Hepatocyte nuclear factor-4α (HNF-4α), a member of the nuclear receptor superfamily, is a crucial regulator of a large number of genes involved in glucose, cholesterol, and fatty acid metabolism. Unlike other members of the superfamily, HNF-4α activates transcription in the absence of exogenously added ligand. Recently published crystallographic data show that fatty acids are endogenous ligands for HNF-4. Transcriptional analysis of point mutations of the residues that are located in helices H3, H5, H10, and H11, which have been shown to come in contact with the ligand, resulted in a dramatic decrease in activity, without affecting DNA binding and dimerization. Our results show the importance of residues Ser-181, Met-182 in H3, Leu-219, Leu-220 and Arg-226 in H5, Ile-338 in H10, and Ile-346 in H11 that line the ligand-binding domain pocket in HNF-4α and impair its transactivation potential. Structural modeling reveals that the mutations do not cause any large scale structural alterations, and the observed loss in transactivation can be attributed to local changes, demonstrating that these residues play a significant role in maintaining the structural integrity of the HNF-4α ligand binding pocket.

The transcription factor HNF-4α (NR2A1), a member of the nuclear hormone receptor superfamily, is a key regulator of diverse metabolic pathways, through regulation of a large number of genes involved in glucose, cholesterol, and fatty acid metabolism (1). Originally identified by its importance in the regulation of liver-specific genes, HNF-4α is also expressed in the pancreas, kidney, stomach, skin, and intestine (2–4). That binds HNF-4α (13). This transcriptional cross-talk between the TGF-β-regulated Smads and HNF-4α may play an important role in various hepatocyte functions that are regulated by TGF-β and Smads (14). Other studies have delineated the key role of HNF-4α in development. In mice, HNF-4α transcripts have been detected as early as day 4.5, whereas knock-out of the gene impairs gastrulation and is embryonic lethal (15). It was shown that HNF-4α, although dispensable for specification of the hepatic lineage, is necessary for the differentiation of hepatocytes in the developing liver (16). Moreover, production of a conditional gene knock-out of HNF-4α has elucidated its central role in the maintenance of the hepatic phenotype in mature hepatocytes and has suggested a putative role for HNF-4α as a lipid sensor for the cell (17).

Like other members of the nuclear receptor superfamily, HNF-4α has a modular structure composed of functional domains. The DNA-binding domain (DBD) C and the ligand-binding domain (LBD) E are homologous to those of other nuclear receptors, sharing the highest degree of similarity with RXRα, with close to 60% sequence identity in the DBD and over 35% identity in the LBD. As shown previously, HNF-4α contains two activation functions, designated AF-1 and AF-2, located in the A/B and D/E regions, respectively. The AF-2 is complex, spanning the LBD region between amino acids 128 and 366. The hinge region (D) of HNF-4α, particularly residues 160–175, was shown to be indispensable for AF-2 activity, as it was the integrity of the AF-2 AD core motif (located in E), which is highly conserved among nuclear receptors (18). Crystallographic studies of nuclear receptor LBDs have revealed an anti-parallel α-helical fold consisting of 12 α-helices (H1–H12) (19, 20). In this model, mainly residues located in helices H3, H5, and H11 form a hydrophobic ligand binding pocket.

HNF-4α activates transcription in the absence of exogenously added ligand. In fact, it was long considered an orphan member of the nuclear receptor superfamily, because a ligand for it had not been definitively identified. Although it was proposed that fatty acyl-CoA thiocysteine acts as ligands for HNF-4α (21), coactivator binding and protease digestion assays suggested that these compounds did not behave as traditional ligands for HNF-4α (22). Nevertheless, when the HNF-4α LBD was crystallized in the absence of added ligands, it was found that the ligand binding pocket contained fatty acids (23). The carboxylic acid head group of the fatty acid ion pairs with the guanidinium group of Arg-226 at one end of the ligand binding pocket, while the aliphatic chain fills a long, narrow channel that is lined with hydrophobic residues. These findings suggest that fatty acids are endogenous ligands for HNF-4.

