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The Orphan Receptor Hepatic Nuclear Factor 4 Functions as a Transcriptional Activator for Tissue-Specific and Hypoxia-Specific Erythropoietin Gene Expression and Is Antagonized by EAR3/COUP-TF1

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The erythropoietin (Epo) gene is regulated by hypoxia-inducible *cis*-acting elements in the promoter and in a 3′ enhancer, both of which contain consensus hexanucleotide hormone receptor response elements which are important for function. A group of 11 orphan nuclear receptors, transcribed and translated in vitro, were screened by the electrophoretic mobility shift assay. Of these, hepatic nuclear factor 4 (HNF-4), TR2-11, RORα1, and EAR3/COUP-TF1 bound specifically to the response elements in the Epo promoter and enhancer and, except for RORα1, formed DNA-protein complexes that had mobilities similar to those observed in nuclear extracts of the Epo-producing cell line Hep3B. Moreover, both anti-HNF-4 and anti-COUP antibodies were able to supershift complexes in Hep3B nuclear extracts. Like Epo, HNF-4 is expressed in kidney, liver, and Hep3B cells but not in HeLa cells. Transfection of a plasmid expressing HNF-4 into HeLa cells enabled an eightfold increase in the hypoxic induction of a luciferase reporter construct which contains the minimal Epo enhancer and Epo promoter, provided that the nuclear hormone receptor consensus DNA elements in both the promoter and the enhancer were intact. The augmentation by HNF-4 in HeLa cells could be abrogated by cotransfection with HNF-4ΔC, which retains the DNA binding domain of HNF-4 but lacks the C-terminal activation domain. Moreover, the hypoxia-induced expression of the endogenous Epo gene was significantly inhibited in Hep3B cells stably transfected with HNF-4ΔC. On the other hand, cotransfection of EAR3/COUP-TF1 and the Epo reporter either with HNF-4 into HeLa cells or alone into Hep3B cells suppressed the hypoxia induction of the Epo reporter. These electrophoretic mobility shift assay and functional experiments indicate that HNF-4 plays a critical positive role in the tissue-specific and hypoxia-inducible expression of the Epo gene, whereas the COUP family has a negative modulatory role.

The biological effects of a variety of hormones, including steroids and thyroxine, and other ligands such as retinoic acid and vitamin D depend upon binding to a superfamily of zinc finger receptor proteins that in turn bind to consensus hexanucleotide repeat elements in responsive genes (12, 15, 52, 53). This family includes an enlarging group of recently cloned genes which have homology to these hormone receptors and encode proteins that bind to consensus elements but lack a known ligand. The biologic functions of most of these orphan receptors are also unknown. In this report, we present evidence that one of them, hepatic nuclear factor 4 (HNF-4) (47, 48), plays a critical role in the regulation of the gene encoding erythropoietin (Epo), a hormone critical for the proliferation and differentiation of red blood cells.

Epo is a 30.4-kDa glycoprotein produced in the kidneys, and to a lesser extent in the liver, in response to hypoxia (21, 37). The Epo gene is an ideal model system for studying induction by a physiologically important stimulus, hypoxia, as well as for investigating tissue and developmental specificity. The regulation of the Epo gene has been examined at the macroscopic level in transgenic experiments which showed that kidney- and liver-specific expression depends on *cis* elements located, respectively, 9.5 to 14 kb and 0.4 to 6 kb upstream of the transcription start site (9, 42, 43, 45). More refined characterization of the *cis* elements involved in Epo gene regulation has been based primarily on reporter gene transient transfections coupled with electrophoretic mobility shift assays (EMSA) and footprint analyses. The human hepatoma cell line Hep3B secretes Epo in a physiologic manner (17) and therefore has been useful for investigating the regulation of the Epo gene. Hypoxic induction of Epo gene expression, both in the kidneys (40) and in Hep3B cells (16), depends primarily upon increased transcription. Considerable progress has been made in the identification and characterization of *cis*-acting elements that contribute to the transcription of the Epo gene (3, 5, 19, 30, 38, 44). In both the kidneys in vivo and in Hep3B cells, hypoxia increases the expression of the endogenous Epo gene 50- to 100-fold. Hypoxic induction of the Epo gene depends on interaction between the Epo promoter and a highly conserved enhancer element located 120 bp downstream of the poly(A) addition site. In a luciferase reporter system, this 3′ enhancer, when placed upstream of the Epo promoter (bp −118 to +26),

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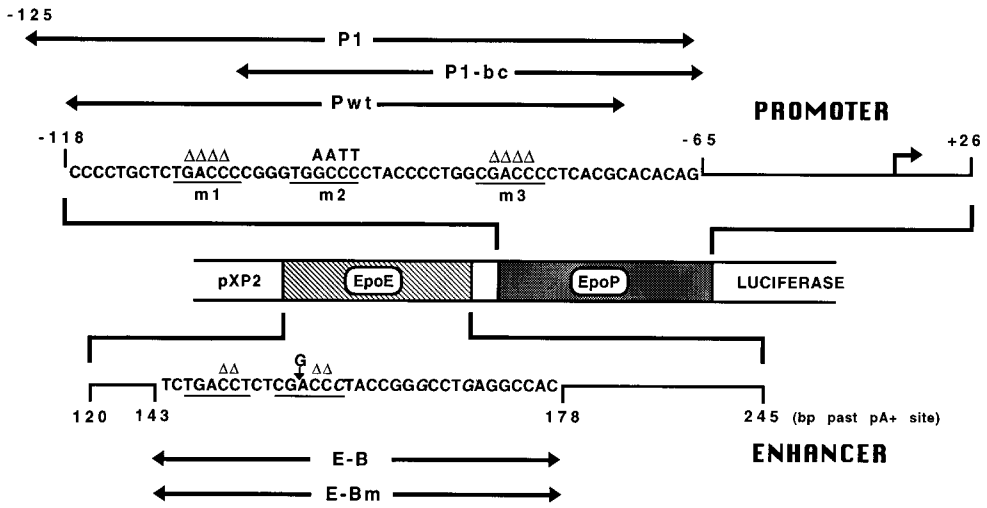

FIG. 1. Diagrams of the reporter constructs and the wild-type and mutant DNA fragments used in this study. The pEPLuc reporter construct is shown in the middle. Shown above it is the 144-bp insert from the Epo promoter (base pairs are numbered with respect to the transcriptional start site [5]), and shown below it is the 126-bp insert from the Epo 3′ enhancer [base pairs are numbered with respect to distance downstream from the poly(A) addition site (20, 29)]. These enhancer fragments contained the additional 3 bases (italics) found previously to be present in our independent human Epo clone assigned GenBank accession number L16588 (5). They were not present in EMSA probes E-B and E-Bm, which are shown below the enhancer. The promoter DNAs used in EMSA reactions are also shown (above the promoter). The putative hexanucleotide repeat elements in the promoter and enhancer are underlined. The mutations are shown either as base substitutions (AATT), insertions (↓), or deletions (△). The G insertion indicated along with the deletions in the 126-bp Em fragment was not in the E-Bm oligonucleotide.

cooperated to produce a 50-fold response in Hep3B cells subjected to hypoxia (5). The Epo promoter contains a minimal sequence, −118 to −65 bp upstream of the transcription start site, necessary to confer a sixfold response to hypoxic induction (5). This region of the promoter contains three hexanucleotide consensus nuclear receptor binding half sites separated by 4 and 10 bp. The 3′ enhancer alone confers a 4- to 14-fold induction of reporter gene expression (3, 5, 30, 38, 44) in a wide variety of cell types (31). Deletion and mutation experiments have established that the minimal enhancer is a 50-bp segment that is composed of three interacting parts (30, 41). A highly conserved 9-bp site near the 5′ end of the enhancer (human and mouse Epo genes) on its own provides weak induction, and when mutated, it abolishes induction by the minimal enhancer. Nuclear extracts from a number of different types of cells contain a 120-kDa hypoxia-inducible factor (HIF-1) which binds to this region (4, 41, 54, 55). The induction depends upon ongoing transcription (54) and protein synthesis (41) and is inhibited by blocking protein phosphorylation (54). The middle portion of the enhancer, consisting of three CA repeats, is not well conserved, but when mutated, it inhibits induction (41). Thus far, specific protein binding to this segment has not been demonstrated. The CA repeats are also present at two sites in the Epo promoter, although their role has not yet been determined. The highly conserved 3′ portion of this enhancer contains a direct repeat of a hexanucleotide consensus nuclear receptor binding half site separated by a 2-bp spacer (DR2) (5).

