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TFIIB-Directed Transcriptional Activation by the Orphan Nuclear Receptor Hepatocyte Nuclear Factor 4

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The orphan nuclear receptor hepatocyte nuclear factor 4 (HNF-4) is required for development and maintenance of the liver phenotype. HNF-4 activates several hepatocyte-specific genes, including the gene encoding apolipoprotein AI (apoAI), the major protein component of plasma high-density lipoprotein. The apoAI gene is activated by HNF-4 through a nuclear receptor binding element (site A) located in its liver-specific enhancer. To decipher the mechanism whereby HNF-4 enhances apoAI gene transcription, we have reconstituted its activity in a cell-free system. Functional HNF-4 was purified to homogeneity from a bacterial expression system. In in vitro transcription assays employing nuclear extract from HeLa cells, which do not contain HNF-4, recombinant HNF-4 stimulated transcription from basal promoters linked to site A. Activation by HNF-4 did not exhibit a ligand requirement, but phosphorylation of HNF-4 in the in vitro transcription system was observed. The activation function of HNF-4 was localized to a domain displaying strong homology to the conserved AF-2 region of nuclear receptors. Dissection of the transcription cycle revealed that HNF-4 activated transcription by facilitating assembly of a preinitiation complex intermediate consisting of TBP, the TATA box-binding protein component of TFIID and TFIIB, via direct physical interactions with TFIIB. However, recruitment of TFIIB by HNF-4 was not sufficient for activation, since HNF-4 deletion derivatives lacking AF-2 bound TFIIB. On the basis of these results, HNF-4 appears to activate transcription at two distinct levels. The first step involves AF-2-independent recruitment of TFIIB to the promoter complex; the second step is AF-2 dependent and entails entry of preinitiation complex components acting downstream of TFIIB.

Liver organogenesis and hepatic differentiation are achieved via a network of transcriptional regulators (for review, see reference 74). Hepatocyte nuclear factor 4 (HNF-4) occupies a primordial position in this hierarchy (6, 38) and disruption of its gene is embryonic lethal (10). In the adult animal, HNF-4 is primarily expressed in liver, intestine, and kidney cells (14, 67, 76). In combinatorial associations with other hepatic and ubiquitous transcription factors (40, 73), HNF-4 is required for tissue-specific expression of numerous liver-restricted genes, including those involved in glucose metabolism (25), urea biosynthesis (63), erythropoesis (19), and cholesterol homeostasis (8, 18, 21, 22, 26, 52, 53).

HNF-4 (reviewed in references 65 and 66) is a member of the nuclear receptor superfamily, which also includes the receptors for retinoids, vitamin D_3, and thyroid and steroid hormones, as well as many orphan receptors whose ligands remain unknown (70, 72). Nuclear receptors are characterized by two zinc finger DNA binding motifs and a large, conserved hydrophobic segment containing the dimerization and putative ligand binding domains (23, 70). In addition, transcription activation and/or repression domains have been localized to various regions of the molecule (70). Typically, one activation function (AF-1) is located in the hypervariable amino-terminal A/B region, and another (AF-2) is located in the highly conserved carboxy-terminal E region (70).

Like other transcription factors, nuclear receptors are thought to exert their regulatory effects (activation or repression) upon binding to their cognate binding elements (58, 69) in the promoter DNA, which consist of hexanucleotide repeats (23, 70). The precise mechanism whereby the regulatory signal is transduced to RNA polymerase II is currently the focus of intense research. For classical steroid hormone receptors, this process is triggered by ligand binding to the receptor (70). On the other hand, the mechanisms by which orphan receptor activity is modulated are not known (55). Given the conservation of the putative ligand binding domain and eventual discovery of ligands for some receptors (28, 43), it has been argued that all orphan receptors rely on similar control mechanisms (55, 70).

The hepatocyte-specific enhancer in the gene encoding apoliprotein AI (apoAI), a major component of high-density lipoprotein (reviewed in reference 34) contains a site (site A) consisting of direct hexanucleotide repeats that can bind and respond to several members of the nuclear receptor family (26, 39, 59). Binding studies have shown that site A is occupied by endogenous HNF-4 in human hepatoma HepG2 cells (50). Moreover, HNF-4 activates transcription through site A (as well as other HNF-4 elements) in a variety of hepatic and nonhepatic cell types (18, 26), as well as in *Saccharomyces cerevisiae* (18), indicating that factors required for transcriptional activation by HNF-4 are ubiquitously distributed. Recent evidence has further imparted a critical role to HNF-4 in synergizing with the hepatocyte-enriched factor HNF-3β for activation of the apoAI liver-specific enhancer (26).

