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Exclusive Homodimerization of the Orphan Receptor Hepatocyte Nuclear Factor 4 Defines a New Subclass of Nuclear Receptors

GUOQIANG JIANG, LUVIMINDA NEPOMUCENO, KERI HOPKINS, AND FRANCES M. SLADEK*

Environmental Toxicology Graduate Program, University of California, Riverside, Riverside, California 92521

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Hepatocyte nuclear factor 4 (HNF-4), a highly conserved member of the steroid hormone receptor superfamily critical for development and liver-specific gene expression, is very similar to another superfamily member, retinoid X receptor α (RXRα), in overall amino acid sequence and DNA binding specificity. Since RXRα is known to heterodimerize with many other nuclear receptors, the formation of heterodimers between HNF-4 and RXRα was examined. With the electrophoretic mobility shift assay, coimmunoprecipitation, and transient transfection assays, it is shown that, unlike other nuclear receptors, HNF-4 does not form heterodimers with RXRα either in the presence or in the absence of DNA. We also show that in vitro-translated HNF-4 does not form heterodimeric complexes on DNA with a number of other receptors, including RXRβ, RXRγ, retinoic acid receptor α, or thyroid hormone receptor α. To investigate the hypothesis that the lack of heterodimerization between HNF-4 and RXRα is due to a strong homodimerization activity of HNF-4, glycerol gradient sedimentation and kinetic analysis were used to show that HNF-4 is in fact a stable homodimer in solution. Finally, immunohistochemistry is used to show that the HNF-4 protein is found exclusively in the nuclei in both HepG2 cells, which express endogenous HNF-4, and transfected COS cells, which overexpress HNF-4. These findings lead us to propose that HNF-4 defines a new subclass of nuclear receptors which reside primarily in the nucleus and which bind DNA and regulate transcription as homodimers.

Hepatocyte nuclear factor 4 (HNF-4) is a positive-acting transcription factor which is expressed very early in embryo development and is essential to liver development and function (reviewed in references 84 and 85). Mouse HNF-4 mRNA appears in the primary endoderm of implanting blastocysts at embryonic day 4.5 and in the liver and gut primordia at day 8.5 (20), while mice deficient in HNF-4 do not survive past day 9 postcoitus (12). HNF-4 has also been proposed to be responsible for the final commitment for cells to differentiate into hepatocytes (68). In adult rodents, HNF-4 is located primarily in the liver, kidney, and intestine, and in insects HNF-4 is found in the equivalent tissues (86, 95). HNF-4 is known to activate a wide variety of essential genes, including those involved in cholesterol, fatty acid, and glucose metabolism; blood coagulation; detoxification mechanisms; hepatitis B virus infections; and liver differentiation (reviewed in references 84 and 85).

HNF-4 is a member of the superfamily of ligand-dependent transcription factors, which includes the steroid hormone receptors, thyroid hormone receptor (TR), vitamin A receptor, and vitamin D receptor (VDR), as well as a large number of receptors for which ligands have not yet been identified, the so-called orphan receptors (reviewed in references 56, 72, 73, and 88). All receptors are characterized by two conserved domains: the zinc finger region, which mediates DNA binding, and a large hydrophobic domain which mediates protein dimerization, transactivation, and ligand binding. Whether HNF-4 responds to a ligand is not known, but it has been shown to activate transcription in the absence of an exogenously added ligand (38, 51, 65, 66, 79, 86). HNF-4 is also highly conserved with the *Drosophila* HNF-4, containing 91% amino acid sequence identity to the rat HNF-4 in the DNA binding domain and 68% identity in the large hydrophobic domain (95).

The members of the receptor superfamily have been classified in a variety of ways, one of which is by their ability to dimerize with themselves and with other members of the superfamily. For example, the steroid hormone receptors, glucocorticoid, mineralocorticoid, and progesterone receptors (GR, MR, and PR, respectively), all bind DNA and activate transcription as homodimers. They are present in the cytoplasm complexed with heat shock proteins (HSP) until the presence of the appropriate ligand disrupts the complex, allowing the receptors to translocate to the nucleus (reviewed in references 26, 71, and 88). On the other hand, the retinoid acid receptor (RAR) and retinoid X receptor (RXR) as well as the VDR, peroxisome proliferator-activated receptor (PPAR), and TR, which do not bind HSP and reside primarily in the nucleus, all bind DNA and activate transcription not only as homodimers but also as heterodimers (reviewed in references 30, 73, and 87). In fact, several of the nuclear receptors bind DNA very inefficiently, if at all, as homodimers (RXRα, RAR, VDR, TR, and PPAR) but bind DNA well as heterodimers (reviewed in references 30 and 87). At least two of the receptors (RAR and TR) form heterodimers in solution with RXRα (42, 53, 94). The most common dimerization partner for all of these receptors is RXRα. The third class of receptors identified to date reside in both the nucleus and the cytoplasm and bind DNA preferentially as monomers (NGFI-B, FTZ-F1, steroidogenic factor 1 [SF-1], and RORα1) (31, 52, 70).

HNF-4 is very similar to the retinoid receptors, in particular to RXRα, in both amino acid sequence and DNA binding specificity. Mouse RXRα is 60% identical to rat HNF-4 in the DNA binding domain and 44% identical in the large hydrophobic domain (see Fig. 1). In comparison, RARα, which readily heterodimerizes with RXRα, is 61% identical to RXRα in the DNA binding domain and only 27% identical in the large hydrophobic domain (63). HNF-4 and RXRα have also been shown to share response elements from at least six dif-

* Corresponding author. Mailing address: 5419 Boyce Hall, Environmental Toxicology Graduate Program, University of California, Riverside, Riverside, CA 92521. Phone: (909) 787-2264. Fax: (909) 787-3087. Electronic mail address: Sladek@mail.ucr.edu.

ferent genes as well as a consensus site of a direct repeat of AGGTCA separated by one nucleotide (referred to as DR+1) (7, 8, 27, 29, 38, 39, 46, 54, 60, 69, 90). The structural and functional similarities of HNF-4 and RXRα suggest that HNF-4 might heterodimerize with RXRα and/or other receptors.

In this report, electrophoretic mobility shift analyses (EMSA) of HNF-4 and RXRα proteins expressed in vivo and in vitro show that HNF-4 in fact does not heterodimerize with RXRα on any one of a number of response elements and that while HNF-4 forms homodimers in solution in the absence of DNA, it does not form heterodimers with RXRα. It is also shown that HNF-4 does not heterodimerize with a number of other receptors on DNA, suggesting that the lack of heterodimerization is a general property of HNF-4. In order to investigate the reason for the lack of heterodimerization, glycerol gradient sedimentation and an order-of-addition experiment are used to show that HNF-4 is a fairly stable homodimer in solution. Finally, immunohistochemical staining verifies that HNF-4 resides exclusively in the nucleus. These results lead us to propose that HNF-4 defines a new subfamily of nuclear receptors which are present exclusively in the nucleus, exist in solution, bind DNA as homodimers, and do not form heterodimers with RXRα or other receptors. The implications of this classification are discussed.

MATERIALS AND METHODS

Reagents and procedures. Light-isomerized retinoic acid (RA+lt) was prepared by exposing all-*trans* retinoic acid (10 mM in 100% ethanol) to incandescent light (100-W bulb with aluminum foil reflectors) for 1 h at 4°C with stirring. Stock solutions were stored under N_2 gas at −20°C in the dark. A similar treatment has been reported to yield ~10% 9-*cis* retinoic acid, the ligand for RXRα (43). Standard PCR, cloning, labeling, DNA sequencing, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) techniques were used throughout this study (4, 82). Fuji medical X-ray film (RX) was used for all autoradiography.

Production and purification of antisera. Antiserum to a glutathione *S*-transferase (GST) fusion protein containing amino acids 84 to 419 of rat HNF-4 (referred to hereafter as α84-419 antiserum) was prepared by subcloning an *Afl*III-*Pfl*MI restriction fragment of rat HNF-4 into pGEX2T (Pharmacia, Piscataway, N.J.) with *Eco*RI linkers. The fusion protein was overexpressed in *Escherichia coli*, purified by using a glutathione agarose column as previously described (4), and subcutaneously injected into a New Zealand White rabbit with complete Freund's adjuvant. The resulting antiserum was prepared by standard procedures (41). Antisera to α445 and RXRα are described in the legend to Fig. 1. Affinity-purified α445 was prepared by passing α445 antiserum over a column containing the peptide to which the antiserum was raised (86). The procedure was done essentially as previously described (41), except that the antiserum was eluted with either 0.1 M glycine (pH 2.5) or 4.3 M $MgCl_2$ (pH 6.5). The final antibody concentration was approximately 0.5 mg/ml.

Oligonucleotides, expression vectors, and reporter constructs. The double-stranded oligonucleotides used as probes were the apolipoprotein AI (ApoAI) site A element (referred to hereafter as ApoAI site A) from −192 to −205 of the human *apoAI* gene (80); APF1, from −66 to −87 of the human *apoCIII* gene (86); and SS TRE, a synthetic thyroid hormone response element containing two strong TR half-sites (AGGTAA) arranged as a direct repeat separated by four nucleotides (24). All probes contained a 4-base overhang at the 5′ end to facilitate labeling. The sequences of the hormone response elements in these oligonucleotides are given in Table 1.

pSG5 expression vectors (35) were used for transient cotransfection experiments, and pMT2 expression vectors (45) were used for overexpression of proteins in COS-7 cells for EMSA and immunoprecipitations. Rat HNF-4 cDNA from the original clone pf7 (86) was subcloned into pSG5 at a unique *Bam*HI site and was previously subcloned into pMT2 at a unique *Eco*RI site (66). Human RXRα cDNA (64) was subcloned into the pMT2 and pSG5 expression vectors at unique *Eco*RI sites. HNF4.390 was constructed by PCR with pf7 as the template and as primers oligonucleotides Npf7, corresponding to the N terminus of the HNF-4 coding sequence (5′-GCGCGGATCCATGGACATGGCTGAC-3′), and C390A, corresponding to amino acids 386 to 390 of rat HNF-4 (5′-GCGGATCCCATGTGTTCTTGC-3′) (added *Bam*HI sites are underlined). The HNF4.390 PCR product was subcloned into the pSG5 vector at the *Bam*HI site, sequenced, and then subcloned into the pMT2 vector at the *Eco*RI site with the use of *Eco*RI adapters (Promega, Madison, Wis.). The reporter construct ApoAI.Luc, consisting of two copies of ApoAI site A linked to positions −41 to +397 of the human *apoAI* gene driving the firefly luciferase gene, was a generous gift from J. F. Grippo (pSP45).