Both the promoter and enhancer nuclear hormone receptor consensus sequences form specific DNA-protein complexes with nuclear proteins of Hep3B cells in EMSA. Specific binding to the enhancer can be displaced by the promoter element, and vice versa, but it cannot be displaced by competitors in which the consensus half site sequences have been mutated. Moreover, mutations at these sites markedly decrease hypoxic induction by the enhancer (5) and the promoter (13). Taken together, these results suggest that the same nuclear hormone receptor(s) binds to the Epo promoter and enhancer, thereby amplifying hypoxic induction.

Two considerations argue strongly that the nuclear hormone receptor(s) that regulates Epo gene induction does not bind to one of the known ligands. First, none of the ligands that were tested (thyroxine, vitamin D_3, dexamethasone, progesterone, beta-estradiol, testosterone, and retinoic acid) significantly affected the induction of a reporter plasmid containing the Epo enhancer with the thymidine kinase promoter (5). Secondly, all of these ligands have well-known, albeit complex biological effects, none of which bear obvious relevance to tissue oxygenation. It is much more likely that the Epo gene is regulated by an orphan receptor. Therefore, we examined a number of cloned orphan receptors in the interest of determining whether any might contribute to Epo gene regulation.

MATERIALS AND METHODS

Plasmid constructions. All molecular biological techniques were based on standard protocols (1). The cDNA encoding full-length rat HNF-4 isoform α1 (455 residues) (47) was inserted into the *Eco*RI site in the polylinker of the expression vector, pCMX (52), and the resulting plasmid was designated pCMX-HNF-4. A plasmid expressing truncated HNF-4 (pCMX-HNF-4ΔC) was made by *Msc*I digestion and religation, which enable excision of a fragment that encodes the 101 C-terminal residues as well as the 3′ untranslated region. Two amino acids are translated in the linker sequence between the *Msc*I site and a stop codon in pCMX. As a result, HNF-4ΔC is 356 amino acids long. Plasmids expressing EAR3/COUP-TF1 (33), RORα1 (14), and other orphan receptors were prepared in a similar way by inserting their cDNAs into pCMX.

In our functional experiments we used the reporter plasmid pEPLuc-version(v)1 described by Blanchard et al. (5), in which the 126-bp 3′ Epo enhancer [120 to 245 bp 3′ of the poly(A) addition site] and the 144-bp minimal Epo promoter (from bp −118 to +26 relative to the transcription initiation site) were placed upstream of the firefly luciferase gene (10) in pXP2 (36). In the mutant constructs, the consensus response elements in either the promoter (pE-PmLuc) or the enhancer (pEm-PLuc) were mutated as shown in Fig. 1. The plasmids containing the wild-type (E) and mutant (Em) 126-bp 3′ Epo enhancers in pBluescript KS(+) (pBKS; Stratagene) were as described previously (5) with a correction to the mutation in the Em sequence consisting of an insertion of a G as indicated in Fig. 1. These enhancer fragments contained the additional 3 bases (italics in Fig. 1) found previously to be present in our independent human Epo clone assigned GenBank accession number L16588 (5). The plasmid pEPluc-v2 was generated by excising E or Em from pBKS with *Ecl* 136 plus *Hin*dII and inserting each 5′ of the promoter in the *Bam*HI (blunted) site of P118-pXP2 (5). The orientation of E within all the pEPLuc constructs had the 3′ end of the E fragment near the 5′ end of the promoter fragment. The Epo promoter (P118) from bp −118 to +26 was excised from pXP2 by using *Bam*HI plus *Eag*I and

subcloned into the corresponding sites in pBKS to create P118wt-pBKS. This plasmid was used to generate the promoter mutants depicted in Fig. 1 either singly or in combination by site-specific mutagenesis (26). The mutations were verified by dideoxy sequencing (39). The mutated promoters (Pm) were then excised by using *Hin*dII plus *Sac*I, exchanged for the wild-type promoters (Pwt) by using the same sites in pEPLuc-v2, and confirmed by sequencing. This generated pE-PmLuc-v3, and so plasmid pEwt-PwtLuc-v3 was also made.

EMSA. Promoter DNA probes were excised with appropriate restriction enzymes from either P1-pBKS which contains the human Epo promoter region from bp −125 to −65 in pBKS (5) or P118-pBKS. The probes generated included the following: P1-bc, bp −102 to −65; Pwt, bp −118 to −70; and Pm, which was identical to Pwt except that the most 3′ response element had been mutated by deletion as shown in Fig. 1. The Epo enhancer DNA probe, E-B [+143 to +178 bp downstream of the poly(A) addition site, not including the 3 extra bases in our genomic DNA], was excised from the pE-B plasmid (5). The DNA fragments described above all contained some polylinker at one or both ends. All of the DNA probes were end labeled by filling in 5′ overhangs with α-^{32}P-deoxynucleoside triphosphates (NEN) and Klenow fragment (Boehringer Mannheim) and purified on a 10% polyacrylamide gel. In addition, the following unlabeled DNA competitors (5) were used in the EMSA experiments: oligonucleotide P1 (bp −125 to −65), E-B, and E-Bm (for which a CC dinucleotide was deleted from each of the two hexanucleotide response elements). The DNA fragments used in EMSA are shown diagrammatically in Fig. 1.

Recombinant nuclear hormone receptor proteins were synthesized in vitro from the pCMX-cDNA constructs by T7 polymerase transcription and rabbit reticulocyte lysate translation (with unlabeled methionine) by using the TnT system of Promega. Nuclear extracts were prepared from normoxic Hep3B cells and Hep3B cells exposed to hypoxia for 6 h according to a protocol based on a combination of several methods (1, 11, 46). Hep3B cells were harvested by scraping and rinsed well with cold phosphate-buffered saline (PBS), and the cell pellet was resuspended with 5 pellet volumes of buffer H {10 mM HEPES [*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.9], 10 mM KCl, 0.75 mM spermidine, 0.15 mM spermine, 0.1 mM EDTA, 0.1 mM EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid], 1 mM dithiothreitol [DTT], protease inhibitors}. All steps were carried out at 4°C. Cells were spun at 1,850 × *g* for 5 min, and the pellet was resuspended to 3 pellet volumes with buffer H. After 10 min of swelling, the cells were sonicated with a Branson Sonifier 250 microtip by using a 10% cycle and 25 pulses at a power setting of 6. The disrupted cells were spun at 3,300 × *g* for 15 min, and the pelleted nuclei were resuspended with 0.5 pellet volumes of buffer H. With stirring, buffer E (25% glycerol, 20 mM HEPES [pH 7.9], 0.85 M KCl, 0.75 mM spermidine, 0.15 mM spermine, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, protease inhibitors) was added dropwise to yield a final concentration of 0.31 M KCl. The nuclei were mixed on a Labquake rotator for 30 min and then spun in a JA-20 rotor at 14,500 rpm (25,000 × *g*) for 30 min. The supernatant was dialyzed twice for 90 min against 200 volumes of nuclear dialysis buffer (20% glycerol, 20 mM HEPES [pH 7.9], 100 mM KCl, 0.2 mM EDTA, 0.2 mM EGTA, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride), spun for 10 min in a microcentrifuge, aliquoted, quick-frozen on dry ice, and stored at −80°C. The protease inhibitors added as indicated above included 1 mM phenylmethylsulfonyl fluoride, 24.4 U of aprotinin per ml, and 2 μg each of pepstatin A, leupeptin, antipain, and bestatin per ml.