A number of site-directed mutagenesis studies performed, among others, in RXRα and RARγ, have demonstrated the
functional importance of several LBD amino acid residues in ligand binding and ligand-dependent transactivation (24, 25). Based on these studies, we have investigated the role of homologous residues in HNF-4α-mediated transcription, outside the boundaries of the D and AF-2 AD regions, which were shown to be indispensable for transcriptional activity. Transcriptional analysis of point mutations of the residues that are located in helices H3, H5, H10, and H11, resulted in a dramatic decrease in activity, demonstrating the importance of these residues in generating the correct fold in HNF-4α. Our results show the importance of residues Ser-181, Met-182 in H3, Leu-219, Leu-220, and Arg-226 in H5, Ile-338 in H10, and Ile-346 in H11 (contiguous with H110 in HNF-4α crystal structure (23)) that line the LBD pocket in HNF-4α and impair its transactivation potential. Interestingly, mutagenesis of Arg-212 present in H4, which was originally shown to participate in the stabilization of H12 in RARγ holo-LBD (25) and was critical for transcriptional activity of RARγ, has no dramatic effect on the transcriptional activity of HNF-4α.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—After cloning the GAL-4DBD1-147 into vector pCDNA 3.1 (+) (Invitrogen) at the HindIII and XbaI sites to generate plasmid pCMXGAL-DBD, the wild type HNF-4α-LBD (amino acids 116–371) was cloned into pCMXGAL-DBD at the EcoRI and BamHI sites. All subsequent constructs were generated by PCR. Oligonucleotide-mediated mutagenesis was used to introduce point mutations in specific amino acid residues of the HNF-4α-LBD. For generating the mutants V178G, S181Y, and M182K, PCR reactions were carried out: one containing the primer HNF-4 AFL3 F and the relevant reverse primer LBDMUTGR1 R and the relevant forward primer LBDMUTGR1 F. Aliquots containing 2% of each of the initial PCR reactions were mixed and used for another round of PCR containing the external primers HNF-4 AFL3 F and HNF-4 CLA1 R. The PCR-amplified fragments were then cloned into the EcoRI/HindIII sites of pCMXGAL-DBD carrying the full-length HNF-4α-LBD. Similarly, mutations R212G, L219Q, L220Q, and R226G were generated using the relevant primers LBDMUTGR2 R and LBDMUTGR2 F, and the external primers HNF-4 AFL3 F and HNF-4 CLA1 R and mutations I338F and I346F were generated using the primers HNF-4 CLA1 F, LBDMUTGR3 R, and HNF-4 MSC1 R, LBDMUTGR3 F. All mutations were verified by DNA sequencing analysis. The point mutations are denoted as the amino acid (in one-letter code), its position, and the amino acid with which it was replaced. Table I shows the sequence of oligonucleotides used. Plasmid pG5CAT containing 5 GAL-4 binding sites upstream of the β-globin promoter and the CAT gene was used as a reporter to assay the degree of transactivation.

The full-length HNF-4α-LBD mutants were generated by PCR-mediated site-directed mutagenesis, using appropriate primers and the rat HNF-4α cDNA as template. To construct the desirable mutants, the internal primers presented in Table II were used along with the amino- and carboxyl-terminal primers, HNF-N and HNF-C. The PCR-amplified fragments were cloned into the vector pCDNA 3.1 (+) (Invitrogen) at the HindIII and BamHI sites. All mutations were verified by DNA sequencing analysis. The wild type apolipoprotein CIII promoter (apoCIII-890/+24 CAT) plasmid (26) and a homopolymeric construct, (BA1),CAT, which contains five copies of the regulatory element BA1 in front of the apoB TATA box and the CAT gene (8), were used as reporters to assay the degree of transactivation.

**Cell Transfections and CAT Assays**—Human hepatoma HepG2 cells, monkey kidney COS-7 cells, and human embryonic kidney 293-T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and L-glutamine at 37 °C in a 5% CO2 atmosphere. Plasmids were transfected into COS-7 and/or HepG2 cells and assayed for their ability to promote transcription of the chloramphenicol acetyltransferase (CAT) reporter gene constructs. All transient transfections were performed using the calcium phosphate DNA coprecipitation method, as described previously (8). The pCMV-β-gal plasmid was used as an internal control. CAT activities were determined using [14]C]chloramphenicol and acetyl-CoA as previously described (8). The β-galactosidase activity of the cell lysates was measured, and the values were used to normalize variability in the efficiency of transfection, as previously described (8). The results represent the mean ± S.E. of at least three independent experiments, each carried out in duplicate.