Transient transfections. COS-7 cells (ATCC CRL 1651) were maintained at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum (BCS). The cells were transiently transfected via $CaPO_4$ precipitation as previously described (86), with some modifications. Cells (10^6) were plated in each well of a six-well plate (Falcon 3046) and incubated in 2.5 ml of DMEM supplemented with 10% BCS at 37°C for 6 to 8 h. A $CaPO_4$ precipitate (0.5 ml) containing the reporter construct (2 μg), the expression vector(s) (1 to 8 μg), the internal control cytomegalovirus β-galactosidase (β-Gal) (1 μg), and pGEM DNA (up to 25 μg total) were then added. After approximately 17 h at 37°C, the cells were shocked with 15% glycerol and then fed 1.5 to 2.0 ml of DMEM plus 10% stripped serum (controlled process serum replacement [CPSR]; Sigma, St. Louis, Mo.), 10 mM sodium butyrate to enhance transcription (34), and either ethanol (to 0.1%) or RA+lt (1 or 5 μM). The cells were harvested 24 h later with a Triton lysis buffer (300 μl). The luciferase activity in the extracts was determined with a luminometer, and the β-Gal activity was determined with a microplate reader, as previously described (6, 82). Relative levels of gene induction were calculated by using relative light units normalized to β-Gal activity.

Overexpression of proteins in COS cells. pMT2 expression vectors containing the various amounts (5 to 25 μg) of cDNA were electroporated at 240 V and 1080 μF (ProGenetor II; Hoefer, San Francisco, Calif.) into COS-7 cells (1.8×10^7/0.8 ml of DMEM), plated in two 100-mm-diameter petri dishes in DMEM plus 10% BCS, and harvested 20 to 24 h later. For experiments with RA+lt, the medium was changed to DMEM plus 10% CPSR and either ethanol (to 0.1%) or RA+lt (1 or 5 μM) approximately 8 h after electroporation. Nuclear extracts were prepared on ice by washing the cells on the plate twice in phosphate-buffered saline (PBS) and then lysing the cells in 0.5 ml of hypotonic solution {0.25× buffer H where 1× buffer H consisted of 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.9), 25 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, and 1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid]}. After 5 to 10 min, the plates were scraped, and the lysates were transferred to tubes containing an equal volume of 2× buffer H plus 20% glycerol and centrifuged at 2,500 rpm for 10 min. After the supernatant (cytoplasmic extract) was removed, the nuclear pellet was resuspended in approximately 30 μl of low-salt buffer (1× buffer H plus 20% glycerol). The nuclei were extracted by adding 0.72 volume of high-salt buffer (1× buffer H plus 1 M KCl and 20% glycerol) dropwise with mixing followed by gentle rocking at 4°C for 45 to 60 min. The extracts were centrifuged at 12,000 rpm for 20 min, and the supernatant (nuclear extract, approximately 75 μl per 100-mm-diameter plate) was removed, assayed for total protein concentration via the Bio-Rad assay (usually 1 to 2 μg/μl), aliquoted, snap frozen on powdered dry ice, and stored at −75°C. For whole-cell extracts, cells harvested in PBS were resuspended in 2× buffer H plus 20% glycerol and then lysed by repeated freeze-thawing (four times) in a dry ice-ethanol bath. The cellular debris was removed by centrifugation at 12,000 rpm for 15 min, and the supernatant was recovered (approximately 300 μl per 150-mm-diameter plate) and processed as described above. All solutions, except PBS, contained fresh 1 mM dithiothreitol and 0.5 mM phenylmethylsulfonyl fluoride. All centrifugations were done in a Sorvall MC 12V microcentrifuge (Du Pont) at 4°C.

In vivo labeling with [^{35}S]methionine. COS cells were plated in DMEM plus 10% BCS after electroporation and then switched to DMEM containing 2% CPSR after approximately 8 h. At approximately 16 h, the medium was removed, and the cells were washed twice with PBS and then incubated with 5 ml of methionine- and cysteine-deficient DMEM (ICN, Costa Mesa, Calif.) for 30 min. Fresh cysteine-deficient medium containing 0.25 mCi of [^{35}S]Met (Trans ^{35}S-label; ICN) per ml was then added, and the incubation was continued for 3 to 4 h. It was previously determined that peak protein expression from the pMT2.HNF4 vector occurs at 20 to 22 h postelectroporation (data not shown). Nuclear and cytosolic extracts were prepared as described above.

Coimmunoprecipitation with HNF-4 protein and antibodies. ^{35}S-labeled extracts from COS cell transfections were precleared as follows. Nuclear extracts (15 μl at approximately 1 μg/μl) and preimmune rabbit antiserum (2 μl) in 50 μl of Ab/Ag buffer (50 mM Tris-HCl [pH 8.0], 1 mM EDTA, 0.2 mM EGTA, 1 mM dithiothreitol, 10% glycerol, 0.5 mM phenylmethylsulfonyl fluoride) were incubated for 30 min. Protein A agarose (37 μl of a 50% suspension; Sigma) was added, the incubation was continued for 30 min, and then the mixture was pelleted at 12,000 rpm for 5 min. The precleared extracts were incubated for 30 min at 4°C (no rocking) with wild-type rat HNF-4 produced in insect cells by using a baculovirus expression system (HNF4.BV) (7 μg of total protein of a nuclear extract prepared as the COS extracts, of which ~10% was rat HNF-4) (44a). Affinity-purified α445 antiserum (2 μl per reaction) was added, and the incubation was continued for 1 h. Protein A agarose (25 μl) was added, and the incubation was continued for 3 h. The precipitates were washed twice with 0.5 ml of NET* buffer (50 mM Tris-HCl [pH 7.5], 50 mM NaCl, 0.1% Nonidet P-40, 1 mM EDTA, 0.2% bovine serum albumin [BSA]) and once in 10 mM Tris-HCl (pH 7.5)–0.1% Nonidet P-40. Precipitates were resuspended in SDS loading buffer and loaded onto a 10% polyacrylamide–SDS gel. After electrophoresis, the gel was treated with En^{3}Hance (NEN/Du Pont, Boston, Mass.), dried for 2 h at 75°C, and autoradiographed as indicated. All centrifugations were done in

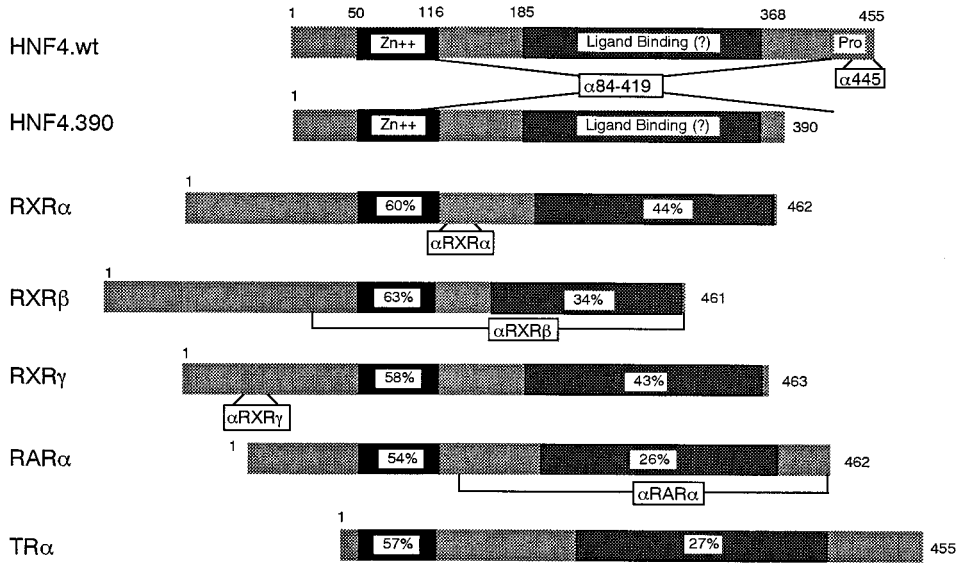

FIG. 1. Schematic representation of receptors used in this study and antisera that distinguish them. Numbers indicate amino acids in HNF4.wt (86), an HNF-4 mutant truncated at amino acid 390 (HNF4.390) (this study), human RXRα (64), human RXRβ (23), mouse RXRγ (63), human RARα (76), and rat TRα (67). Percentages indicate amino acid identity with HNF-4 in the conserved zinc finger-DNA binding and the ligand binding-dimerization domains. Antisera to α445 (86), αRXRα (48), and αRXRγ were raised against synthetic peptides to the regions indicated; α84-419 was raised against a GST fusion protein containing amino acids 84 to 419 of HNF-4. Antisera against RXRβ and RARα (49) were raised against bacterial fusion proteins corresponding to large portions of the receptors. All antisera recognize only the receptors shown. Antisera to RXR and RAR were gifts from J. Dyke and R. Evans, Salk Institute.

a Sorvall MC 12V microcentrifuge at 4°C. All incubations were done at 4°C with gentle rocking on a Nutator unless noted otherwise.