EMSA reaction mixtures (15 μl) contained 10 mM Tris HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, 0.5 mM $MgCl_2$, 40 mM NaCl, 5% glycerol, 0.2 to 2 μg of poly(dI-dC), 2 μl of either nuclear extract (3 μg of protein) or an in vitro transcription-translation reaction mixture of a pCMX-cDNA construct, and 40 fmol of ^{32}P-labeled DNA probe. The reaction mixtures were incubated at 30°C for 15 min or at room temperature for 25 min. Competition reaction mixtures included a 25- to 50-fold molar excess of unlabeled DNA added before the protein. Antisera against retinoic acid receptors (23, 24) and HNF-4 (provided by Frances Sladek [49]) were diluted fivefold in antiserum dilution buffer (3% bovine serum albumin, 0.02% sodium azide) before addition of 1 μl to EMSA reactions. The polyclonal antiserum (provided by Ming-Jer Tsai) against the COUP family (8, 28, 33) (consisting of EAR3/COUP-TF1, EAR2, and ARP-1/COUP-TF2) was used undiluted (0.3 μl per reaction). In supershift assays, the antisera were added to the EMSA reactions halfway through the incubation. The DNA-protein complexes were resolved on 4% polyacrylamide gels (acrylamide/bisacrylamide ratio, 29:1) in 0.5× TBE (1× TBE is 90 mM Tris, 90 mM boric acid, and 2 mM EDTA) at 250 V and 27 mA at room temperature. The gels were dried, and autoradiography was performed.

RNA analysis. Unfractionated RNAs from normoxic and hypoxic Hep3B and HeLa cells were analyzed by Northern (RNA) blot hybridization with labeled full-length rat HNF-4 as a probe. RNase protection analyses were performed on unfractionated RNA from normoxic and hypoxic Hep3B cells, either untransfected or transfected with either full-length HNF-4 or HNF-4ΔC. These RNAs were probed with (i) a human Epo ^{32}P-riboprobe transcribed from a 240-bp segment of a marked genomic DNA within exon 5 (18), protecting a 185-base portion of Epo mRNA, or (ii) a rat HNF-4 ^{32}P-riboprobe transcribed from a 457-bp segment of cDNA extending from the 3′ end of the ligand binding-dimerization domain to the 5′ end of the C-terminal proline-rich region and including 61 bp of polylinker for a total probe length of 518 bases, protecting 457 bp of full-length HNF-4 and 248 bp of HNF-4ΔC. The linearized templates were transcribed in vitro by using T7 RNA polymerase in the presence of 50 μCi of [α-^{32}P]GTP. After hybridization overnight at 50°C and RNase digestion, the samples were denatured and analyzed by electrophoresis on a 4% or 6% polyacrylamide–8 M urea gel, which was then subjected to autoradiography.

Transfections and functional assays. Hep3B cells were cultured in alpha-modified Eagle medium with 10% iron-enriched calf serum in 95% air and 5% CO_2 at 37°C. HeLa cells were cultured under similar conditions in Dulbecco's modified essential medium with 10% heat-treated fetal calf serum. For transient transfections, HeLa cells were harvested and resuspended to a final concentration of 10^7 cells per ml with 30 μg of reporter plasmid and various amounts of effector plasmids in a total volume of 0.8 ml per cuvette. Electroporation was performed in a Gene Pulser cuvette (Bio-Rad) at 200 V and 960 μF. The electroporated cells were suspended in 20 ml of medium and split equally to two 10-cm-diameter dishes. After 1 h of incubation at 37°C in normoxia (95% air and 5% CO_2), one plate was moved to hypoxia (1% O_2, 94% N_2, and 5% CO_2) for 48 h.

Hep3B cells were transfected both transiently and stably by using DOTAP transfection reagent as recommended by the manufacturer (Boehringer Mannheim). For transient transfections, 10 μg of reporter plasmid and various amounts of effector plasmids in DOTAP reagent were incubated on 6-cm-diameter dishes of Hep3B cells for 24 h. Then each dish was split equally to two 10-cm-diameter dishes and both were incubated at 37°C for 48 h, one dish in normoxia (95% air and 5% CO_2) and the other dish in hypoxia (1% O_2, 94% N_2, and 5% CO_2). Each DNA sample was transfected in triplicate within an experimental set. For stable transfections, two 6-cm-diameter dishes of Hep3B cells were incubated for 24 h each with 5 μg of pCDNA3 (Invitrogen), which contains a neomycin resistance gene, plus 150 μg of either pCMX, HNF-4-pCMX, or HNF-4ΔC-pCMX mixed with DOTAP reagent. Each dish was then expanded to a 10-cm-diameter dish, and after 2 to 3 days one dish of each set was split 1:3 and all dishes were treated with 400 μg of G418 per ml. After 2 to 3 weeks of selection, independent colonies were expanded.

Cells were rinsed with PBS and lysed directly on the dish with 500 μl of Cell Lysis Buffer (Analytical Luminescence Laboratory). Luciferase activity in 100 μl of extract was assayed with an enhanced luciferase assay kit and luminometer (Analytical Luminescence Laboratory). Each measurement of relative light units was corrected by subtraction of the background. Hypoxic inducibility was defined as the ratio of corrected relative light units of the hypoxic dish to corrected relative light units of the normoxic dish prepared from the same transfection. We made no correction for transfection efficiency because all experiments compared measurements for split cells and there was excellent agreement between duplicate transfections.

RESULTS

Analysis of the binding of nuclear hormone receptors to the Epo promoter and enhancer. Both the Epo promoter and enhancer contain nuclear hormone receptor consensus DNA sites (see Fig. 1) which have been demonstrated to bind to proteins from Hep3B cell nuclear extract and to cross-compete for these proteins (5, 13). Since maximal hypoxic induction of the Epo gene requires these elements in both the promoter (13) and the 3′ enhancer (5), we have utilized probes from both of these regions in EMSA studies in order to examine binding to nuclear proteins from Hep3B cells in comparison with binding to in vitro-transcribed and -translated nuclear hormone receptor proteins. Figure 1 shows diagrams of the wild-type and mutant probes that were employed. The orphan hormone receptors that were tested either alone or with RXRα in an initial screen for specific binding to the wild-type Epo enhancer probe (E-B) were as follows: HNF-4, TR2-11, RORα1, EAR3/COUP-TF1, RARα, RXRα, rTRα, mPPAR, NGFI-B, XR5-1, and mERR2. RXRα, in particular, is known to form heterodimers with a variety of nuclear receptors (15, 23–25, 50, 53, 56). The following receptors failed to bind specifically to E-B by themselves or in combination with RXRα (i.e., there was no specific gel shift with the probe): NGFI-B, XR5-1, mERR2, rTRα, and mPPAR. In addition, RARα and RXRα also failed to bind individually, but they did bind as a heterodimer. In contrast, TR2-11 (7), HNF-4, RORα1, and EAR3/COUP-TF1 all produced DNA-protein complexes that could be displaced by unlabeled E-B but not by a fragment mutated at the hexanucleotide repeats (E-Bm) (data not shown; see Fig. 3 and 4). Moreover, as shown in the left panel of Fig. 2, TR2-11 and HNF-4 generated DNA-protein complexes with mobilities similar to those of complexes obtained with nuclear extract from