**Western Blot Analysis**—COS-7 and 293-T cells were transfected with the various pCMXGAL-LBD mutants and the pCDNA 3.1-LBD mutants, respectively. Nuclear extracts containing 0.5–10 μg of protein were prepared as described previously (27) and combined with 2× loading buffer (200 mM Tris-HCl, pH 6.5, 4% SDS, 20% glycerol, 10% β-mercaptoethanol, and 0.2% bromphenol blue) in a total volume of 60 μl, 30 μl of which was loaded and electrophoretically separated on a 10% SDS-polyacrylamide gel. Proteins were transferred to an Immobilon P membrane (Millipore, Bedford, MA) by electroblotting. Membranes were precubed in PBS containing 0.1% Tween 20 (PBST), 5% nonfat dry milk, and 0.5% bovine serum albumin for 1 h at 25 °C. Subse-

| Construct | 5′ Primer |
|-----------|-----------|
| LBDMUTGR1F | V178G S181Y M182K |
| LBDMUTGR1R | V178G S181Y M182K |
| LBDMUTGR2F | R212G L219Q L220Q R226G |
| LBDMUTGR2R | R212G L219Q L220Q R226G |
| LBDMUTGR3F | I338F I346F |
| LBDMUTGR3R | I338F I346F |
| HNF-4 AFL3 F | 5′-AGG AAG AAC CAC ATG TAC TCC TGC AGG TTT AGC-3′ |
| HNF-4 CLA1 R | 5′-TTC ATT ATC ATC GAT CTG CAG CTC TTG GAA GGG-3′ |
| HNF-4 CLA1 F | 5′-GCC TTC CAG GTC CAG CTC CTG TGG GAA GGG-3′ |
| HNF-4 MSC1 R | 5′-CAG CAG GTC CAG CTC CTG TGG GAA GGG-3′ |
**LBD Mutations of HNF-4α**

**Table I**

| Construct  | 5’ Primer |
|------------|-----------|
| MUTGR1F    | 5’-GGT TGT GAG TAG ATG AAG GAG CAG-3’ |
| S181Y      | 5’-GGT TGT GAG TCT AAG AAG GAG CAG-3’ |
| M182K      | 5’-CTG CTC TTT CAT ATG ACA ACA CAC-3’ |
| MUTGR1R    | 5’-CTG CTC TTT CAT ATG ACA ACA CAC-3’ |
| S181Y      | 5’-CTG CTC TTT CAT ATG ACA ACA CAC-3’ |
| M182K      | 5’-CTG CTC TTT CAT ATG ACA ACA CAC-3’ |
| MUTGR2F    | 5’-CTG CTC TTT CAT ATG ACA ACA CAC-3’ |
| L219Q      | 5’-CTG CTC TTT CAT ATG ACA ACA CAC-3’ |
| R226G      | 5’-CTG CTC TTT CAT ATG ACA ACA CAC-3’ |
| MUTGR2R    | 5’-CTG CTC TTT CAT ATG ACA ACA CAC-3’ |
| L219Q      | 5’-CTG CTC TTT CAT ATG ACA ACA CAC-3’ |
| R226G      | 5’-CTG CTC TTT CAT ATG ACA ACA CAC-3’ |
| MUTGR3F    | 5’-T CTG CAG AGC TTT ACC TGG CAG AT-3’ |
| I338F      | 5’-G ATC GAG CAG TTT CAG TTT ATC AA-3’ |
| I346F      | 5’-G ATC GAG CAG TTT CAG TTT ATC AA-3’ |
| MUTGR3R    | 5’-ATG AGG TAG GAT GCT CAG A 3’ |
| III38F     | 5’-ATG AGG TAG GAT GCT CAG A 3’ |
| I346F      | 5’-TT GAT GAG CAG TTC CAG TTC ATC AA-3’ |
| HNF-N      | 5’-GAT ATC GAG CCC CCT GGT GGC T-3’ |
| HNF-C      | 5’-TCC AAG CAG CTG GTG CTC ACC AG-3’ |