EMSA. Shift reactions and gel analysis were carried out as previously described, with minor modifications (86). A standard mobility shift reaction mixture (15 μl) contained crude nuclear or cytoplasmic extract (1 to 5 μg of total protein) incubated at room temperature (RT) with 0.5 ng of ^{32}P-labeled probe and nonspecific DNA [1 μg of poly(dIdC), 0.1 μg of sonicated denatured salmon sperm DNA, 25 ng of the nonspecific oligonucleotide −175 TTR] (86). The probes were double-stranded oligonucleotides treated with T4 polynucleotide kinase and [γ-^{32}P]ATP (ICN). Antisera (1 μl, undiluted or diluted up to fourfold in 3% BSA in PBS plus 0.02% azide) were added after 15 min, and the incubations were continued for another 15 to 30 min before 3 μl was loaded onto an 8% polyacrylamide gel in 25 mM Tris-borate–0.25 mM EDTA. Gels were preelectrophoresed at 300 V at 4°C for approximately 40 min, loaded, and run for 1.5 h at RT at a constant current of 12 mA (300 to 400 V). After drying, the gels were subjected to autoradiography for 14 h to 3 days at RT unless noted otherwise.

Western blot (immunoblot) analysis. Proteins separated by SDS–10% PAGE were transferred to a polyvinylidene difluoride membrane (Immobilon; Millipore) in a Bio-Rad Minigel Transfer Apparatus set at 20 V for 10 min and then at 60 V for 30 min at 4°C as previously described (4). The blot was probed essentially as previously described (4), with the following modifications. The membrane was preincubated in blocking buffer (5% nonfat dried milk, 2.5% Na deoxycholate, 1.0% Nonidet P-40, and 0.1% SDS in 1× TTBS [4]) and then incubated for 1 h with a 1:2,500 dilution of α445 antiserum in blocking buffer. The blot was washed in blocking buffer without milk and then in 5% milk in 1× TTBS and incubated for 1 h in the same buffer with a 1:5,000 dilution of goat anti-rabbit immunoglobulin G Fab′ conjugated to alkaline phosphatase (Jackson Laboratories). The blot was washed three times in 1× TTBS and developed with nitroblue tetrazolium and BCIP (5-bromo-4-chloro-3-indolylphosphate toluidinium).

RESULTS

HNF-4 and RXRα from liver produce overlapping shift complexes. Previous reports (29, 42, 90) and our own unpublished data demonstrated that both HNF-4 and RXRα bind to the same element (site A) in the *apoAI* promoter. Therefore, crude rat liver nuclear extracts were examined for the presence of HNF4:RXRα heterodimers by EMSA with the ApoAI site A as a probe and antisera specific to the two receptors, as described in the legend to Fig. 1. The results in Fig. 2 show that the extracts yielded two prominent shift complexes as well as one diffuse complex, all of which competed with the unlabeled ApoAI site A (lanes 1 and 2, respectively). Antiserum specific to HNF-4 shifted even further (supershifted) the top prominent complex, leaving the bottom complex and the diffuse complex (lane 4). Antiserum specific to RXRα, on the other hand, eliminated at least part of the diffuse complex but did not affect most of the top complex or any of the bottom complex (lane 5). Nuclear extracts from COS cells in which RXRα was overexpressed also showed a diffuse complex that migrated in a fashion similar to that from the rat liver extracts, further identifying the diffuse complex as containing RXRα (lanes 6 and 7). The fact that the RXRα antiserum appeared to eliminate part of the complex with which the HNF-4 antiserum reacted suggested that there might indeed be HNF-4:RXRα heterodimers present in the rat liver extracts. However, it was also possible that the intensity of the HNF-4 shift complex was diminished by the RXRα antiserum simply because it overlapped the diffuse complex.

A truncated HNF-4 protein, HNF4.390, readily forms dimers with full-length HNF-4 but not with RXRα. In order to better distinguish the HNF-4 and the RXRα shift complexes, a truncated HNF-4 protein that contained the first 390 amino acids of rat HNF-4, HNF4.390, was generated (Fig. 1). When HNF4.390 was overexpressed in COS cells, it yielded a shift complex which migrated substantially faster than that of wild-type rat HNF-4 (HNF4.wt) and which, as expected, reacted with an antiserum raised to a fusion protein containing amino acids 84 to 419 of rat HNF-4, α84-419, but not with an antiserum raised to the C terminus of HNF-4, α445 (Fig. 3, lanes 1 to 6) (see Fig. 1 for a description of the antisera). When HNF4.390 and HNF4.wt were expressed together in COS cells, a shift complex with an intermediate mobility that reacted with both HNF-4 antisera was produced, indicating the presence of HNF4.wt:HNF4.390 heterodimers (lanes 7 to 9).

In order to determine whether RXRα heterodimerizes with HNF-4, an analysis similar to that done for HNF4.wt and HNF4.390 was done for RXRα and HNF4.390. The results,

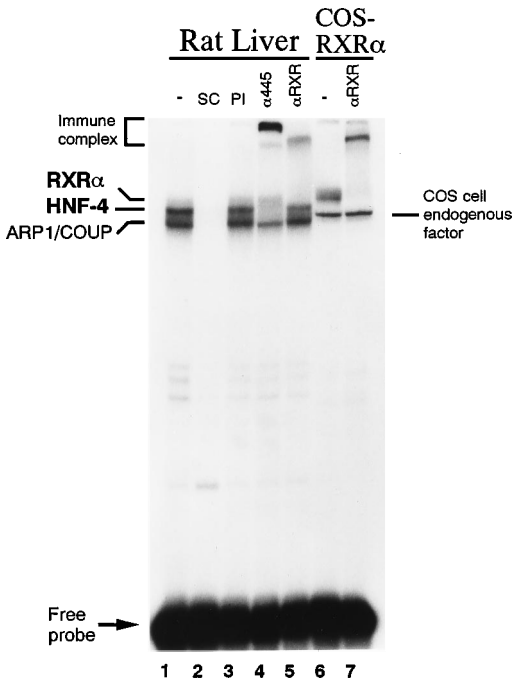

FIG. 2. HNF-4 and RXRα from rat liver produce partially overlapping shift complexes on ApoAI site A. EMSA of crude rat liver nuclear extracts (1 μg of total protein; lanes 1 to 5) and COS cell nuclear extracts containing overexpressed RXRα (1.5 μg of total protein; lanes 6 and 7) with the ApoAI site A probe as described in Materials and Methods. −, no competition; SC, specific competition with 25 ng of unlabeled ApoAI site A; PI, preimmune antisera; α445 and αRXRα, antisera to HNF-4 and RXRα, respectively, as described in the legend to Fig. 1; immune complex, supershift containing probe, receptor, and antisera; endogenous COS cell factor, a complex from COS cell nuclei that is partially inhibited by ApoAI site A and that does not react with HNF-4, RXRα, or COUP-TF antisera (data not shown). The identity of the proteins in the shift complexes, RXRα, HNF-4, and ARP1/COUP-TF, as indicated, was determined by specific competitions, reaction with antisera and comigration with complexes formed with in vitro-synthesized protein and nontransfected COS cell extracts (data not shown). The free probe is ^{32}P-labeled oligonucleotide that was not bound by protein. Rat liver nuclear extracts were prepared as previously described (86). Shown is the autoradiogram of the shift gel. wt, wild type.

also shown in Fig. 3, indicate that while there were clearly two distinct shift complexes, one corresponding to RXRα and one corresponding to HNF4.390, there was no complex of intermediate mobility that reacted with either the RXRα or the α84-419 antiserum (lanes 10 to 15). This was also true when the proteins were expressed in the presence of RA+lt (lanes 16 to 21). RA+lt was used instead of purified 9-*cis* retinoic acid, the ligand for RXRα, so that there would be a variety of retinoic acid isomers present in case an isomer other than 9-*cis* was necessary for heterodimerization.

The probe used in the experiment for which the results are shown in Fig. 3, APF1, contains the HNF-4 site from the human *apoCIII* promoter (66, 86). Our results show that RXRα overexpressed in COS cells clearly binds APF1, and this constitutes the first report of RXRα binding to this element. Since heterodimer formation can depend on the element to which the proteins bind (75, 93), several other HNF-4 and RXRα response elements were analyzed for heterodimerization activity. The results show that no heterodimers between HNF-4 and RXRα were detected on any other element examined, including ApoAI site A, ApoB, ACO (acetyl coenzyme A oxidase), PEPCK (phosphoenolpyruvate carboxykinase), α1AT (α1-antitrypsin), 151 TTR (transthyretin), TREpal, and a VDRE (vitamin D response element) (see data summarized in Table 1).

HNF-4 and RXRα do not heterodimerize at any protein concentration. Since it was possible that the lack of HNF-4:RXRα heterodimer formation in Fig. 3 was due to inappropriate protein concentrations of the two receptors, mixing experiments were performed. Extracts containing one overexpressed protein (HNF4.wt or RXRα) were incubated with an increasing amount of extract containing overexpressed HNF 4.390 and vice versa. The results are shown in Fig. 4. In Fig. 4A, it can be seen that an increasing amount of wt:390 heterodimer was formed between HNF4.wt and HNF4.390 under both mixing conditions. However, in Fig. 4B, it is equally evident that no heterodimers were formed between RXRα and HNF4.390 under either condition: no shift of intermediate mobility appeared and the α84-419 antiserum reacted only with the HNF4.390 complex (lane 8), while the RXRα antiserum reacted only with the RXRα complex (lane 16). The faint complex migrating between the RXRα and the HNF4.390 complexes is endogenous to COS cells (Fig. 2). Similar results were obtained with a probe containing an HNF-4–RXR site from ACO (data not shown).

HNF-4 and RXRα do not form heterodimers in the absence of DNA. Whereas no heterodimerization between HNF-4 and RXRα was detected on DNA, it was still possible that heterodimerization occurred in solution (i.e., in the absence of DNA). In order to investigate this possibility, HNF4.wt, HNF4.390, and RXRα were overexpressed in COS cells in the presence of [^{35}S]Met. The labeled nuclear extracts were then incubated with excess unlabeled HNF4.wt produced in a baculovirus system (HNF4.BV) and subsequently precipitated with affinity-purified α445 antiserum, which recognizes HNF4.wt but not HNF 4.390. The precipitates were analyzed by SDS-PAGE followed by autoradiography, as shown in Fig. 5A. As expected, ^{35}S-HNF4.wt was precipitated well in this system since it contains the α445 epitope (top gel, lanes 7 and 8). HNF4.390 was also precipitated although to a much lesser extent (top gel, lanes 3 and 4). Since HNF4.390 does not react with the α445 antiserum (Fig. 3), this precipitation must have been due to interaction, presumably dimerization, with HNF4.BV, which does contain the α445 epitope. Although only a fraction of the HNF4.390 protein was precipitated, the effect was very reproducible (Fig. 5A, lanes 1 to 4). These results indicate that HNF-4 is capable of forming homodimers in solution.