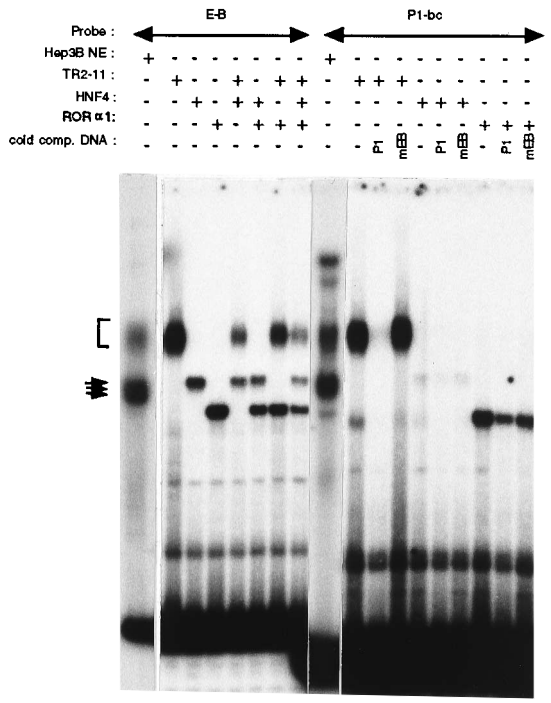

FIG. 2. The orphan receptors TR2-11, HNF-4, and RORα1 bind specifically to both the Epo enhancer and promoter. Results of EMSA analysis of the binding to Epo enhancer (E-B) and promoter (P1-bc) probes by Hep3B nuclear extract and by in vitro-transcribed and -translated orphan receptors TR2-11, HNF-4, and RORα1 as indicated above each lane are shown. Cold competitor DNAs were added at a 50-fold molar excess where noted. See Fig. 1 for diagrams of the probes and unlabeled competitor DNAs. The arrowheads and bracket indicate the sequence-specific DNA-protein complexes formed by Hep3B nuclear extract on both probes as described by Blanchard et al. (5). The complexes indicated by the three arrowheads have since been shown to be composed of at least three bands. All lanes are from the same gel.

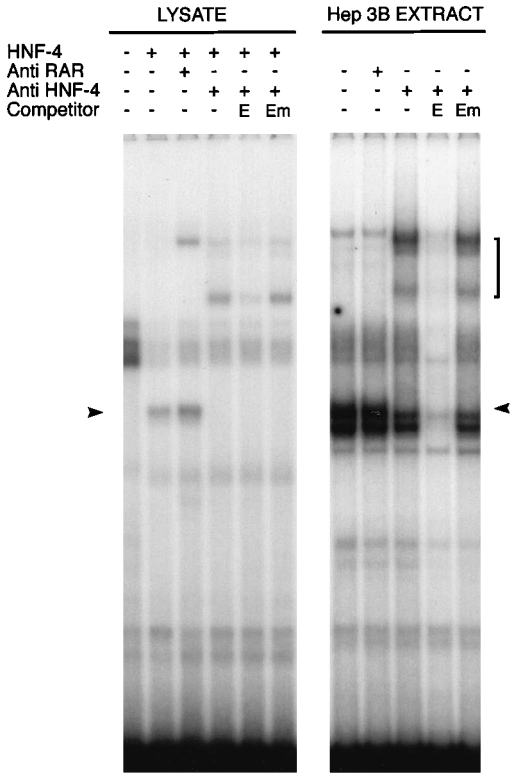

FIG. 3. Epo enhancer binds HNF-4 in Hep3B nuclear extract. EMSA analysis of the binding of Epo enhancer (E-B) (Fig. 1) by HNF-4 expressed in vitro in reticulocyte lysate (left) and nuclear extract from hypoxia-treated Hep3B cells (right), in the absence or presence of anti-RARα or anti-HNF-4 antisera as well as 50-fold-molar-excess unlabeled wild-type E-B (E) and mutant E-B (Em) competitors, as indicated above each lane, is shown. The far left lane contains unprogrammed lysate. The arrowheads indicate the complexes formed by HNF-4 and supershifted to the regions labeled with brackets by the anti-HNF-4 antiserum. Competition by unlabeled E-B and mutant E-B in the absence of anti-HNF-4 antiserum was also examined (data not shown), and it clearly demonstrated that HNF-4 binding was specific. All lanes are from the same gel.

Hep3B cells, whereas the migration of the DNA-protein complex observed with RORα1 did not match the migration of any complex formed by the Hep3B nuclear extract. TR2-11 comigrated with the diffusely migrating complex observed with Hep3B nuclear extract (bracket), whereas HNF-4 comigrated with the top complex of a faster triplet (triple arrowheads). We were unable to demonstrate heterodimer formation when probes were added to binary mixtures of those orphans which alone showed specific binding, e.g., HNF-4 plus TR2-11, HNF-4 plus RORα1, and TR2-11 plus RORα1, nor did these orphans or EAR3 form heterodimers with RARα or RXRα (data not shown). In like manner, when the promoter probe P1-bc (containing two of the three promoter half-sites) was tested, TR2-11 and HNF-4 prepared in vitro again generated specific DNA-protein complexes that migrated similarly to those observed with Hep3B nuclear extract, whereas RORα1 did not (right panel of Fig. 2). The DNA-protein complex observed with HNF-4 was consistently weaker when the promoter probe (P1-bc) was used than when the enhancer probe E-B was used (left panel). This was also true when the full-length P1 probe (containing all three sites) was used (data not shown).

HNF-4 and members of the COUP family present in Hep3B nuclear extract bind to the Epo promoter and enhancer. We used antisera to some of the receptors tested as described above that bound the Epo probes to determine if the DNA-protein complexes observed with Hep3B nuclear extract contained those receptors. In EMSA of both normoxic and hypoxic Hep3B nuclear extract, no supershifts were noted with antisera to RARα, RARβ, RARγ, RXRα, and RXRγ (anti-RARα data are shown in Fig. 3 and 4; data for the others are not shown). As shown in Fig. 3, when in vitro-expressed HNF-4 was mixed with the Epo enhancer probe E-B, the complex formed corresponded closely to the uppermost band of a strong triplet seen with both normoxic and hypoxic Hep3B nuclear extract. The addition of anti-HNF-4 antisera to Hep3B nuclear extract resulted in the supershifting of the uppermost complex in the triplet to novel positions of reduced mobility that again comigrated with the supershifted in vitro-expressed HNF-4, demonstrating that the proteins from Hep3B nuclear extract that bind Epo probes include HNF-4. Moreover, these interactions could be readily displaced by unlabeled E-B (E) but not by E-Bm mutated at the hexanucleotide repeats (Em). Similar analysis with promoter probes demonstrated HNF-4 binding, albeit weaker, in Hep3B nuclear extract (data not shown).