**RESULTS**

The assignment of Helices H3 to H12 shown in Fig. 1A is based on the crystal structures of HNF-4α (23), HNF-4γ (30), and the highly homologous RXRα (24, 31) and RARγ (25). We examined the role of three residues present in H3, namely Val-178, Ser-181, and Met-182, in HNF-4α-mediated transactivation by mutating residue 178 to Gly, residue 181 to Tyr, and residue 182 to Lys. As seen in Fig. 1B, mutagenesis of the amino acid residues S181Y and M182K caused a severe reduction in the transcriptional potential of HNF-4α in COS-7 cells. Indirectly, the effect of mutation V178G can be seen in the case of the two double mutants V178G/S181Y and V178G/M182K where a further suppression of transactivation is noticed in comparison with the effect of the single mutants. Analysis of these point mutants in HepG2 cells gave similar results to those obtained in COS-7 cells (Fig. 1C).

We also introduced mutations into residues present in helices H4 and H5, namely R212G, L219Q, L220Q, and R226G. Mutagenesis of the Arg-212 present in H4, which was originally predicted to participate in the stabilization of H12 in the RAR holotype (25), had a moderate effect on the transcriptional activity of HNF-4α in COS-7 and HepG2 cells (Fig. 2, A and B). However, loss of transcriptional activity was observed in mutants L219Q, L220Q, and R226G. It is interesting to note that the effect of single mutations in residues 219, 220, and 226 is so severe that the study of the double mutants R212G/L219Q, L219Q/L220Q, and L220Q/R226G and the triple mutant L219Q/L220Q/R226G could not add further information.

Finally, the importance of amino acid residues Ile-338 in H10 and Ile-346 in H11 was examined. Mutagenesis of I338F also resulted in a significant decrease in transcriptional activity in both cell lines (Fig. 3, A and B). However, mutant I346F present in H11 retained 20% of the observed HNF-4α transcriptional activity in COS-7 cells, and 18% in HepG2 cells. Moreover, the effect of the double mutant I338F/I346F represents the effect of I338F, explaining the loss in transcriptional activity. Western blot analysis in COS-7-transfected cells revealed that the wild type and mutant proteins were expressed although in variable amounts (Fig. 4).

Having identified critical amino acid residues with the pCMXGAL-LBD chimera constructs, we then wished to study the effects of these residues in the context of the intact HNF-4α molecule and test their effect on transcription, using the natural apolipoprotein CIII promoter. For this purpose, mutants S181Y, M182K, L219Q, R226G, I338F, and I346F were introduced into the HNF-4α full-length cDNA and cloned into the pcDNA3.1 vector. The pcDNA3.1-LBD mutants were first ex-
amined for their DNA binding and dimerization properties. It is known that HNF-4α/H9251 binds DNA exclusively as a homodimer, and to investigate whether these point mutants retain proper DNA binding, nuclear extracts from cells transfected with the various mutants were used in electrophoretic mobility shift assay experiments, employing the CIIIB element of the apoCIII promoter as a probe, which is a high affinity binding site for HNF-4α (8). This analysis showed that mutants S181Y, L219Q, M182K, I338F, I346F, S181Y/H11001, L219Q/H11001, M182K/H11001, I338F/H11001, and I346F/H11001 do not bind to the probe.

### TABLE III

| Mutation | Space requirements | Hydrogen bonding | Ion pairing | Hydrophobicity | Activity |
|----------|--------------------|------------------|-------------|---------------|----------|
| WT       |                    |                  |             |               | 100      |
| S181Y    | + Clashes with DAO |                  | No          | Polar → nonpolar | 30   |
| M182K    |                    |                  | + With DAO carboxyl | No          | 4   |
| L219Q    | No                 | No               | No          | No            | 6   |
| R226G    | No                 | No               | + With DAO, Met-182 | No          | 4   |
| I338F    | No                 | No               | - With DAO, Leu-235 | No          | No    |
| I346F    | No                 | No               | No          | No            | 70 |

* The mutation causes increase (+) and reduction (−) of space requirements.
* The mutation causes formation (+) and destruction (−) of hydrogen bonds.
* The mutation causes appearance (+) and disappearance (−) of ion pairing.
* Percent transcriptional activities in the range of experimental error.