Unlike HNF4.390, ^{35}S-RXRα was not immunoprecipitated at all by HNF4.BV and α445 (Fig. 5A, middle gel, lanes 5 to 8). The addition of RA+lt to the extracts prior to precipitation also did not cause heterodimer formation in solution with RXRα or alter homodimer formation between HNF4.BV and HNF4.390 (Fig. 5A, lane 8 in each panel). Similar experiments with another orphan receptor, ARP-1, a close relative of COUP-TF which binds many HNF-4–RXR sites (78, 85), also failed to show heterodimer formation with HNF-4 (Fig. 5A, bottom gel, lanes 5 to 8). These results are in contrast to others that showed considerable heterodimerization in solution between RXRα and TR or RAR by similar coimmunoprecipitation techniques (42, 49, 58, 94).

In order to determine whether the presence of DNA enhances the homodimerization of HNF-4, coimmunoprecipitations were done in the presence of the APF1 oligonucleotide. The results, shown in Fig. 5B, demonstrate that APF1 does not enhance dimerization of HNF-4 (compare lanes 4 and 5). This result argues against the possibility that the precipitation of HNF4.390 by HNF4.BV is due to contamination of the extract with nucleic acids, as others have seen with other transcription factors (55).

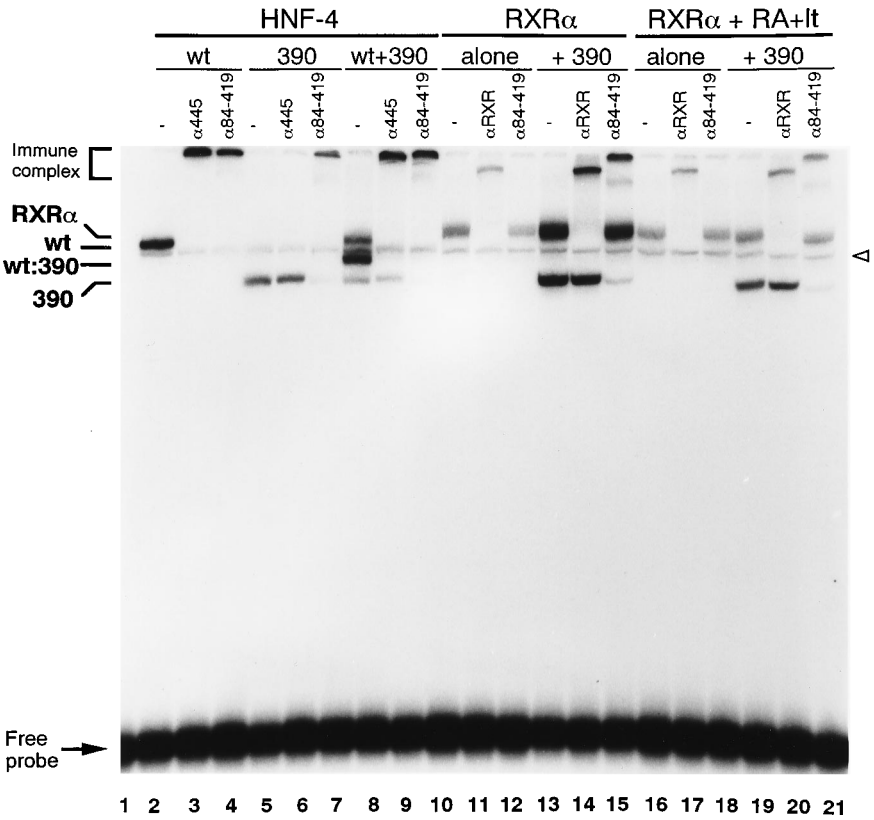

FIG. 3. COS cell-expressed proteins yield HNF4.wt:HNF4.390 dimers but no RXRα:HNF4.390 heterodimers. EMSA, as described in the legend to Fig. 2, of crude nuclear extracts from COS cells (1 μg of total protein) transfected with 5 μg of pMT2 expression vectors carrying HNF4.wt (wt), HNF4.390 (390), or RXRα cDNA in the absence or presence of RA+lt (1 μM), as indicated, were done. wt:390 denotes a heterodimer between HNF4.wt and HNF4.390 (lanes 7 to 9). Open arrowhead, endogenous COS cell factor as described in the legend to Fig. 2. The ^{32}P-labeled probe is APF1 from the *apoCIII* promoter. −, no antiserum; α445, α84-419, and αRXR, antisera described in the legend to Fig. 1 and added to the shift reaction mixture.

In vitro-synthesized HNF-4 does not form heterodimers with other RXR isoforms, RAR isoforms, or TRα. Since there are reportedly significant amounts of RAR in COS cells (75) and since RXRα is known to heterodimerize with RAR on ApoAI site A (42), it is very probable that at least part of the shift complex recognized by the RXRα antiserum in Fig. 2 to 4 is in fact a heterodimer between RXRα and RAR. Therefore, in order to avoid any possible interference of potential HNF4:RXRα heterodimer formation by the presence of receptors endogenous to COS cells, in vitro-synthesized proteins

TABLE 1. Analysis of HNF-4 RXRα heterodimer formation on various hormone response elements

Binding site element[a]	Sequence[b]	Binding by receptor dimer[c]		
		HNF-4:HNF-4	RXRα:RXRα[d]	HNF-4:RXRα
ApoAI site A	5′-AGGGCAGGGGTCAAGGGTTCAGT-3′	+++	+++	−
APF1 (ApoCIII)	5′-CGCTGGGCAAAGGTCACCT-3′	+++	+++	−
ACO	5′-ACCAGGACAAAGGTCACGT-3′	++	+++	−
PEPCK	5′-CCACGGCCAAAGGTCATGA-3′	++	+++	−
ApoB	5′-AAAGGTCCAAAGGGCGCCT-3′	+++	−	−
α1AT	5′-ACAGGGGCTAAGTCCACTG-3′	++	−	−
151 TTR	5′-CTAGGCAAGGTTCATATTT-3′	++	−	−
TREpal	5′-CTCAGGTCATGACCTGA-3′	−	++	−
VDRE	5′-CGCAGTTCAAGGAGTTCAGAACT-3′	−	+	−

[a] The name of the element represents the gene from which the probe was derived except for TREpal, which is a synthetic element, and VDRE (vitamin D response element), which is derived from the osteopontin gene (13). The hormone binding sites not referenced in the text are ACO (89), PEPCK (39), ApoB (65), α1AT (86), and 151 TTR (86).

[b] Sequence of the response element and flanking regions contained within the oligonucleotides used as probes. See the original references or reference 85 for the positions of the sites within the promoters. All of the sites can be classified as DR+1 except TREpal and VDRE, which are IR+0 and DR+3, respectively, as described in footnote *c* of Table 2.

[c] The relative binding affinities of the receptors expressed in COS cells were analyzed by EMSA (Fig. 3) (HNF4.390 was used). All data are from gels that are not shown except those with the APF1 and ApoAI site A probes, which are shown in Fig. 3 and 6, respectively. (Receptors synthesized both in vitro and in COS cells were examined on ApoAI site A.) Binding affinity is indicated as strong (+++), moderate (++), weak (+), or none (−).

[d] The RXRα dimer may contain receptors endogenous to COS cells (see the text for details).

TABLE 2. Analysis of heterodimer formation between HNF-4 and RXRα and other receptors

Receptor[a]	Element[b]	Site type[c]	Heterodimer with[d]:	
			RXRα	HNF-4
hRXRβ	ApoAI site A	DR+1	ND	–
mRXRγ	ApoAI site A	DR+1	ND	–
hRARα	ApoAI site A	DR+1	+	–
hRARβ2	ApoAI site A	DR+1	+	–
hRARγ	ApoAI site A	DR+1	+	–
rTRα	SS TRE	DR+4	+	–
rTRα	APF1	DR+1	ND	–
hARP1	APF1	DR+1	ND	–
hARP1	ApoAI site A	DR+1	+	–
mPPARα	ACO	DR+1	+	–
hVDR	VDRE	DR+3	+	–
hER	ERE	IR+3	ND	–
hER	APF1	DR+1	ND	–

[a] Receptors were synthesized in vitro as described in the legend to Fig. 6, except ARP-1, PPARα, and ER (estrogen receptor), which were expressed in COS cells as described in the legend to Fig. 3. h, human; m, mouse; r, rat.

[b] Elements used as probes in EMSA. See Table 1 for the specific sequences. SS TRE and ERE from the vitellogenin gene were essentially as previously described (24, 33).

[c] Site types are classified as direct repeat (DR) or indirect repeat (IR), with the spacing of nucleotides between the half-site indicated (e.g., DR+1 is a direct repeat separated by one nucleotide).

[d] Heterodimerization was determined as described in footnote *c* of Table 1, except that HNF-4 and RXRα were synthesized in vitro for all reactions except those with ARP-1, PPARα, VDR, and ER, for which they were expressed in COS cells. All data are from gels not shown except those with RXRγ, RARα, and TRα (SS TRE), which are from Fig. 6. + indicates the presence of heterodimers only. ND, not done.

were used to look for heterodimers. RXRα, RXRβ, RARα, TRα, and HNF4.wt were synthesized in vitro in the presence of [^{3}H]leucine, normalized to the same relative molar amount of protein, and tested for heterodimer formation on ApoAI site A and a thyroid hormone response element (SS TRE).