Therefore, as part of our ongoing effort to recreate apoAI liver-specific transcription in vitro and to establish the mechanism of action of HNF-4 in this process, we have employed cell-free systems to assess the transcriptional activation potential of recombinant HNF-4. Our results suggest that HNF-4 does not require a conventional ligand to activate genes under its control. We have mapped the activation domain of HNF-4 and have shown that enhancement of the preinitiation complex (PIC) is effected in part through direct physical interactions

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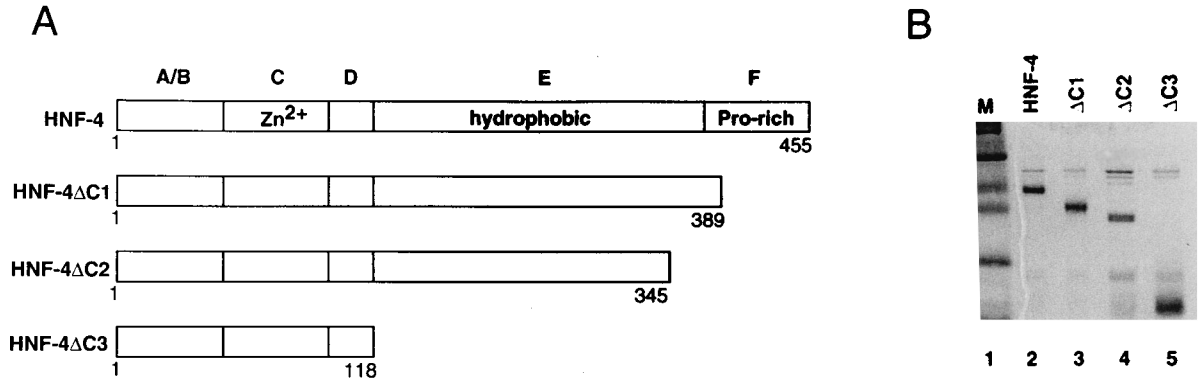

FIG. 1. Purification of recombinant HNF-4 and deletion derivatives. (A) Schematic representation of full length HNF-4 (top) and the carboxy-terminal deletions generated in this study. Salient features of the molecule are marked (5). Numbers to the right indicate the carboxy-terminal residues of the truncated proteins. (B) Bacterially expressed HNF-4 (lane 2) and its truncated versions (lanes 3 to 5) tagged with six histidine residues were purified over Ni^{2+}-nitrilotriacetic acid-agarose resin. Samples (0.5 μg) were analyzed by SDS-PAGE and visualized by Coomassie blue staining. Molecular weight markers (M [lane 1]) are also included.

with the basal transcription factor TFIIB. We further suggest that during HNF-4-mediated transcription activation, recruitment of TFIIB and assembly of downstream basal factors into the PIC are distinct steps controlled by different activities in HNF-4.

MATERIALS AND METHODS

Bacterial expression of HNF-4 and its derivatives. Standard molecular biology procedures were employed for all recombinant DNA work (61).

Rat HNF-4 cDNA (67) was amplified by oligonucleotide primers which introduced an *Nde*I site at the 5′ (amino-terminal) end and a *Bam*HI site at the 3′ (carboxy-terminal) end. The PCR product was subcloned into *Nde*I- and *Bam*HI-cleaved expression vector 6His-pET11d (29). The resultant plasmid was transformed into *Escherichia coli* BL21 (DE3) (pLysS) (Novagen). After induction, histidine-tagged HNF-4 was purified over Ni^{2+} affinity resin as described previously (51). The HNF-4 derivatives (Fig. 1A) HNF-4ΔC1, HNF-4ΔC2, and HNF-4ΔC3 were similarly expressed, except that different 3′ oligonucleotide primers for PCR amplification were used.

