous transactivator, in the context of the intact GAL-HNF-4 chimera elicits a weaker transactivation than when it is separated from the rest of the HNF-4 sequences. It also appears that in the context of GAL-CD2 to GAL-CD4 chimeric constructs, the activity of AF-1 is inhibited by sequences in region E, and at least a portion of these sequences is located in the 175–239 region.

A Second Transactivator within the HNF-4 LBD Is Negatively Regulated by Region F—To investigate the possibility that HNF-4 contains a second transactivation domain, we generated N-terminal deletions of HNF-4 fused to GAL4 DBD-(1–147). Deletion of the AF-1 domain resulted in 5-fold reduction of transcriptional activity of the otherwise intact HNF-4B (con-
struct GAL-D1HNF-4 in Fig. 4). Remarkably, deletion of region F resulted in strong transactivation (construct GAL-D1CD1 in Fig. 4), indicating the existence of a second activation domain spanning residues 48–370, whose activity is inhibited by F.

Additional cotransfection experiments using a series of N- and C-terminal deletion mutants of HNF-4 fused to GAL4 DBD-(1–147), revealed that the regions D and E contain a powerful transactivator, designated AF-2 (Fig. 4). Comparison of the activities of constructs GAL-D2HNF-4 and GAL-D2CD1 indicates that the activation potential of AF-2 is strongly inhibited by F.

The importance of region 360–366, which contains the conserved motif $\phi dE d\phi d$, on the AF-2 activity was assessed with mutants GAL-D2CD1a and GAL-D2CD1b. Deletion of residues 367–370 (mutant GAL-D2CD1a) did not affect the transactivation potential of AF-2. However, further deletion of residues 361–366 (mutant GAL-D2CD1b) resulted in abolishment of transactivation, indicating that this region contains an activation domain critical for AF-2 activity, designated AF-2 AD. To assess the contribution of the conserved glutamic acid Glu$^{363}$ in the transactivation potential of HNF-4 AF-2, we changed it to a lysine (mutant GAL-E363K). This mutant was transcriptionally inactive, indicating that Glu$^{363}$ is critical for the HNF-4 AF-2 activity. Notably, although HNF-4 is a strong transactivator, it has a leucine at position 366, similar to nuclear receptors ARP-1, EAR-2, and EAR-3, which are negative regulators of transcription, and in contrast to the RAR, RXR, and TR families of receptors, which contain a glutamic acid residue in the analogous position critical for transactivation (Fig. 5). We therefore addressed the importance of the leucine Leu$^{366}$ in the transactivation potential of HNF-4 AF-2, by changing it to glutamic acid (mutant L366E). Surprisingly, the AF-2 activity was abolished (Fig. 4), indicating that Leu$^{366}$ is critical for the HNF-4 AF-2 activity. Taken together, these observations imply that the HNF-4 AF-2 AD may interact with a coactivator(s) different from those that interact with RARs, RXRs, and TRs.

To further delineate the N-terminal boundaries of AF-2, we

**Fig. 5. Sequence alignment of the AF-2 AD regions of members of the nuclear receptor superfamily.** Amino acid numbers are indicated, and hydrophobic residues (denoted by $\phi$) are boxed. The highly conserved glutamic acid residues in the AF-2 AD of HNF-4 and all ligand-activated nuclear receptors are shown by white on black. The leucine residue at position 366 of HNF-4, which is critical for its AF-2 AD activity, and the conserved leucine residues of ARP-1, EAR-2, and EAR-3, which are negative regulators of transcription, are shaded. Species designation prefixes are as follows: human (h); rat (r); mouse (m); Drosophila (d). References and accession numbers for the indicated receptors are given in Ref. 1.

**Fig. 6. Transactivation potential of the HNF-4 AF-1 and AF-2 activation domains in HeLa and Caco-2 cells.** The reporter plasmid pG5CAT was co-transfected into HeLa and Caco-2 cells with pBSV-$\beta$-gal and effector plasmids expressing the indicated HNF-4 deletion mutants fused in-frame to the yeast GAL4 DBD-(1–147). Cells were harvested 48 h later and assayed for CAT and $\beta$-galactosidase activities. The normalized CAT values in each cell type are shown as the percentages of the activity obtained with the pG5CAT reporter cotransfected with pGAL-HNF-4B. The bar graphs show mean values of at least three independent experiments.
analyzed mutants GAL-D3CD1 to GAL-D6CD1, harboring progressive 10-amino acid deletions in region D (Fig. 4). The AF-2 activity decreased gradually in GAL-D3CD1, GAL-D4CD1, and GAL-D5CD1, and it was abruptly abolished in GAL-D6CD1, indicating that the region 128–175 is critical for HNF-4 AF-2 activity. However, this region did not function as an autonomous transactivation domain in construct GAL-C9 (Fig. 3). Our attempts to further dissect the AF-2 domain using a series of C-terminal and internal deletions of the D/E regions fused to GAL4 DBD-(1–147) were unsuccessful (Fig. 4), leading us to conclude that AF-2 is a complex domain, requiring the presence of the entire 128–366 region for full activity. Interestingly, construct GAL-D7 had no transactivation potential, indicating that the HNF-4 AF-2 AD cannot function as an autonomous transactivator, in contrast to AF-2 ADs of other nuclear receptors, which can activate transcription autonomously (2, 5–8). Moreover, construct GAL-D10 had no transactivation potential (Fig. 4), consistent with our previous results that region F is not an activation domain.