The results, shown in Fig. 6, indicate that on ApoAI site A, at equimolar amounts of receptor, RXRα produced no detectable shift complex while HNF-4 did (Fig. 6A, lanes 1 and 3). Furthermore, the RXRα-programmed lysate did not significantly enhance the HNF-4 shift complex or create any new bands (panel A, lane 5), as it did with in vitro-translated TRα (panel C, lanes 1 to 4). Furthermore, RXRγ, which readily formed heterodimers with RARα (panel B, compare lane 5 with lanes 1 and 3), did not affect the HNF-4 shift complex (compare lanes 10 and 13). Finally, no evidence of heterodimers with HNF-4 was seen with TRα on SS TRE (panel C), with RXRβ, RARα, RARβ2, or RARγ on ApoAI site A, or with RXRα or TRα on APF1 (summarized in Table 2).

HNF-4 and RXRα activate transcription in vivo independently of one another. The studies described above indicate that HNF-4 and RXRα bind to DNA response elements independently of each other in vitro. In order to determine whether the two receptors act independently of each other in vivo, transient cotransfection experiments were performed with a reporter construct consisting of site A and part of the *apoAI* promoter linked to the firefly luciferase gene (ApoAI.Luc). The results are shown in Fig. 7. In the absence of RA+lt, an increasing amount of RXRα expression vector gave only minimal luciferase gene activity (fivefold maximum), while similar amounts of the HNF-4 expression vector yielded significantly more luciferase activity, up to 28-fold (Fig. 7A). When an increasing amount of RXRα was cotransfected with a constant amount of HNF-4, the amount of activation induced by HNF-4 was marginally reduced. Likewise, an increasing amount of HNF-4 was not significantly affected by the presence of a small amount of RXRα (Fig. 7B).

The presence of RA+lt during the transfection gave remarkably different results. The level of transcription activated by RXRα was approximately twofold higher than that with HNF-4, up to 70-fold induction, while the HNF-4 activity itself was not affected by the presence of RA+lt, confirming previous results that HNF-4 does not respond to retinoids (Fig. 7C) (69, 85a). Greater transactivation by RXRα in the presence of RA+lt was also seen in experiments in which an increasing amount of RXRα increased the luciferase activity above that of the HNF-4 level. In contrast, increasing amounts of HNF-4 slightly reduced activation by RXRα (Fig. 7D).

These results indicate that in the absence of the RXRα ligand, HNF-4 is a much more potent activator of *apoAI* transcription than is RXRα and that large amounts of RXRα only partially inhibit HNF-4 activity. In contrast, in the presence of the RXRα ligand, RXRα is a more potent activator of *apoAI* transcription than is HNF-4, but large amounts of HNF-4 can reduce this activation somewhat. This reduction could be due to HNF-4 preferentially binding the site and activating transcription to a moderate degree, thereby preventing RXRα from binding and activating transcription to a greater degree. The results from Fig. 6A, and other data not shown, indicate that HNF-4 binds ApoAI site A with a much higher affinity than RXRα. The question remains, however, as to whether the enhanced transcriptional activation by RXRα in the presence of RA+lt is due to enhanced DNA binding not detected in vitro or whether the RXRα that does bind is simply a better activator of transcription than HNF-4. In any case, there was

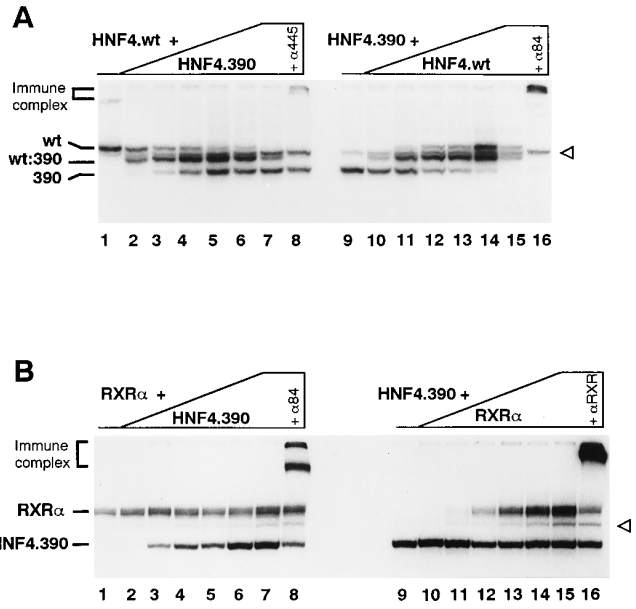

FIG. 4. RXRα does not form heterodimers with HNF-4 at any receptor concentration. EMSA of COS cell extracts from single transfections with HNF4.wt and HNF4.390 (A) or RXRα and HNF4.390 pMT2 expression vectors (B). The extracts were mixed and incubated at RT for 5 min prior to the addition of the shift mix containing a ^{32}P-labeled ApoAI site A probe. A constant amount of one extract was incubated with an increasing amount of the second extract as indicated. Antiserum was added after 15 min, as indicated and as described in the legend to Fig. 1 (lanes 8 and 16; α84 is α84-419). The amount of extracts used were purposely chosen to yield roughly equal amounts of shift complex (compare lane 1 to lane 9). Total protein was kept constant by the addition of BSA at the time of mixing the extracts. HNF4.wt and HNF4.390 were from nuclear extracts. RXRα was from a whole-cell lysate in order to reduce the amount of the shift complex from the endogenous COS cell factor (open arrowheads).

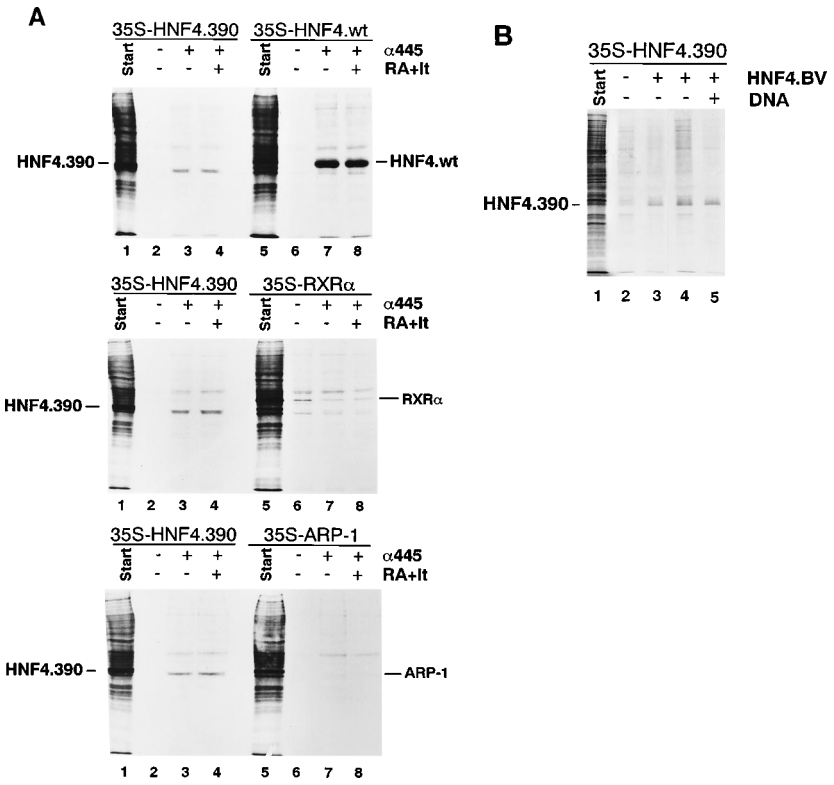

FIG. 5. Analysis of dimer formation in solution by using coimmunoprecipitation of extracts from ^{35}S-labeled COS cells containing overexpressed HNF4.wt, HNF4.390, RXRα, or ARP-1 proteins as indicated. The autoradiogram of the SDS–10% PAGE analysis of the precipitates and starting extract (Start) is shown. (A) HNF-4 forms homodimers in solution but not heterodimers with RXRα or ARP-1. Precipitations were done with unlabeled HNF4.BV and affinity-purified α445 antiserum as described in Materials and Methods. The absence (−) and presence (+) of α445 antiserum and RA+lt (0.1 μM) during the incubation of the indicated ^{35}S-labeled extract with HNF4.BV are indicated. All lanes contained 2.5 μl of nuclear extract, except the start, which contained 0.25 μl. ^{35}S-labeled HNF4.390 was precipitated by HNF4.BV and α445, indicating the formation of HNF-4 homodimers in solution (top gel, lanes 1 to 4). ^{35}S-HNF4.wt was precipitated much better than HNF4.390 because it reacts with α445 (top gel, lanes 5 to 8). ^{35}S-HNF4.390 was subsequently precipitated in parallel with ^{35}S-RXRα and ^{35}S-ARP-1 as a positive control (middle left and bottom left gels). Faint bands of unknown identity migrating above and below the position of HNF4.wt (lanes 3, 4, 7, and 8) serve as internal controls for the precipitation and loading. RXRα and ARP-1 were not precipitated above background levels, but they can be detected in the lanes labeled Start (middle and bottom gels, lanes 5 to 8). The gels were treated with EnHance, dried, and exposed to film for 9.5 h at −70°C with two intensifying screens. (B) Formation of HNF-4 homodimers in solution is not enhanced by the presence of DNA. Precipitations were done with unlabeled HNF4.BV and α445 as described in Materials and Methods, except that nonpurified α445 (3 μl per reaction mixture) was used, the extracts were not precleared and incubation with the labeled extract and the unlabeled HNF4.BV was done at RT for 30 min. The reaction mixtures were subsequently placed on ice (lanes 2 and 3), or incubation was continued at RT for another 30 min in the absence (lane 4) or presence (lane 5) of excess DNA containing an HNF-4 binding site (180 nM APF1 oligonucleotide versus ~50 nM HNF-4 total protein, as determined by kinetic analysis, data not shown). The gel was dried and exposed to film at RT for 80 h.

no evidence of cooperative, synergistic, or significant inhibitory interaction between HNF-4 and RXRα, in either the absence or the presence of RA+lt, indicating that HNF-4 and RXRα do not heterodimerize in vivo. In contrast, much greater levels of competition have been detected in vivo between RXRα and other receptors with which it has been shown to heterodimerize (57, 61).