### FIG. 1

**Transcriptional activity of HNF-4α LBD and its point mutants in helix 3, fused to GAL-4 DBD-(1–147).**

A, schematic representation of the LBD of HNF-4α (helices H3–H12). The positions of the α-helices and corresponding point mutations are indicated. B, the reporter plasmid pG5CAT (3 μg) was transfected into COS-7 cells with pCMV-β-gal (1 μg) and effector plasmids expressing HNF-4α and the indicated point mutants in helix 3, fused in-frame to the yeast GAL-4 DBD-(1–147) (2 μg each). Cells were harvested 36 h later and assayed for CAT and β-galactosidase activities. The relative CAT activity (±S.E.) of three independent experiments is shown in the form of a bar graph, as the percentage of the activity obtained with the pG5CAT reporter construct transfected with WT. A representative CAT assay of one of three experiments is shown at the bottom. C, same as B, in HepG2 cells.
M182K, L219Q, R226G, I338F, and I346F yielded bands of identical electrostatic mobility as the wild type, proving that, like wild type HNF-4α, all mutants bind DNA as homodimers (Fig. 5A). Despite the mutations introduced, it can also be observed that the proteins bind equally well to the probe as the wild type. Furthermore, nuclear extracts from transfected cells expressing CD1b, a truncated HNF-4α protein that was previously shown to retain binding and dimerization (18), were included in the binding assay. The position of CD1b homodimers is indicated in Fig. 5A. To investigate the dimerization properties of the mutants, we employed EMSA analysis to monitor the formation of heterodimers between wild type HNF-4α or the LBD mutant proteins and CD1b. Equal amounts of nuclear extracts from transfected cells expressing the wild type or the various point mutants and CD1b were mixed and then tested for binding to the CIIBB probe. A complex with intermediate electrophoretic mobility (heterodimer) was formed in addition to complexes that corresponded to the homodimers of either HNF-4α wild type/HNF-4α LBD point mutants or CD1b (Fig. 5B). These results demonstrated that one molecule each of LBD mutant and CD1b bound concomi-

tantly to element CIIB and indicated that the point mutations introduced into the LBD of HNF-4α do not affect binding and dimerization. The expression of wild type and mutant proteins was monitored by Western blot analysis in 293-T transfected cells and is shown in Fig. 5C.

Because these mutants are able to heterodimerize with the wild type, we also examined whether they can abrogate endogenous HNF-4α activity in cells where the receptor is expressed naturally. HepG2 cells were transfected with a homopolymeric promoter construct that is responsive to HNF-4α only, either alone, or in cotransfection with the LBD point mutant plasmids. Mutants 182, 219, 226, and 338 drastically reduced the (BA1)-CAT basal activity in HepG2 cells (Fig. 7), suggesting that they can suppress endogenous HNF-4α and act as dominant negative mutants, possibly through heterodimerization with endogenous HNF-4α molecules. This ability of the mutants to suppress endogenous HNF-4α strongly suggests that the mutants affect the activation function of the receptor without interfering with its ability to dimerize and bind target promoters. Interestingly, transfection with mutants 181 and 346 resulted only in a moderate reduction in transcription, consistent with our previous results (Fig. 6), where these mutants retain 30 and 70% of wild type activity, respectively.

Because the DNA binding and dimerization properties are not affected by the mutations, it is implied that the mutations do not cause any large scale structural alterations retaining the overall shape of the molecule. Therefore we can attribute any effects on the activity to local structural changes extending their effects in a distance of up to 8 Å, and to changes in the chemical and physical properties of the mutated residues. We have modeled mutations S181Y, M182K, L219Q, R226G, I338F, and I346F to examine their effects on the environment in respect to (a) changes in the interactions with the fatty acid and (b) changes in interactions not including the fatty acid. Table III summarizes these results. The model structure obtained for each mutation indicates the expected changes in the local environment and allows conclusions about how the mutation can be related to the functional effect.

Regarding S181Y, we realized that tyrosine forms clashes with the fatty acid (Fig. 8) that can lead to a small change in the shape of the LBD pocket. Moreover, in contrast to serine, the nonpolar tyrosine does not hydrogen bond with the fatty acid, rendering the capturing and correct positioning of the ligand less possible. This can explain the observed reduction in transcriptional activity to 30% of the wild type (Fig. 6).