The AF-1 and AF-2 Domains of HNF-4 Can Activate Transcription in a Cell Type-independent Manner—Because the expression of HNF-4 is restricted primarily in hepatic, intestinal, and renal cells, we addressed the question whether the activator and inhibitory domains of HNF-4 can function exclusively in cell types where HNF-4 is expressed or in other cell types as well. To investigate this question, we performed cotransfection experiments with the above described GAL4-HNF-4 chimeric constructs in Caco-2 and HeLa cells. These experiments showed that the pG5CAT transactivation pattern in Caco-2 and HeLa cells was remarkably similar to that observed in HepG2 cells (Fig. 6). Taken together, these results suggest that AF-1 and AF-2 domains can activate transcription through communication with ubiquitous rather than cell-specific basal transcription factors and coactivators.

Effects of HNF-4 Deletion Mutants on ApoB and ApoCIII Gene Transcription—We showed previously that HNF-4 binds with high affinity to regulatory elements BA1 (41) and CIIIB (34) of the apoB and apoCIII promoters, respectively, and activates the transcription of these promoters (22). Having identified the HNF-4 functional domains with the GAL-HNF-4 chimeric constructs, we then wished to study the effects of these domains on the transcription of native apoB and apoCIII promoters. For this purpose, we cotransfected selected HNF-4 mutants (shown in Fig. 7A) with the reporter constructs apoB-1800CAT (22), (BA1)5CAT (42), and apoCIII-890CAT (34) in HepG2 cells. To verify the nuclear localization of these mutants, we performed immunofluorescence analysis in COS-1. These HNF-4 mutants (carrying a FLAG peptide at their N termini) were transiently transfected in COS-1 cells, and the expressed proteins were detected with an anti-FLAG M2 monoclonal antibody. This analysis showed that HNF-4B and its deletion mutants CD1, CD2, CD3, CD4, CD6, and ND1 were localized in the nucleus (Fig. 8 and data not shown). As seen in Fig. 7B, HNF-4A and HNF-4B slightly reduced the activity of the apoB-1800CAT construct, in agreement with previous studies (22). Mutants HNF-HF and CD1 increased the activity of apoB-1800CAT by approximately 2-fold (Fig. 7B), consistent with the results obtained from the GAL-HNF-4 chimeras, indicating that region F functions as a negative regulatory do-
The effects of HNF-4 deletion mutants on the activity of apoCIII-890CAT reporter were also tested in a similar series of experiments. As shown in Fig. 7E, HNF-4A, HNF-4B, and HNF-HF activated the apoCIII promoter by 7-, 9-, and 11-fold, respectively. Furthermore, a 60-fold enhancement in transactivation was obtained by CD1, demonstrating that region F has a negative effect on HNF-4 activity in the context of native promoters (Fig. 7E). In contrast, cotransfections with CD1b and ND1 resulted in loss of apoCIII promoter transactivation, similar to that observed with the apoB reporter constructs. Interestingly, cotransfections with D1HNF-4 resulted in 40% reduction of transactivation, indicating that the contribution of AF-1 to the overall HNF-4 activity may depend on promoter context. Moreover, CD1b reversed the HNF-4B-dependent transactivation of apoCIII-890CAT in a dose-dependent manner (Fig. 7F), demonstrating that it is a potent dominant negative mutant of HNF-4 on a native promoter.

**DISCUSSION**

In this report, we have performed a systematic structural-functional analysis of the orphan nuclear receptor HNF-4. We have mapped the HNF-4 DBD between residues 48–128, which corresponds to the well-studied DBD of members of the nuclear receptor superfamily (2). This domain consists of a 66-residue highly conserved core containing two zinc finger modules (region C), followed by a 9-residue carboxyl-terminal extension. Crystallographic studies of the RXRα-TRβ heterodimer bound on a direct repeat DR4 response element have revealed a polar head-to-tail assembly of the two DBDs on the two repeats (43). The two monomers occupy adjacent major grooves on one side of the DNA double helix, with the carboxyl-terminal extension making extensive minor groove contacts. Since HNF-4 also binds to direct repeats as a dimer, it is reasonable to expect that the HNF-4 DBDs may have a similar structural arrangement on the cognate response elements.