EMSA analysis also indicated that a COUP family member, EAR3/COUP-TF1, binds to both the Epo enhancer and the Epo promoter and comigrates with a complex observed with Hep3B nuclear extract (the middle band of the triplet) (Fig. 4, arrow on the left). This particular complex formed by Hep3B nuclear extract (Fig. 4, arrow on the left) was supershifted in

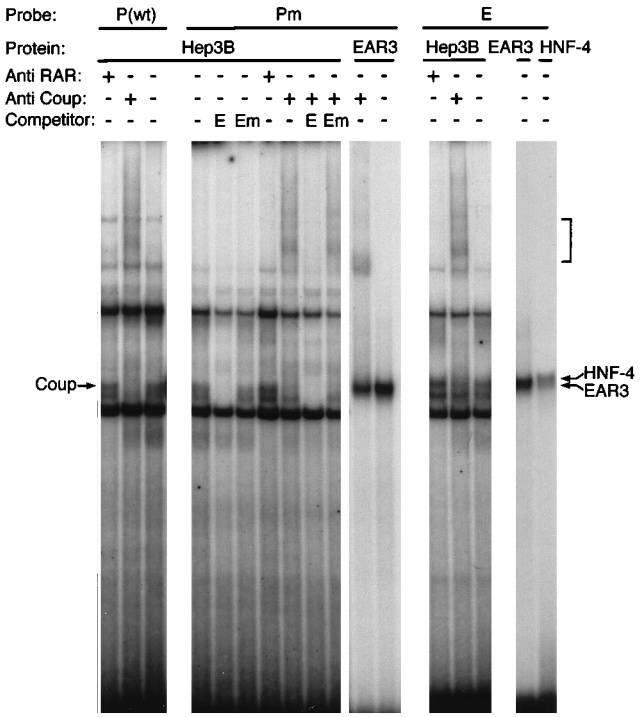

FIG. 4. An EAR3/COUP-TF1-related factor from Hep3B nuclear extract binds both the Epo enhancer and promoter. Results of EMSA analysis of the binding of Epo promoter probes Pwt and Pm and Epo enhancer probe E-B (E) (Fig. 1) to in vitro-expressed EAR3/COUP-TF1 and nuclear extract from hypoxia-treated Hep3B cells as indicated above each lane are shown. Probe Pm has the 4-bp deletion in the most 3′ of the putative response elements in the promoter (m3 in Fig. 1). Also added as indicated were anti-RARα antiserum, polyclonal anti-hCOUP antiserum, and/or a 25-fold molar excess of unlabeled wild-type E-B (E) or mutant E-B (Em) oligonucleotide as a competitor. The HNF-4 and EAR3 arrows indicate the complexes formed by the respective in vitro-synthesized proteins. The Coup arrow indicates the complex formed by an EAR3/COUP-TF1-related factor in Hep3B nuclear extracts and supershifted to the region labeled with the bracket by the anti-hCOUP antiserum. The in vitro-expressed EAR3/COUP-TF1 was incompletely supershifted because of the large amount of protein produced relative to the amount of antiserum used, although this amount of antiserum was sufficient to completely shift the complex in the nuclear extract. All lanes are from the same gel.

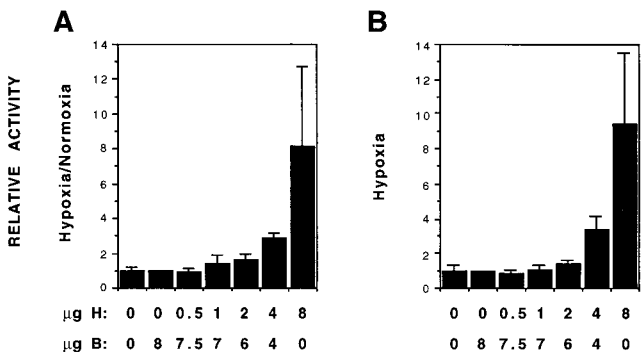

FIG. 5. HNF-4 enhances the hypoxic induction of the reporter pEPLuc in transient transfection experiments with HeLa cells. HeLa cells were cotransfected with 30 μg of pEPLuc-v1 and increasing amounts (shown in micrograms) of pCMX-HNF-4 (H) as indicated, balanced with pCMX (B) to equal totals of 8 μg. The transfected cells were split and then incubated for 40 to 44 h either at 21% O_2 or at 1% O_2. The results have been normalized within each experiment to the activity observed for the reporter in the presence of 8 μg of B and averaged over at least three independent experiments for each point with error bars designating standard deviations. (A) The normalized ratio of luciferase expression at 1% O_2 (hypoxia) to that at 21% O_2 (normoxia) is shown. The absolute ratio of hypoxic luciferase activity to normoxic luciferase activity observed in the presence of reporter plus 8 μg of B was 221 ± 48. (B) Normalized expression of luciferase in cells exposed to hypoxia (1% O_2). The average luciferase activity observed in these experiments with hypoxia treatment in the presence of 8 μg of HNF-4 was (6.15 ± 1.85) × 10^5 relative light units per 100 μl of extract.

the presence of anti-hCOUP antiserum. Because of the weak binding of HNF-4 to the promoter probes, the top complex in the triplet is barely visible. Therefore, the complex comigrating with EAR3/COUP-TF1 appears as the upper complex of a doublet. The interpretation of this supershift is confounded by wild-type (Pwt) complexes with similar mobility observed even in the absence of antibody. A clearer pattern of the novel complexes of reduced mobility generated by anti-hCOUP antiserum (Fig. 4, bracket) was obtained with a mutant promoter probe (Pm), in which the most 3′ putative response element in the promoter was mutated as shown in Fig. 1 (m3). Both the unshifted and antibody-supershifted COUP complexes formed on the promoter probes were displaced specifically by the unlabeled enhancer oligonucleotide E-B (E). The Epo enhancer as a probe yielded similar results. The supershifting of the COUP-related complex by the anti-COUP antiserum enabled clearer observation of the closely migrating HNF-4-containing complex. Thus far, we have no information concerning the identity of the DNA-binding factor in the fastest migrating sequence-specific complex of the triplet (specific for the nuclear hormone receptor response elements in the Epo promoter and enhancer). In conclusion, we have demonstrated that HNF-4 and a member of the COUP family (possibly EAR3/COUP-TF1) are both involved in binding to the Epo enhancer and promoter in Hep3B cells.

Expression of HNF-4 in HeLa cells enhances the hypoxia induction of an Epo gene reporter. The EMSA results reported above prompted us to perform reporter gene experiments in order to examine the effects of selected nuclear hormone receptor family members on Epo gene expression. We focused our attention on HNF-4, since it is known to be expressed in the intestines, kidneys, and liver (48). The latter two organs are the principle sites of Epo production. In order to assess a possible functional role of HNF-4 in the regulation of the Epo gene, a vector expressing full-length rat HNF-4 was cotransfected along with Epo-luciferase reporter constructs into Hep3B cells. However, the addition of HNF-4 had little effect on reporter gene expression (data not shown), perhaps because of the ability of Hep3B cells to produce adequate amounts of endogenous HNF-4. Because HNF-4, as mentioned above, is expressed in the liver, we performed Northern blot hybridization and RNase protection assays on Hep3B cell RNA and documented that HNF-4 is expressed in both normoxic and hypoxic cells (data not shown). In contrast, there was no expression of HNF-4 in HeLa cells. Since hypoxic induction can occur in many cell types, including HeLa cells (31, 55), we examined the effect of expression of HNF-4 on the induction of Epo-luciferase reporter genes in HeLa cells. The construct pEPLuc-v1 contained both the Epo promoter and 3′ enhancer as previously described (5). As shown in Fig. 5A, cotransfection with HNF-4 markedly increased hypoxic induction in a dose-dependent fashion. In these (Fig. 5B) and subsequent experiments whose results are shown in Fig. 6, 7, and 9B, transfection of HNF-4 affected the expression of the Epo-luciferase reporter in hypoxic cells, but not the low-level expression in normoxic cells. Thus, in all cases, the hypoxic induction of luciferase (ratio of expression at 1% O_2 to that at 21% O_2) closely paralleled the luciferase expression in hypoxic cells. Transfection of HeLa cells with the promoterless and enhancerless pXP2 luciferase vector yielded