Mutation M182K also alters a residue that comes in direct contact with the ligand. Lysine occupies less space than the bulky methionine and due to its ability to hydrogen bond with Met-342 (Fig. 9) an approaching of helices H10 and H3 becomes possible. On the other hand the charged lysine has the potential to ion pair to the fatty acid head group, thus competing with Arg-226. This can lead to a mispositioning of the fatty acid. These changes can affect both the way the ligand is captured and positioned and the shape of the LBD pocket. As expected, this mutation abolishes transcriptional activity (Fig. 6).
Fig. 3. Transcriptional activity of HNF-4α LBD and its point mutants in helices 10 and 11, fused to GAL-4 DBD-(1–147). A, the reporter plasmid pG5CAT (3 µg) was transfected into COS-7 cells with pCMV-β-gal (1 µg) and effector plasmids expressing HNF-4α and the indicated point mutants in helices 10 and 11, fused in-frame to the yeast GAL-4 DBD-(1–147) (2 µg each). Cells were harvested 36 h later and assayed for CAT and β-galactosidase activities. The relative CAT activity (±S.E.) of three independent experiments is shown in the form of a bar graph, as the percentage of the activity obtained with the pG5CAT reporter construct transfected with WT. A representative CAT assay of one of three experiments is shown at the bottom. B, same as A, in HepG2 cells.

Fig. 4. Detection of the expression of wild type pCMXGAL-LBD and mutated proteins in COS-7 cells by Western blot analysis. COS-7 cells were transfected with the indicated pCMXGAL-LBD chimeras (6 µg). Protein extracts corresponding to ~0.5 × 10⁶ cells/lane were analyzed by SDS-polyacrylamide gel electrophoresis, followed by transfer to a polyvinylidene difluoride membrane. The expression of pCMXGAL-LBD chimeric proteins was detected by using a rabbit anti-GAL-4 DBD polyclonal antibody and, as secondary antibody, goat anti-rabbit IgG conjugated to horseshadish peroxidase, as described under “Experimental Procedures.” Numbers indicate molecular mass protein markers in kilodaltons, and each chimeric protein expressed is marked at the top of the corresponding lane. The arrow indicates the position of pCMXGAL-LBD chimeras (upper band). The first lane contains extract from cells transfected with vector pCMXGAL-DDB alone.

Regarding L219Q, which also leads to a dramatic loss of activity (6%), we realized that the polar glutamine does not introduce significant changes in space requirements. However, it can form hydrogen bonds with the fatty acid and Met-182 in H3 that can lead to a mispositioning of the fatty acid, as well as a deformation of the LBD pocket due to an approaching of H5 and H3.

Mutation R226G changes the positively charged arginine to nonpolar glycine, which does not hydrogen bond or ion pair with the fatty acid head group. This can lead to a loss of the ligand binding capacity of the pocket or a mispositioning of the ligand. As expected, this mutation also leads to a severe loss of transcriptional activity (Fig. 6).

Mutations I338F and I346F do not introduce any changes in the direct interaction with the ligand. In the case of I338F, the expected increase in distance between H3, H5, and H10 due to clashes formed by phenylalanine with Leu-220 and Val-255 (Fig. 10) seems to be critical, because this mutation abolishes activity (Fig. 6). In contrast, I346F has a moderate effect, retaining 70% of the activity, because phenylalanine forms only minor clashes with Met-182 (H3). Our results lead to the conclusion that both the structural integrity of the LBD pocket and the appropriate positioning of the ligand are imperative for HNF-4α transcriptional activity.

DISCUSSION

The discovery of the prototypic fold of nuclear receptor ligand-binding domains was a major breakthrough in the understanding of ligand-receptor interactions and the mechanisms, which underlie the activation of transcription of target genes in response to various stimuli. It was thus of special interest to investigate how the conformation predicted by the mousetrap model (32), would apply to the so-far considered orphan receptor HNF-4α. Although it was suggested that fatty acyl CoA thioesters could act as potential ligands for HNF-4α (21), these compounds were not examined for their ability to alter the conformation of HNF-4α or the interaction with coregulatory molecules, such as SRC-1, GRIP-1, and CBP/p300 (33, 34). Such properties are considered to be important for traditional ligands, so the previous proposal was questioned and the existence of a ligand remained an open question. In this context it was challenging to pinpoint specific amino acid residues, which are important for generating the correct fold in the LBD of HNF-4α and could also come in contact with a potential endogenous ligand.