HNF-4 is a stable homodimer in solution. The results of the coimmunoprecipitation experiment shown in Fig. 5 suggest that HNF-4 exists as a homodimer in solution. In order to confirm that result, glycerol gradient sedimentation, a more rigorous test, was performed. The results, depicted in Fig. 8, show that more than 90% of native HNF-4 has a molecular mass close to that of β-galactosidase, 120 kDa. Since the amino acid sequence of HNF-4 predicts a molecular mass of 50.6 kDa, this result suggests that HNF-4 exists predominantly as a homodimer in solution. Since the gradient was performed under relatively low-salt conditions (50 mM KCl), an alternative explanation is that HNF-4 is complexed with another protein, such as an HSP, which causes it to have a larger native molecular mass.

In order to determine whether HNF-4 might be complexed with an HSP and to assess the stability of the HNF-4 homodimer, an order-of-addition experiment was performed. This type of assay has been used by others to determine whether protein dimerization of a given factor is the rate-limiting step in binding DNA (9, 18). The binding kinetics of a concentrated solution of protein, which favors the formation of dimers, is compared with that of a dilute solution, which should favor disaggregation of dimers. When this experiment was done with HNF-4, the kinetics of homodimers binding DNA in the concentrated solution was identical to that in a solution which had been diluted 100-fold 15 min prior to the addition of the probe (Fig. 9). These results indicate that a dissociation step from a putative complex with an HSP is not required for DNA binding. They also suggest that dimer formation is not a rate-limiting step in the binding of HNF-4 to DNA and that HNF-4 dimers are fairly stable in solution. Finally, the results are consistent with the fact that full-length HNF-4 monomer binding to DNA has never been detected under any circumstance whatsoever (85a).

HNF-4 is located exclusively in the nucleus. Since to date the only other receptors reported to exist predominantly as homodimers in solution are the steroid receptors, and since

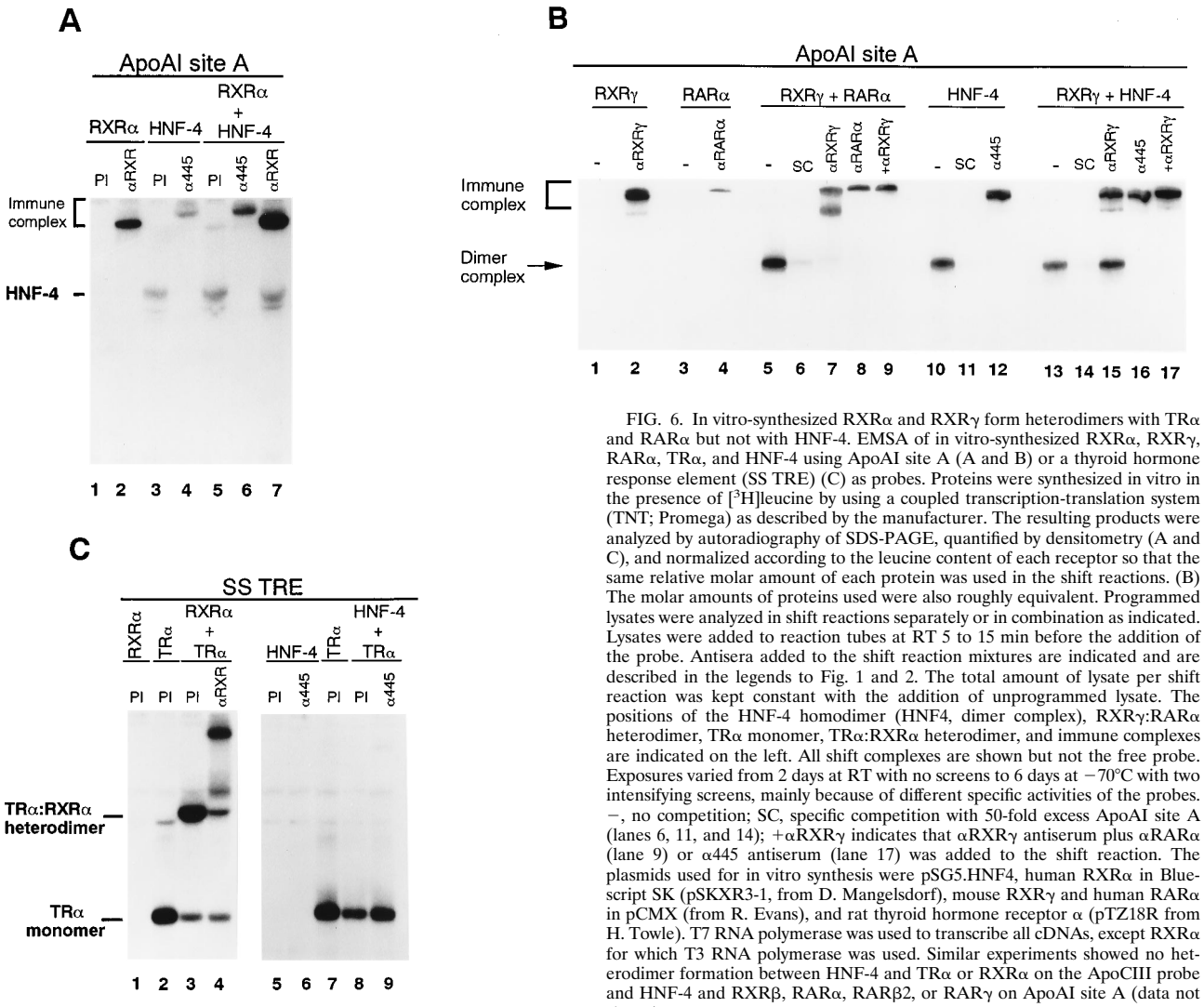

FIG. 6. In vitro-synthesized RXRα and RXRγ form heterodimers with TRα and RARα but not with HNF-4. EMSA of in vitro-synthesized RXRα, RXRγ, RARα, TRα, and HNF-4 using ApoAI site A (A and B) or a thyroid hormone response element (SS TRE) (C) as probes. Proteins were synthesized in vitro in the presence of [^{3}H]leucine by using a coupled transcription-translation system (TNT; Promega) as described by the manufacturer. The resulting products were analyzed by autoradiography of SDS-PAGE, quantified by densitometry (A and C), and normalized according to the leucine content of each receptor so that the same relative molar amount of each protein was used in the shift reactions. (B) The molar amounts of proteins used were also roughly equivalent. Programmed lysates were analyzed in shift reactions separately or in combination as indicated. Lysates were added to reaction tubes at RT 5 to 15 min before the addition of the probe. Antisera added to the shift reaction mixtures are indicated and are described in the legends to Fig. 1 and 2. The total amount of lysate per shift reaction was kept constant with the addition of unprogrammed lysate. The positions of the HNF-4 homodimer (HNF4, dimer complex), RXRγ:RARα heterodimer, TRα monomer, TRα:RXRα heterodimer, and immune complexes are indicated on the left. All shift complexes are shown but not the free probe. Exposures varied from 2 days at RT with no screens to 6 days at −70°C with two intensifying screens, mainly because of different specific activities of the probes. −, no competition; SC, specific competition with 50-fold excess ApoAI site A (lanes 6, 11, and 14); +αRXRγ indicates that αRXRγ antiserum plus αRARα (lane 9) or α445 antiserum (lane 17) was added to the shift reaction. The plasmids used for in vitro synthesis were pSG5.HNF4, human RXRα in Bluescript SK (pSKXR3-1, from D. Mangelsdorf), mouse RXRγ and human RARα in pCMX (from R. Evans), and rat thyroid hormone receptor α (pTZ18R from H. Towle). T7 RNA polymerase was used to transcribe all cDNAs, except RXRα for which T3 RNA polymerase was used. Similar experiments showed no heterodimer formation between HNF-4 and TRα or RXRα on the ApoCIII probe and HNF-4 and RXRβ, RARα, RARβ2, or RARγ on ApoAI site A (data not shown).

most of the steroid receptors are found in the cytoplasm as well as the nucleus (reviewed in references 36 and 73), we determined whether HNF-4 might also be found in the cytoplasm as well as the nucleus. Immunohistochemical staining by using the α445 antiserum showed that HNF-4 resides exclusively in the nucleus in COS cells transiently transfected with an HNF-4 expression vector as well as in a cell line expressing endogenous HNF-4 (HepG2) (Fig. 10). These data are consistent with those showing that HNF-4 binding activity is found in nuclear fractions but not cytosolic fractions of COS cells transfected with pMT2.HNF4 (data not shown).

DISCUSSION

The results presented in this study indicate that HNF-4 does not heterodimerize with RXRα in either the presence or the absence of DNA. They also show that HNF-4 is a stable homodimer in solution, a finding which could at least partially explain the lack of heterodimerization. These results, in conjunction with immunohistochemical staining localizing HNF-4 to the nucleus, led us to propose that HNF-4 represents a new subclass of nuclear receptors.

HNF-4 does not heterodimerize with any other receptor analyzed thus far. The results of this study show that HNF-4 and RXRα do not heterodimerize with each other if they are synthesized in vitro or in vivo. The results with the in vivo production (i.e., in COS cells) argue against the likelihood that some type of posttranslational modification or endogenous ligand or protein factor is necessary for heterodimerization. The results with the in vitro-produced proteins, on the other hand, argue against the likelihood that heterodimerization between overexpressed RXRα and some factor endogenous to COS cells (e.g., another nuclear receptor such as RAR) impedes heterodimerization with HNF-4.