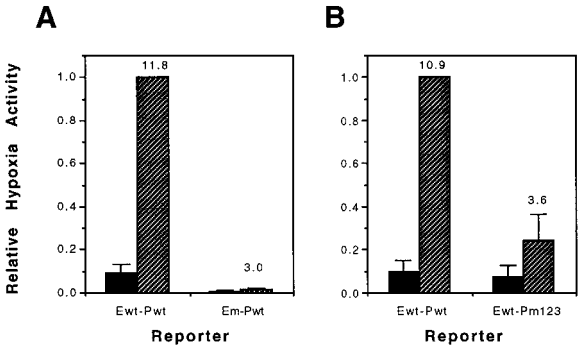

FIG. 6. Mutation of the nuclear hormone receptor response elements in the Epo enhancer and Epo promoter affects HNF-4 enhancement of the hypoxia response in HeLa cells. (A) The wild-type EPLuc-v2 reporter gene (Ewt-Pwt-v2) compared with a reporter construct mutated in the nuclear hormone receptor response elements in the enhancer (Em-Pwt-v2). (B) The wild-type EPLuc-v3 reporter gene (Ewt-Pwt-v3) compared with a reporter construct mutated in all three of the nuclear hormone receptor response elements in the promoter (Ewt-Pm123-v3). See Fig. 1 and Materials and Methods for descriptions of the reporter constructs. Each EPLuc reporter (30 μg) was cotransfected into HeLa cells with 8 μg of either pCMX (filled bars) or pCMX-HNF-4 (hatched bars). Cells were split and exposed to normoxia or hypoxia for 40 to 44 h. The results have been normalized within each experiment to the activity observed for the wild-type reporter in the presence of 8 μg of HNF-4 and averaged over at least three independent experiments for each point with error bars designating standard deviations. Numbers above the bars indicate for each reporter the enhancement of the hypoxia response due to the presence of HNF-4 (the ratio of the hypoxia response in the presence of HNF-4 to the hypoxia response in the presence of the pCMX vector).

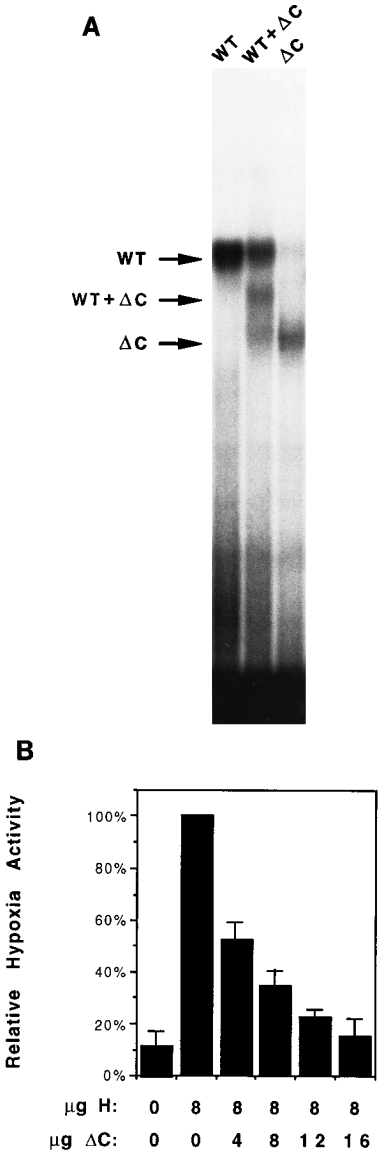

FIG. 7. Cotransfection of the C-terminal truncation mutant of HNF-4 (HNF-4ΔC) inhibits the enhancement of the hypoxic induction of the reporter pEPLuc-v1 by wild-type HNF-4. (A) Truncation of the C-terminal 101 amino acids of HNF-4 does not interfere with DNA binding. Results of EMSA analysis of in vitro-synthesized full-length HNF-4 (WT) and/or HNF-4ΔC (ΔC) binding to the Epo enhancer probe E-B as indicated above each lane are shown. The proteins involved in each complex formed are labeled with arrows. (B) HeLa cells were cotransfected with 30 μg of pEPLuc-v1 and various amounts (shown in micrograms) of pCMX-HNF-4 (H) and pCMX-HNF-4ΔC (ΔC) as indicated, balanced with pCMX to equal totals of 24 μg. Cells were split and exposed to normoxia or hypoxia for 40 to 44 h. The results are expressed as average percentages of the hypoxia-induced activity observed within each experiment for the reporter in the presence of 8 μg of H plus 16 μg of pCMX and are averaged over three independent experiments with error bars designating standard deviations. The average luciferase activity observed in these experiments for the reference sample was $(2.79 \pm 1.53) \times 10^5$ relative light units per 100 μl of extract.

no luciferase activity irrespective of whether HNF-4 was cotransfected or whether the cells were treated with hypoxia (not shown).

Binding sites in both the Epo enhancer and promoter are important for HNF-4 enhancement of hypoxia induction. In order to delineate the mechanism of HNF-4 enhancement and to determine whether the nuclear hormone receptor response sites in both the Epo enhancer and promoter are required for the HNF-4 effect on hypoxia induction in HeLa cells, we compared reporters mutated (Fig. 1) in either the enhancer (Em-Pwt-v2; Fig. 6A) or the promoter (Ewt-Pm123-v3; Fig. 6B) with the corresponding wild-type reporters. The nuclear hormone receptor response sites in the enhancer (Em-Pwt-v2; Fig. 6A) were important for the basal hypoxia induction as well as the enhancement by HNF-4. The basal (in the absence of HNF-4) hypoxia activity of the mutant enhancer reporter was 10% of the wild-type level, and the ability of HNF-4 to boost the hypoxia activity was only 27% of the wild-type level. However, the nuclear hormone receptor response sites in the promoter were less important for the basal hypoxia activity (75% of the wild-type level) but very important for the enhancement by HNF-4 (33% of the wild-type level). Thus, HNF-4 augments the hypoxic induction of pEPLuc in HeLa cells by binding to the nuclear hormone receptor response elements in the Epo enhancer and promoter.

Cotransfection with a dominant negative HNF-4 expression vector inhibits HNF-4 enhancement of hypoxia induction of the Epo gene reporter. The HNF-4 enhancement of hypoxia induction was further examined by cotransfection in HeLa cells of an expression vector coding for a truncated HNF-4 protein lacking the C-terminal 101 amino acids (HNF-4ΔC) along with pEPLuc-v1 and pCMX-HNF-4. By analogy with other well-characterized nuclear receptors, this truncated protein is predicted to retain the DNA binding domain and to lack a small portion of the ligand binding domain and the entire C-terminal activation domain (12, 15, 53). Accordingly, as expected, EMSA with in vitro-transcribed and -translated HNF-4ΔC demonstrated binding to the Epo enhancer and promoter probes with the formation of complexes that migrated faster than complexes containing the full-length HNF-4 (HNF-4wt). HNF-4 binds DNA as a homodimer, and a mixture of HNF-4wt and HNF-4ΔC was demonstrated to form a heterodimer