Importantly, while we were carrying out experiments to examine the effect of LBD mutations on transcriptional activation, the crystal structures of the HNF-4α (30) and HNF-4α (23) LBDs were resolved. Interestingly, the HNF-4 LBD crystals contained endogenous fatty acid ligands, which could not be displaced from the crystals. Thus ligand binding by HNF-4 sets a novel paradigm in the nuclear receptor superfamily, because in this case the ligand appears to serve as a structural prerequisite for the correct folding of the constitutively active protein, rather than serving as a switch for transition to its active state. In the crystals HNF-4α forms homodimers, and the two molecules in each homodimer adopt distinct conformations, the so-called “open” and “closed” forms, in which H12 is either fully extended into the solvent and collinear with H10 or packed against the body of the receptor, respectively (23).

In our study mutations in residues Val-178, Ser-181, and Met-182, located in helix 3, impaired HNF-4α-mediated transcriptional activation (Figs. 1 and 6). These residues are involved in ligand binding, as shown by crystal structure analysis of the HNF-4α LBD (23). The homologous residue to Met-182 in RXRα, Ala-272, was shown to participate in the correct positioning and stabilization of H12 following ligand binding in the crystal structure of the receptor, although its effect on transcriptional activity was not examined (31).

Interestingly, crystallographic analysis demonstrated Met-182 to have a unique role among nuclear receptors in the
HNF-4 LBD. In the ligand binding pocket of HNF-4, a direct contact was found between Met-143 in H3 and Met-302 in H11. This contact bridged the binding pocket forming a cleft onto which H12 is packed, thus possibly stabilizing the LBD in its active conformation and effectively blocking direct ligand access to H12 (30). This effect comes in contrast with other nuclear receptors where H12 contacts the ligand. As it was further found in the crystal structure of HNF-4 LBD, the side chains from the homologous residues Met-182 and Met-342 fill the upper portion of the HNF-4 LBD ligand binding pocket and prevent the ligand from adopting the particular shape identified in the 9-cis RA (23). Consequently, the two methionine residues may play a significant role in determining ligand binding specificity in HNF-4. As mentioned above, mutagenesis of one of these residues, M182K, leads to severe loss of transcriptional activation indicating the importance of this residue in the stabilization of the HNF-4 structure. Structural modeling of the mutation Lys-182 in Fig. 9 shows that a lysine in position 182 can form a hydrogen bond with Met-342 in H10, enhancing the existing interaction between H3 and H10, without causing any change in space requirements. Therefore, the loss in transcriptional activity can only be attributed to one of the following: in the first case, similar to the WT, Arg-226 ion pairs with the fatty acid head group contributing to its correct positioning, but the positive charge of Lys-182 prevents the correct positioning of the hydrophobic tail in the rest of hydrophobic pocket (Fig. 9). On the other hand, Lys-182 can ion pair with the carboxylic acid head group. This would lead to a mispositioning of the fatty acid, and as a result H3 and H5 would approach each other, thus changing the shape of the pocket.

Changes in amino acid residues Leu-219, Leu-220, and Arg-226, located in helix 5, played also a significant role in HNF-4 mediated transactivation (Figs. 2 and 6). Importantly, residues Leu-219, Leu-220, and Arg-226 come into direct contact with the ligand in both open and closed forms of the receptor (23). On the other hand, the homologous residues Leu-309, Ile-310, and Arg-316 in RXRα were shown to interact directly with the ligand in the holo-RXRα crystal (31). In particular, Arg-316 was shown to be involved in an ionic interaction with the carboxylate group of 9-cis-RA. The RA carboxylate group was also found to participate in a water-mediated hydrogen bond network involving, among others, the backbone carbonyl group of Leu-309. Furthermore, mutation of residues Ile-310 and Arg-316 in human RXRα showed that they severely affect the...
affinity for the RA ligand and are thus critical for RA-dependent transactivation (19).

From structural modeling analysis it is obvious that in mutation 219 changing the hydrophobic leucine into a polar glutamine does not introduce significant changes in space requirements. However, the glutamine can hydrogen bond with the fatty acid and the residue Met-182 in H3, which can lead to a mispositioning of the ligand, as well as a deformation of the ligand binding pocket, because H5 and H3 approach each other. This can explain the marked effect of mutation L219Q in HNF-4α transcriptional activity.