Our results also show that both HNF-4A and HNF-4B isoforms bind to DNA as dimers and that the dimerization domain spans residues 175–360 in region E. Recently, the crystal structure of the unliganded RXRα LBD homodimer has revealed a novel antiparallel α-helical fold consisting of 12 α-helices (H1–H12) (9). The breakpoints between the α-helices are conserved throughout the nuclear receptor superfamily, and this structure evidently represents a prototypic fold of nuclear receptor LBDs (9–11, 44). The dimerization interface is formed primarily by α-helix H10 and to a lesser degree by H9 and a loop between H7 and H8 (9). Based on the high degree of similarity between the RXRα and HNF-4 LBDs and on the predicted high α-helical content of the HNF-4 LBD (data not shown), we have assigned the putative α-helices H3–H12 in the region E of HNF-4 (Fig. 9). According to this analysis, C-terminal deletions up to residue 360 have no effect on dimerization of HNF-4 because they lie carboxyl to H10 and leave the dimerization interface intact. However, the additional deletion of residues 340–360 in mutant CD2 results in abolishment of dimerization. We attribute this loss of dimerization primarily to the removal of tryptophan 340 in H10, because the residue in the corresponding position in the RXRα LBD (Leu340) participates in hydrophobic van der Waals contacts (contacts H10–H10) and is critical for dimerization (9). Interestingly, deletion of residues 175–240 in mutant ND2 removes the α-helices H3, H4, H5, and the β-strand s1, resulting also in loss of dimerization. Although the corresponding region in the RXRα LBD does not participate directly in the formation of the dimerization interface, its effect on HNF-4 dimerization may be attributed to the requirement of the secondary elements encompassed in this region for the correct architectural fold of the HNF-4 LBD.

The loss of DNA binding activity in mutants CD2, CD3, and CD4 and the recovery of this activity in CD5 and CD6 is surprising because all of these mutants contain an intact DBD. These results indicate that, in the absence of the 340–465 region, sequences located carboxyl to residue 174 inhibit efficient DNA binding of HNF-4 monomers. Although disruption of the α-helices that compose the dimerization interface can account for the loss of dimerization, it cannot explain why mutants CD2–CD4 do not bind to DNA as monomers. A similar inhibitory effect of C-terminal sequences on the DBD activity was described previously for the nuclear receptor ARP-1, and a model was proposed to explain the DNA binding and dimerization behavior of this receptor (45). In this model, the DBD is masked by a region in the C-terminal domain of the receptor, thus inhibiting efficient DNA binding of receptor monomers. Upon dimerization, the DBD is unmasked and binds to DNA. It is therefore possible that a similar masking of the DBD by sequences in the E region may also be operational in HNF-4. Dimerization of HNF-4, an event that requires the 334–360 region, will then result in unmasking of the DBD and strong binding to DNA.

Several different types of activation domains have been identified and classified according to the frequency of certain amino acids, including acidic, proline-rich, glutamine-rich, and serine/threonine-rich activators (46). The HNF-4 AF-1 is located in the first 24 amino acids of the receptor, and it is classified as an acidic activator based on the net charge of its amino acid composition. Although it has the potential to adopt an amphipathic α-helical structure, its exact structure within the context of the intact receptor or during its interaction with other proteins is unknown. Interestingly, it has been reported recently that the AF-1 of RARβ2 is also an acidic activator (47), raising the possibility that the AF-1s of HNF-4 and RARβ2 may transduce related signal pathways and may function by similar mechanisms.

In contrast to the small size of AF-1, the HNF-4 AF-2 spans residues 128–366 and is not amenable to further dissection. AF-2 is very complex, and it cannot be classified in any of the
Its high content (23%) in proline residues led to the early identification of a novel negative regulatory function within region \( F \) on the HNF-4 activity. Although the physiologic significance of the inhibitory function of region \( F \) on the HNF-4 activity has not yet been established, we envision that this may define a novel level of regulation of the HNF-4 activity. Although the precise mechanism for this inhibition is unclear, it is conceivable that region \( F \) may mask critical residues of the AF-2 transactivation surface, either by itself or through interaction with another protein(s). An attractive target for such masking is the AF-2 AD because of its absolute requirement for AF-2 function and its proximity to region \( F \). Since the AF-2 AD is essential for AF-2 function, even partial masking of this domain would be sufficient to diminish AF-2 activity. The significant inhibition of the AF-2 activity by the first half of region \( F \) in mutant HNF-HF is consistent with a model involving masking of the surface close to AF-2 AD by sequences within the segment 371–410. Notably, deletion of region \( F \) in mutant CD1 results in an apparent synergistic transactivation of the apoB promoter by AF-1 and AF-2 domains (compare activities of constructs HNF-4B, D1HNF-4, CD1, and DCD1 in Fig. 7B). Interestingly, evidence has been presented recently for a ligand-dependent functional interaction between AF-1 and AF-2 in the estrogen receptor (48). It is therefore possible that a similar mechanism is operational in HNF-4, where region \( F \) may play a role in modulating the degree of interaction between AF-1 and AF-2, thus controlling the transcriptional synergism between these domains. Conceivably, alleviation of the F-mediated inhibition in vivo could be brought about by disruption of its interaction with the protein(s) that causes masking, proteolytic cleavage of \( F \), or conformational changes induced by posttranslational modification(s) or upon binding to a putative ligand. Although the physiologic significance of the inhibitory function of region \( F \) on the HNF-4 activity has not yet been established, we envision that this may define a novel regulatory mechanism that may modulate the transactivation potential of HNF-4 in response to certain signals.
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