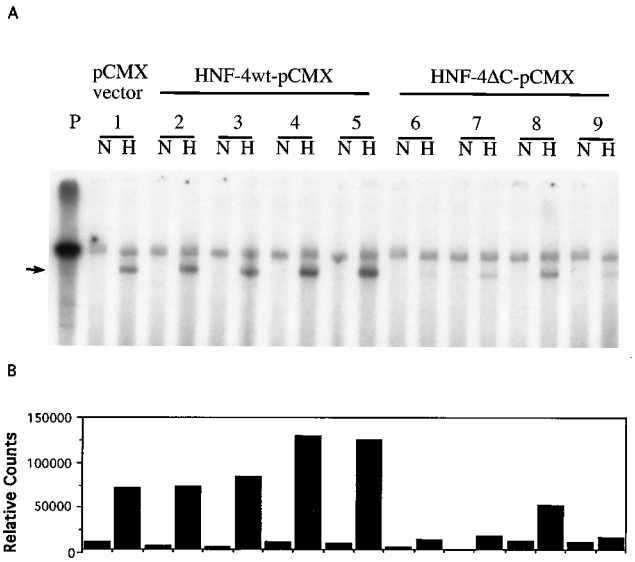

FIG. 8. Stable transfection of the C-terminal truncation mutant of HNF-4 (HNF-4ΔC) into Hep3B cells inhibits the hypoxic induction of the endogenous Epo gene. Hep3B cells were stably transfected with either pCMX vector, full-length pCMX-HNF-4(wt), or pCMX-HNF-4ΔC, and individual colonies were isolated and expanded as described in Materials and Methods. For each cell line tested as indicated, RNA was made from both cells incubated in normoxia (N) and cells incubated in hypoxia (H) for 6 h. The RNA samples (40 μg each) were analyzed by an RNase protection assay with an Epo riboprobe. (A) Autoradiogram of endogenous Epo-protected fragment (indicated by the arrow). Sample P is the probe, and the more slowly migrating band above the indicated Epo-protected fragment is the undigested probe. (B) The results obtained by quantitation with a Molecular Dynamics PhosphorImager of the indicated Epo band.

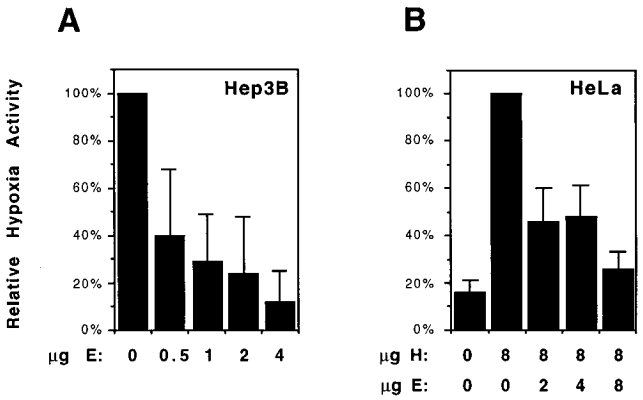

FIG. 9. EAR3/COUP-TF1 inhibits Epo expression. (A) Cotransfection of EAR3/COUP-TF1 inhibits the expression of the reporter pEPLuc-v1 in Hep3B cells. Hep3B cells were cotransfected with 10 μg of pEPLuc-v1 and various amounts (shown in micrograms) of pCMX-EAR3 (E) as indicated, balanced with pCMX to equal totals of 4 μg. Cells were split and exposed to normoxia or hypoxia for 40 to 44 h. The results are expressed as average percentages of the hypoxia-induced activity observed within each experiment for the reporter in the presence of 4 μg of pCMX averaged over four independent experiments with error bars designating standard deviations. The average luciferase activity observed in these experiments with hypoxia treatment for the reference sample was $(8.15 \pm 2.63) \times 10^5$ relative light units per 100 μl of extract. (B) EAR3/COUP-TF1 suppresses the enhancement by HNF-4 of the hypoxic induction of the reporter pEPLuc in HeLa cells. HeLa cells were cotransfected with 30 μg of pEPLuc-v1 and various amounts (shown in micrograms) of pCMX-HNF-4 (H) and pCMX-EAR3 (E) as indicated, balanced with pCMX to equal totals of 16 μg. Cells were split and exposed to normoxia or hypoxia for 40 to 44 h. The results are expressed as average percentages of the hypoxia-induced activity observed within each experiment for the reporter in the presence of 8 μg of H plus 8 μg of pCMX and are averaged over three independent experiments with error bars designating standard deviations. The average luciferase activity observed in these experiments for the reference sample was $(5.58 \pm 2.27) \times 10^4$ relative light units per 100 μl of extract.

on the Epo enhancer that migrates between the complexes formed by each homodimer (Fig. 7A). HNF-4ΔC had no effect on the basal (in the absence of HNF-4wt) level of hypoxia induction (data not shown). However, the enhancement in the hypoxic induction of the Epo-luciferase reporter construct in HeLa cells by cotransfection with pCMX-HNF-4wt could be suppressed by the addition of increasing amounts of pCMX-HNF-4ΔC (Fig. 7B). This demonstrates that in HeLa cells the truncated HNF-4 receptor can compete for binding to the nuclear hormone receptor response elements in the Epo reporter gene and that the C-terminal activation domain is required for HNF-4's enhancement of hypoxic induction.

Stable transfection of HNF-4ΔC into Hep3B cells inhibits the hypoxia induction of the endogenous Epo gene. We were not able to observe a significant effect of transient cotransfection of either HNF-4wt or the truncated HNF-4ΔC with an Epo reporter construct into Hep3B cells, and so we created stable Hep3B lines expressing either HNF-4wt or HNF-4ΔC. We then assessed the impact of the stably transfected HNF-4 on the hypoxia induction (at 6 h) of the endogenous Epo gene by RNase protection assay (Fig. 8A) and analysis by PhosphorImager quantitation (Fig. 8B). Expression of the transfected HNF-4 was also assessed by RNase protection assay (data not shown). When the single cell line transfected with vector alone was challenged with hypoxia, Epo mRNA was expressed at a level of 6.9×10^4 (arbitrary units), whereas the four cell lines expressing HNF-4wt had a mean hypoxia value of $(10.2 \pm 2.9) \times 10^4$, indicating a slight enhancement of Epo expression when HNF-4wt levels were elevated. However, in keeping with the transient transfection results with HeLa cells shown in Fig. 7B, the four Hep3B cell lines expressing HNF-4ΔC, when subjected to hypoxia, had a mean Epo mRNA expression level of only $(2.3 \pm 1.8) \times 10^4$, or a 78% decrease in hypoxia induction compared with that observed in the cells transfected with HNF-4wt. Therefore, this experiment demonstrates that in Hep3B cells the truncated HNF-4 receptor can inhibit the induction of the endogenous Epo gene by competing for binding to the nuclear hormone receptor response elements.

EAR3/COUP-TF1 represses HNF-4 enhancement of hypoxia induction. The COUP family of nuclear receptors is known to compete with other receptors, thereby imposing negative regulation (22, 27, 32, 34, 35, 51). In particular, the stimulation of the transcription of a number of reporter genes by HNF-4 can be suppressed by COUP family members, suggesting competitive binding to the same regulatory element (27, 32). In order to determine whether EAR3/COUP-TF1 plays a functional role in Epo regulation, experiments involving cotransfection with the Epo reporter into Hep3B cells (Fig. 9A) or into HeLa cells together with HNF-4wt (Fig. 9B), similar to that described above for HNF-4ΔC, were done. The addition of even 0.5 μg (1/20th of the amount of Epo reporter) of pCMX-EAR3 resulted in a significant inhibition of the hypoxic expression of the Epo reporter in Hep3B cells (Fig. 9A), demonstrating that EAR3/COUP-TF1 plays a negative modulatory role in the regulation of Epo expression. The addition of increasing amounts of pCMX-EAR3 resulted in a nearly complete suppression of the enhanced hypoxia induction by HNF-4 in HeLa cells (Fig. 9B). In contrast, cotransfection of EAR3/COUP-TF1 with the reporter in the absence of HNF-4 had only a small effect on the basal level of hypoxia induction observed (data not shown), indicating that the suppressive effect is probably due to competition with HNF-4 for the nuclear hormone receptor consensus sites.