The DNA response elements used in this study, ApoAI site A and APF1, can be classified as DR+1 elements—direct repeats of a hexamer half-site (AGGTCA or some variation thereof) separated by one nucleotide. These elements were used because (i) both HNF-4 and RXRα bind to them as homodimers; (ii) they are natural sites implicated in transcriptional control by HNF-4 and/or RXRα; and (iii) if an HNF4:RXR heterodimer were to exist, it would, in all likelihood, bind a DR+1 element with the greatest affinity since HNF-4 homodimers bind to DR+1 elements. The DNA binding speci-

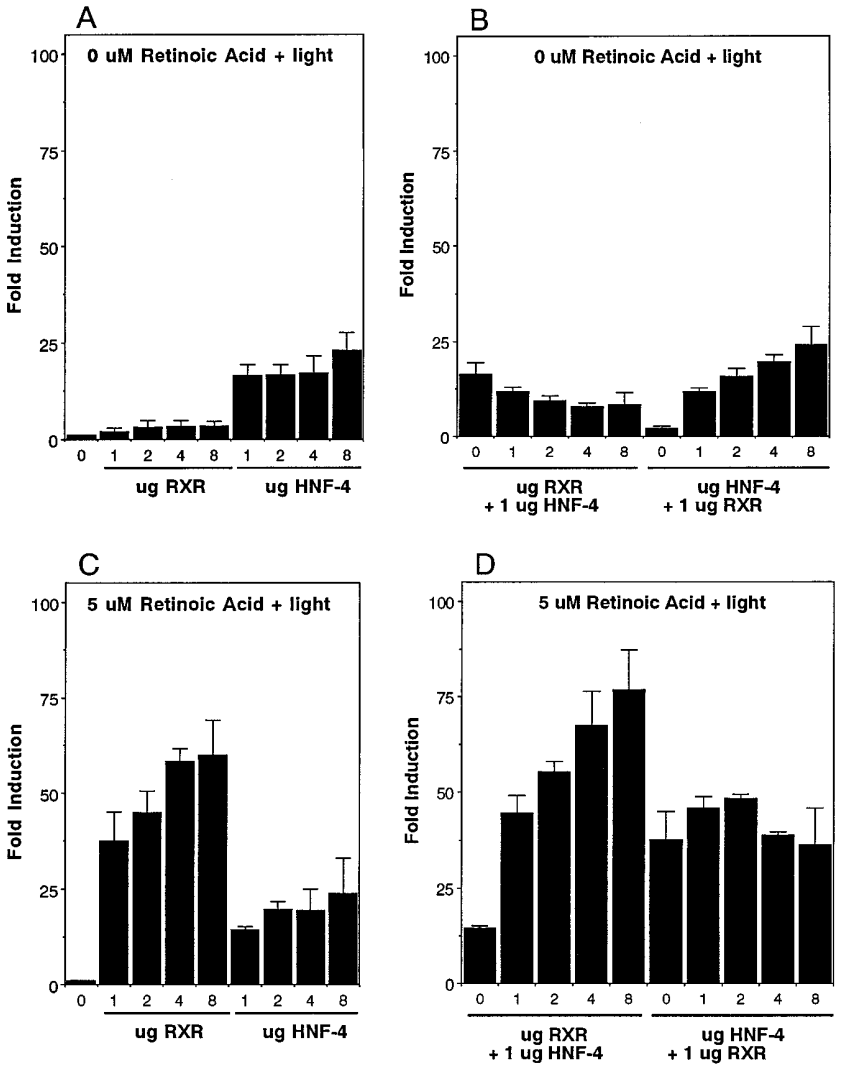

FIG. 7. HNF-4 and RXRα do not heterodimerize in vivo. (A and C) Transient transfection analysis of COS cells cotransfected with the ApoAI.Luc reporter construct and increasing amounts of expression vector (pSG5) containing the human RXRα cDNA or the rat HNF-4 cDNA in the absence and presence of RA+lt as indicated and as described in Materials and Methods. (B and D) Cotransfections as described for panels A and C but with a constant amount of one expression vector and an increasing amount of the other vector as indicated. The average fold-induction from two independent transfections done in duplicate and normalized to β-Gal activity is shown. Error bars indicate range. A third experiment done in duplicate gave similar results (data not shown).

ficity of heterodimers with RXRα has been found to be dictated by the partner of RXRα and, in general, differs from homodimer binding specificity only in that it is usually broader (37, 42, 62, 83).

There are several reasons to believe that the results which we have found with ApoAI site A and APF1 will hold true in general: (i) HNF-4 does not heterodimerize with RXRα on seven other elements (Table 1); (ii) HNF-4 does not heterodimerize with a variety of other receptors on an assortment of elements, including DR+1, DR+4, DR+3, and IR+3 (Table 2) and DR+2 and DR+5 (69); and (iii) HNF-4 does not need RXRα to bind DNA since it binds very well as a homodimer and is in fact a stable homodimer in solution (Fig. 6, 8, and 9). With the exception of ARP-1/COUP-TF, nearly all of the heterodimer partners of RXRα require RXRα for efficient binding as dimers (e.g., Fig. 8) (62). One could speculate even that the lack of heterodimerization with RXRα is due, at least partially, to the fact that HNF-4 is a stable homodimer in solution—HNF-4 monomers are simply not available for heterodimerization.

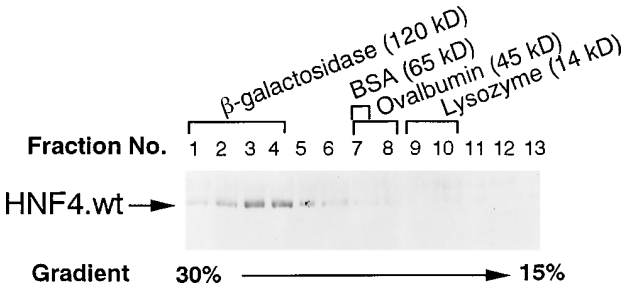

FIG. 8. HNF-4 exists predominantly as a homodimer in solution. The results of Western blot analysis of glycerol gradient sedimentation of wild-type HNF-4 are shown. Crude whole-cell extract from insect cells overexpressing HNF-4 protein (HNF4.BV, 300 μg of total protein of which ~10% is HNF-4) was mixed with the molecular mass markers (indicated above the lanes) shown and sedimented through a 15 to 30% glycerol gradient as previously described (47). Thirteen fractions were collected, analyzed by SDS-PAGE, stained with Coomassie brilliant blue to visualize the markers, destained, and analyzed by Western blot analysis as described in Materials and Methods. Since the amino acid sequence of HNF-4 predicts a 50.6-kDa protein and since HNF-4 sedimented in the range of β-galactosidase (120 kDa), these results indicate that HNF-4 exists in solution predominantly as a homodimer. kD, kilodaltons.

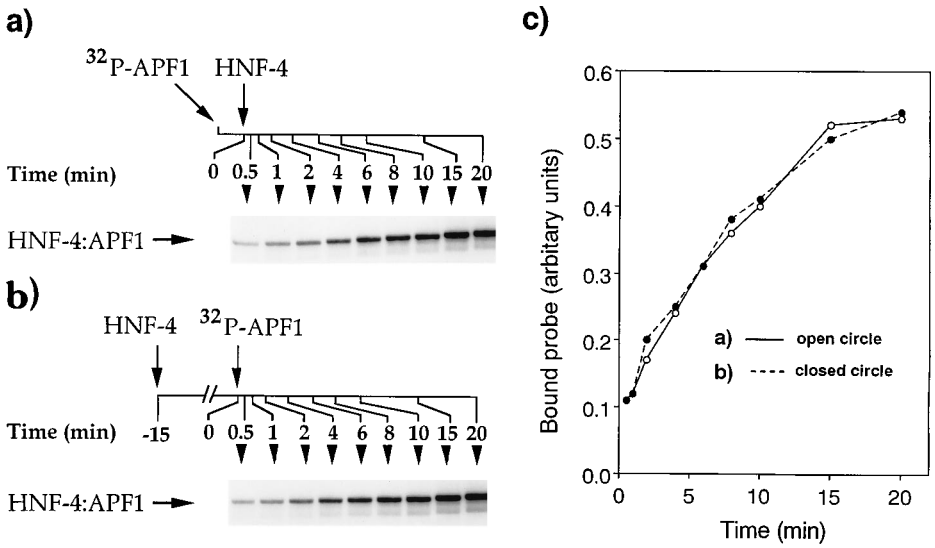

FIG. 9. HNF-4 exists in solution as a stable homodimer. An order-of-addition experiment was performed essentially as previously described (18). (a) HNF4.BV was added after the APF-1 probe (1 ng/15 μl); (b) HNF4.BV was first diluted 100-fold in a shift mix without the probe and incubated for 15 min at RT, and then the probe was added. Aliquots were removed at the indicated times and loaded onto a mobility shift gel. Shown are both the autoradiogram of the shift gels (a and b) and the corresponding densitometric readings of the HNF4 homodimer-DNA complex (c). The experiment was repeated three times with similar results. The results show that HNF-4 binds DNA relatively rapidly and that a prior dilution of the protein does not affect the rate of binding, suggesting that the HNF-4 homodimer is stable in solution.

HNF-4 defines a new subclass of nuclear receptors. If the members of the steroid hormone receptor superfamily are categorized on the basis of protein dimerization, the structure of the cognate DNA binding site, and intracellular localization, four groups are apparent, as depicted in Fig. 11 and as previously reviewed (30, 32, 73, 87). The receptors in group I are bound to HSP in the cytoplasm or the nucleus in the absence of a ligand. In the presence of a ligand, HSP are dissociated and the receptors form homodimers which bind DNA elements composed of indirect repeats. The receptors of group II are located in the nucleus (28, 59) and tend to exist as monomers or heterodimers in solution. Depending on the receptor, the ligands present, and the response element, group II members bind DNA either as monomers, homodimers, or heterodimers. The hallmark of this group is the preference to form heterodimers on DNA, especially with RXRα. With the exception of certain thyroid hormone response elements which are indirect repeats, the binding sites of group II receptors are composed of direct repeats. The receptors of group III are located in both the cytoplasm and the nucleus, as are the group I receptors. However, in contrast to the group I receptors, group III receptors exist as monomers in solution and bind extended half-sites as monomers (31, 40, 70, 91). There is, however, a recent report showing that at least two of the group III receptors (NGFI-B and NURR1) also form heterodimers with RXRα. Those heterodimers, however, are distinct from those formed entirely by group II receptors (74).