Also, amino acid Arg-226, indicated by our study to eliminate HNF-4α transactivation potential when mutated to glycine, is shown to be a key residue in the correct positioning of the ligand in the LBD pocket, because both oxygen atoms of the fatty acid head group are ion paired to the guanidium group of Arg-226 (23). Structural modeling reveals that the hydrophobic glycine is unable to capture the ligand in the correct position. In addition, its presence poses less space requirements, which can shift the ligand’s head group closer to the polar Ser-181 and eventually cause the hairpin Asp-232 to Arg-244 and helix H5 to approach each other, resulting again in a change of the shape of the pocket. Therefore, it is not surprising that we found a drastic reduction in activity.

As can be seen in the crystal structure, residue Ser-181 forms additional hydrogen bonds with one of the oxygens of the carboxylic acid head group. By mutating this residue into tyrosine we observed a significant loss of transcriptional activity (by 70%), which can be attributed both to a loss of the capturing and orientation capacity of the pocket (no hydrogen bonding with the fatty acid carboxylic group) and to the clashes between the aromatic ring of the tyrosine and the fatty acid (Fig. 8). As a result, the LBD cannot be folded correctly to adopt a transcriptionally active conformation. Analysis of residues Ile-338 in H10 and Ile-346 in H11 showed that, although mutant I338F completely lost its ability to activate transcription, mutant I346F maintained a high degree of transcriptional activity compared with the wild type (Figs. 3 and 6).

Mutation I338F abolishes activation, although it does not change direct interaction with the fatty acid. However, as seen...
in different structural requirements for each receptor.

This conclusion is further supported by mutation I346F that leads to an activity loss of 30% (Fig. 6), although it introduces only minor clashes with Met-182. The corresponding residue to Ile-346 in RXRα is Leu-436, which forms part of the lid of the ligand binding cavity and stabilizes H12 in the presence of the ligand (31). As mentioned earlier, Ala-272 is also involved in a similar H12-stabilizing interaction. Thus, both residues have a similar function, stabilizing H12 in the active state position in the RXRα LBD, whereas their homologous residues, Ile-346 and Met-182, appear to have a different role in HNF-4α, as indicated by the different effects of these mutations on trans-activation. The HNF-4α crystal structure shows that, in the closed conformation, H12 is packed onto the body of the receptor, where it is partly stabilized through interactions with hydrophobic residues present in other helices like Met-182 in H3 and Leu-219 in H5 (23). However, no such interactions are observed with Ile-346. This supports the suggestion that there may be specific differences in the ligand binding characteristics of even highly homologous nuclear receptor LBDs, which result in different structural requirements for each receptor.

This notion is further supported by mutation R212G in H4 of HNF-4α. The corresponding amino acid residue in RARγ, Lys-264, was shown to form a crucial salt bridge with Glu-414 in H12, thus stabilizing the H12 lid onto the ligand binding pocket (25). In HNF-4α, H12 is anchored by two hydrogen bonds in the closed conformation, one between Glu-362 in H12 and Lys-350 in H10, and the other between Glu-363 in H12 and Arg-212 in H4 (23). Despite the similar role of the homologous residues in the two receptors, mutation of Lys-264 was shown to abolish RARγ activity (25), whereas mutation R212G maintains transcriptional activation potential in our study (Fig. 2). This difference may be explained by the contribution of the additional hydrogen bond between H10 and H12 in HNF-4α, which can still anchor H12 in the closed conformation.

In conclusion, our mutagenesis results clearly demonstrate the overall resemblance of the amino acid residues that are critical for generating the correct fold in the LBDs of the nuclear receptor superfamily, while pointing to certain differences that also exist, related to the ligand binding specificity of each receptor. Furthermore, they can help verify and expand predictions based on the crystallographic data, as to the importance of specific amino acid residues in the functional role of HNF-4α. Given the crucial role that HNF-4α plays in mature liver function and regulation of metabolism, it would be interesting to investigate further the involvement of specific amino acid residues in the role that other important regulatory molecules may have on HNF-4α activity, such as coactivators.

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