DISCUSSION

HNF-4 and members of the COUP family bind to sites in the Epo promoter and enhancer. We have examined a set of in vitro-transcribed and -translated cloned orphan receptors for binding to the Epo promoter and enhancer nuclear hormone receptor consensus sequences (Fig. 2; see Materials and Methods). We found that RARα:RXRα heterodimer, TR2-11, HNF-4, RORα1, and EAR3/COUP-TF1 could each bind to the Epo probes. We then compared these complexes with those formed by Hep3B nuclear extract. Only recombinant TR2-11, HNF-4, and EAR3/COUP-TF1 formed complexes that comigrated with complexes formed on Epo probes by Hep3B nuclear extract. None of the antisera to various members of the RAR and RXR families supershifted any of the complexes formed by Hep3B nuclear extract, nor did the RARα:RXRα heterodimer comigrate with any Hep3B complexes. An antiserum specific for HNF-4 supershifted one of the complexes formed by Hep3B nuclear extract (Fig. 3), and an antiserum specific for all COUP family members supershifted another of the Hep3B nuclear extract complexes (Fig. 4). We focused on HNF-4 and EAR3/COUP-TF1, as they are known to be expressed in the liver and kidneys. Indeed, HNF-4 is an especially attractive candidate, since two of its three major sites of expression, liver and kidney cortex (32), correspond to the principle sites of in vivo Epo production. In contrast, TR2-11 is expressed relatively weakly in the liver and not at all in the kidneys (6).

HNF-4 enhances hypoxia induction. Among the growing number of orphan nuclear receptors that have been cloned, HNF-4 is one of the best understood and characterized (32, 39, 47, 48, 49). Recently, a number of laboratories have demonstrated that HNF-4 contributes to the regulation of a diverse group of biologically important genes, including those encoding apolipoproteins, coagulation factors, serum proteins, and cytochrome p450s as well as those involved in metabolism of fatty acids, amino acids, and glucose (39, 47). We tested the function of HNF-4 in HeLa, a naive cell line that does not express either this orphan receptor or Epo. Transient transfection of the pEPLuc reporter plasmid along with increasing amounts of HNF-4 resulted in a marked enhancement of luciferase production in hypoxic cells but not in normoxic cells (Fig. 5). This up-regulation by full-length HNF-4 could be abrogated by cotransfection with pCMX-HNF-4ΔC, which contains the DNA binding domain and most of the ligand binding-dimerization domain but lacks the C-terminal activation domain (Fig. 7). Thus, the enhancement by HNF-4 of hypoxic induction depends on the interaction of HNF-4 with other transcription factors through its transactivation domain. Although transient cotransfections of the Epo reporter with HNF-4ΔC in Hep3B were uninformative, stable transfection of Hep3B cells with HNF-4ΔC significantly inhibited the hypoxia-induced expression of the endogenous Epo gene (Fig. 8). This strongly suggests that HNF-4 plays a positive regulatory role in the hypoxia responsiveness of the Epo gene in liver cells.

The ability of HNF-4 to enhance the hypoxia induction of the Epo reporter in HeLa cells required intact nuclear hormone receptor consensus sites in both the enhancer (DR2) and promoter (Fig. 6). The basal level of hypoxic induction of the pEPLuc reporter in HeLa cells in the absence of HNF-4 was quite significant and was dependent on the presence of the DR2 site in the enhancer (Fig. 6). This indicates that another unidentified positively acting factor that can bind this site is present in HeLa cells. HNF-4 may act to enhance hypoxia induction via cooperative interaction and binding with this other factor, or it may compete for the DR2 site as a stronger positive activator. Although many members of the nuclear hormone receptor family are known to form heterodimers with other family members, HNF-4 binds well as a homodimer and has not been demonstrated to heterodimerize with any of the known factors. Therefore, it is more likely that HNF-4 is binding the DR2 directly and cooperating via its transactivation domain with the transcription apparatus.

In the presence of the intact enhancer, mutation of all three of the nuclear receptor binding half sites in the promoter interfered significantly with HNF-4 enhancement but only partially with basal hypoxia induction in HeLa cells. Therefore, these sites are not absolutely required for synergy with the enhancer when a factor(s) other than HNF-4 is binding to the DR2, and other sites within the promoter may be involved (perhaps the CA repeats). However, HNF-4 enhancement is much better if the binding sites in both the promoter and enhancer are present. Mutation of either promoter or enhancer sites deceases the HNF-4 enhancement by 70%. Therefore, the importance of the promoter half sites may depend on which of the nuclear hormone receptor family members are present.

The DR2 site without the other two regulatory regions in the enhancer (the HIF-1 site and the CA repeats) cannot synergize with the Epo promoter in a luciferase reporter in Hep3B cells, nor does the enhancer function well without the DR2 site (reference 5 and Fig. 6). Thus, the proteins binding at the DR2 site, including HNF-4, may be involved in direct protein-protein interactions with the factors binding to the HIF-1 and CA repeat sites to form the fully active complex upon hypoxia challenge. The proteins binding to the Epo promoter may also cooperate in the formation of this active complex.

EAR3/COUP-TF1 negatively regulates hypoxic induction. In a number of systems, transcriptional regulation by HNF-4 can be antagonized by COUP family nuclear receptors (22, 27, 32, 35). Our gel shift experiments showed that one of the nuclear proteins from Hep3B cells that bind specifically to both the Epo enhancer and the Epo promoter has a gel mobility shift identical to that seen with EAR3/COUP-TF1, one of the COUP family members (Fig. 4). Moreover, cotransfection of EAR3/COUP-TF1 and the Epo reporter either alone into Hep3B cells (Fig. 9A) or with HNF-4 into HeLa cells (Fig. 9B) suppressed the hypoxia induction of the Epo reporter. The latter result suggests that the repression by EAR3/COUP-TF1 involves specific competition with HNF-4 for binding to the Epo gene. Since EAR3/COUP-TF1 and ARP-1/COUP-TF2 receptors are expressed in a broad variety of tissues and EAR2 is expressed primarily in the liver (33), it is likely that one or more of the COUP family members modulate the expression of Epo in vivo. As yet we have no evidence that EAR3/COUP-TF1 is a more likely contributor than other COUP family members. HNF-4 does not form heterodimers with ARP-1/COUP-TF2 (27, 32). Thus, EAR3/COUP-TF1 is more likely to antagonize HNF-4 action by competing for binding to the DNA than by formation of inactive heterodimers with HNF-4. The relative levels of HNF-4 and the COUP family members may control the fine-tuning of Epo production in response to hypoxia.

In conclusion, the experiments reported in this paper suggest that HNF-4 contributes importantly to the physiologic regulation of the Epo gene as well as its tissue specificity. Although it is possible that HNF-4 acts on another gene required for hypoxic induction, the simplest and most heuristic interpretation of our experiments is that HNF-4 binds to response elements in the Epo promoter and enhancer, thereby up-regulating hypoxic induction. In view of the proximity of the HIF-1 binding site on the 3′ enhancer to the HNF-4 binding

site and the requirement that both of these regions be intact for a fully functional enhancer, we are now exploring whether there is direct interaction between these two DNA-binding proteins.

By definition, orphan nuclear receptors such as HNF-4 lack a known ligand. Although there is evidence that some receptors function in the absence of any ligand (reference 2 and references therein), this is probably not true for HNF-4, one of the most highly conserved of all the nuclear receptors. Preservation of a high degree of homology in the ligand binding domain argues strongly that the function of this receptor depends on its binding to a ligand which is yet to be discovered. In view of its role in the hypoxic induction of the Epo gene, it is tempting to speculate that the ligand for HNF-4 may participate in oxidation-reduction chemistry.

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The first two authors contributed equally to this work.

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