HNF-4 is similar to group II receptors in amino acid sequence (Fig. 1), preference for DNA binding sites composed of direct repeats (Fig. 2 to 4 and 6), and exclusive nuclear localization (Fig. 10). On the other hand, HNF-4 is similar to group I receptors in that it exists in solution as a stable homodimer and binds DNA exclusively as a homodimer (Fig. 3 to 9). The other defining feature of group I receptors is that they are complexed with HSP. There is no evidence to suggest that HNF-4 is complexed with HSP, and there is in fact considerable evidence indicating that it is not complexed with HSP: (i) rat HNF-4 overexpressed in insect cells by using the baculovirus system (HNF4.BV) and extracted by a freeze-thaw procedure in the presence of a low-salt concentration (25 mM KCl) does not require activation to bind DNA, as do several of the steroid hormone receptors (1, 2, 14); (ii) kinetic studies (saturation curves and order-of-addition experiments) show that HNF-4 extracted from COS cell nuclei with 0.42 M KCl has

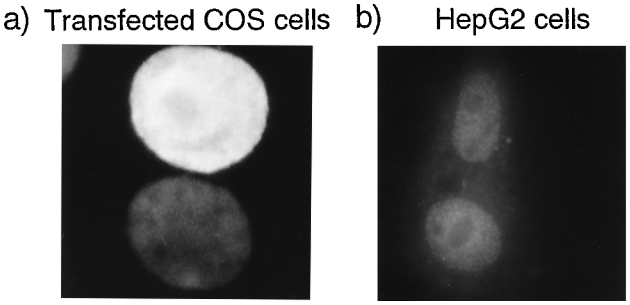

FIG. 10. HNF-4 is found exclusively in the nucleus. Immunocytochemical localization of HNF4.wt in COS cells transiently transfected with 25 μg of pMT7.HNF4 (a derivative of pMT2.HNF4 containing a T7 promoter site (44b) (a) and HepG2 cells, a human hepatocarcinoma cell line which expresses endogenous HNF-4 (b), is shown. Cells were grown on glass slides for 24 h in DMEM plus 10% BCS, prefixed with 2% paraformaldehyde for 5 min, rinsed in PBS, fixed in 100% acetone for 30 s, and air dried. The fixed cells were incubated for 1 h in blocking solution (1× TTBS plus 3% BSA) and then for 2 h with α445 antiserum diluted 1:5,000 in blocking solution, washed three times with 1× TTBS, incubated for 1 h with donkey anti-rabbit immunoglobulin G fluorescein isothiocyanate (FITC) (Jackson Laboratories) diluted 1:200 in blocking solution, washed once with 1× TTBS, and drained. Two drops of 4,6-diamidino-2-phenylindole (DAPI) (2.5 μg/ml in phenylenediamine antifade) were used to counterstain the DNA. Slides were scored by using a Nikon Optiphot II microscope (magnification, ×1,250) with a fluorescence attachment equipped with a triple-band-pass filter (a) or a single blue filter (b) as previously described (81). (a) Nuclei of COS cells expressing HNF-4 were bright white from FITC plus DAPI staining, while nuclei lacking HNF-4 were light gray from DAPI staining alone. The colocalization of the FITC fluorescence with the DAPI stain indicates that HNF-4 is present exclusively in the nucleus of transfected cells, even when expressed at high levels; (b) HepG2 cells show FITC fluorescence primarily in the nucleus. The fluorescence has a punctuated appearance, the significance of which is not known. No significant FITC staining is seen in the cytoplasm. The COS cell photo was slightly enlarged relative to the HepG2 photo.

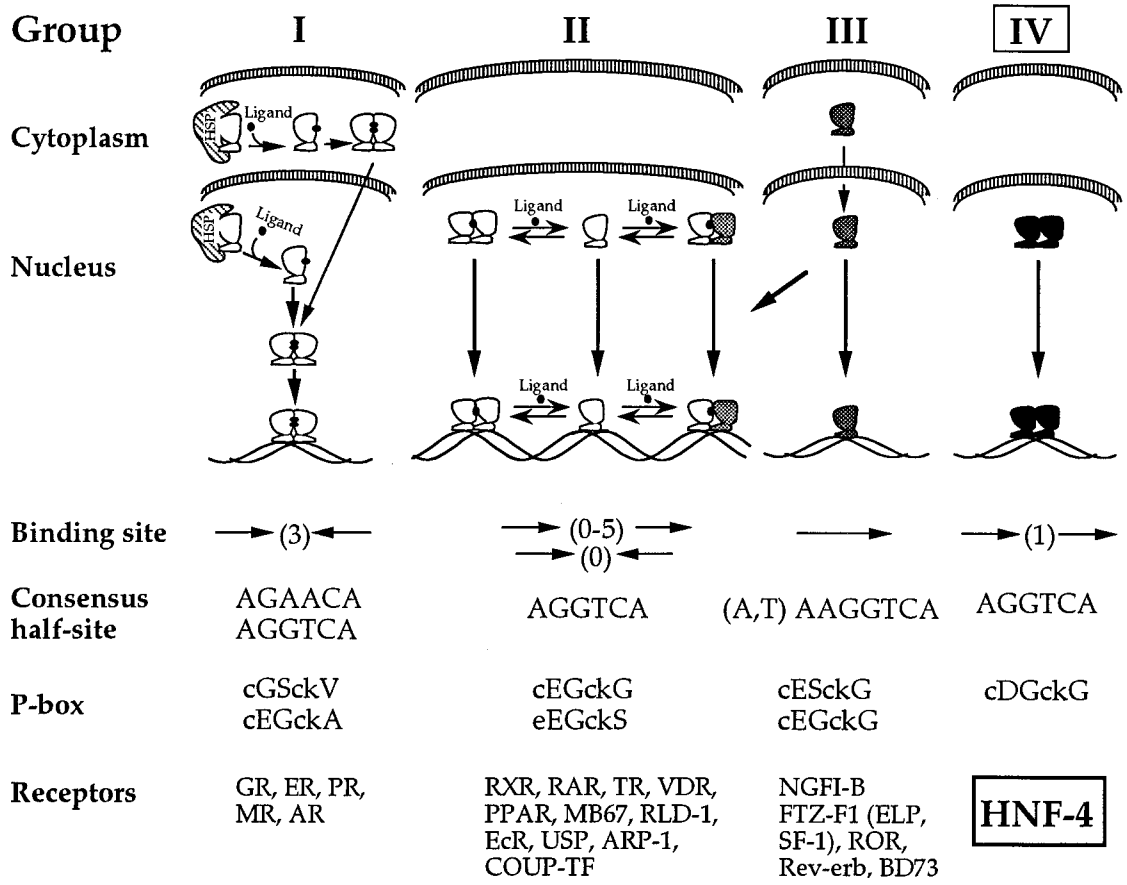

FIG. 11. HNF-4 defines a new subclass of nuclear receptors. Receptors are classified according to current knowledge of their dimerization potential, DNA binding site specificity, and cellular localization. A general description of the different groups is given in the text. In group I, GR, MR, and PR are bound to HSP in the cytoplasm while estrogen receptor (ER), PR, and androgen receptor (AR) are reportedly bound to HSP in the nucleus (14, 21, 77). In group II, a ligand (stoichiometry not specified) may promote the association or dissociation of homo- and heterodimers in solution and on DNA depending on the ligand, the receptors, and the response elements involved. No ligands have been identified for receptors in group III or IV. A consensus half-site for the receptors of each group is given, and the orientation of each half-site, inverted, direct, or no repeat, is shown with arrows. The orientations of monomers within the receptor dimer (symmetric in group I and asymmetric in group II) are indicated. The orientation of HNF-4 monomers in the homodimer are presumed to be asymmetric on the basis of the structure of the HNF-4 binding site. In group I, AGGTCA is the half-site for ER. In group III, (A,T) signifies an AT-rich sequence which flanks the 5′ side of the extended half-site. The P-box sequence (given in single-letter amino acid code) refers to the region between the third and fourth cysteines of the first zinc finger and is thought to play a role in DNA binding specificity. The predominant sequence for the group is listed, with exceptions listed below. Representative receptors for each group are given; those for which the dimerization state in solution has been proven are listed in the top line. Standard abbreviations are used for all receptors. Only those receptors for which sufficient information is available are shown. Information from a variety of sources, in addition to those listed in the text, was used (3, 5, 13, 15, 17, 19, 22, 25, 42, 50, 53, 92).

DNA binding properties similar to those of HNF4.BV extracted in low-salt concentrations (44a); and (iii) whereas at least one group I receptor associates with HSP90 in rabbit reticulocyte lysate during in vitro translation and requires an activation step in order to bind DNA, the group II receptors and HNF-4 do not (Fig. 6) (16). Therefore, since HNF-4 differs significantly from group I, II, and III receptors, we propose that HNF-4 defines a new subclass of receptors, group IV. Group IV receptors are characterized by stable homodimerization, a preference for direct repeats, and predominantly nuclear localization.

HNF-4, a prototype for a larger group of receptors? Although HNF-4 possesses other distinctive structural features, such as a unique P-box sequence (Fig. 11) and genomic structure (95), HNF-4 also could be the prototype of a larger class of receptors. There are at least two other orphan receptors that are candidates for group IV classification. Germ cell nuclear factor (10, 11) and TAK1 (44) have both been shown to bind DNA well as homodimers and do not appear to heterodimerize with RXR. Although the P-box sequence of both TAK1 and germ cell nuclear factor is like that of the group II receptors, the P-box sequence may not be a critical determinant for this categorization. Determination of dimerization in solution and cellular localization are more important parameters to study.

The observation that HNF-4 is a stable homodimer in solution raises some questions about the role of a putative ligand for this group of receptors. For group I receptors, ligands are required to dissociate the receptors from HSP so that they can subsequently form homodimers and bind DNA. For group II receptors, ligands play a crucial role in determining dimerization partners. What then would be the role of a ligand for HNF-4 and other group IV, and group III, receptors? A ligand evidently is not required to dissociate HNF-4 from HSP or to form heterodimers with other receptors. This leads one to speculate that either HNF-4 does not have a ligand or, if it does, the role of the ligand is different from that of the other receptors. Clearly, in order to understand fully how members of this large superfamily regulate gene expression, more work is needed not only to characterize the DNA binding activities of HNF-4 and other orphan receptors but also to identify potential ligands for group III and group IV receptors.

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