Templates for in vitro transcription. Plasmid construct pA_{4x}MLΔ53, containing four copies of site A upstream of the adenovirus major late (ML) core promoter, has been reported previously (50). It contained a 320-bp G-free cassette (64). Plasmids pA_{1x}AI and pA_{2x}AI were derived from a pUC19 vector containing a ca. 380-bp G-free cassette downstream from the apoAI core promoter sequences (positions −41 to −7) at the *Pst*I site (62). One (pA_{1x}AI) or two (pA_{2x}AI) copies of site A were cloned into the *Sph*I site. Plasmid pML200, containing the wild-type ML promoter upstream of a 200-bp G-free cassette, was obtained from U. Schibler.

Nuclear extracts and transcription factors. Nuclear extracts were obtained from HeLa cells by the procedure of Dignam et al. (13). Chromatography over a phosphocellulose P11 column was performed as described previously (54). Fractions eluting at 0.5 and 0.85 M KCl were extensively dialyzed against buffer A (20 mM Tris-HCl [pH 7.9], 0.1 M KCl, 20% glycerol, 0.1 mM EDTA, 1 mM dithiothreitol). The flowthrough fraction containing TFIIA was purified further by chromatography over DEAE-52 followed by MonoS fast-performance liquid chromatography (FPLC) (Pharmacia) (20). Recombinant TFIIB expressed as a histidine-tagged protein was purified to homogeneity as described previously (49, 51). Recombinant TATA-box-binding protein (TBP) was obtained from Promega Corporation, Madison, Wis.

EMSA. Reaction and electrophoresis conditions for site A interactions were as reported previously (26). The conditions for electrophoretic mobility shift assay (EMSA) involving basal transcription factors were as described previously (47). The A-TATA probe was electrophoretically purified from plasmid pA_{1x}AI after digestion with *Hin*dIII and *Sst*I.

In vitro transcription. Reaction conditions for in vitro transcription assays were as described previously (50). Briefly, 25-μl reaction mixtures contained 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [pH 7.9]), 11 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM *o*-methylguanosine, 2 U of RNase T_1, 20 U of RNasin, 500 mM (each) ATP and CTP, 25 mM [α-^{32}P]UTP at 800 Ci/mmol, 5 mM dithiothreitol, and (except for Fig. 4A) 75 μg of HeLa nuclear extract proteins. Variations (template concentrations and incubation times) are specified in the figure legends. Reactions were terminated by addition of 10 mM EDTA–0.5% sodium dodecyl sulfate (SDS)–40 μg of yeast tRNA–0.3 M sodium acetate (pH 5.2). After extraction with phenol, RNA was precipitated by ethanol and resuspended in sample buffer containing 80% formamide and bromophenol blue in 10 mM Tris-HCl (pH 7.4). Electrophoresis was in 5% polyacrylamide gels containing 7 M urea in Tris-borate buffer. Gels were autoradiographed for various times. When indicated, autoradiograms were scanned and band intensities were quantitated in a Molecular Dynamics laser densitometric scanner.

Protein-protein interaction assay. Histidine-tagged HNF-4, its deletion derivatives, and control apoAI were immobilized on Ni^{2+}-nitrilotriacetic acid-agarose (Qiagen) by incubation in buffer B (10 mM Tris-HCl [pH 7.9], 0.1 M KCl, 10% glycerol, 0.125% β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride) for 30 min at 4°C. After being washed three times with buffer B, the resin was incubated with [^{35}S]methionine-labelled in vitro-expressed TFIIB in binding buffer (10 mM Tris-HCl [pH 7.9], 60 mM KCl, 4 mM $MgCl_2$, 2% polyethylene glycol 8000, 0.125% β-mercaptoethanol) for 30 min on ice and 10 min at 30°C. The resin was washed extensively with buffer B containing 30 mM imidazole and 0.1% Nonidet P-40. After the final wash, the samples were resuspended in Laemmli sample buffer, boiled for 10 min, and resolved by SDS-polyacrylamide gel electrophoresis (PAGE).

RESULTS

Bacterially produced HNF-4 binds to site A as a homodimer. HNF-4 cDNA was subcloned into a bacterial expression vector (Materials and Methods), and the expressed protein was purified to near homogeneity by affinity chromatography. The recombinant protein was visualized as a ca. 58-kDa protein (Fig. 1B, lane 2). The slower apparent mobility of the bacterially expressed HNF-4 relative to that of the native protein (54 kDa) may be attributable to the 20 additional amino acids (including 6 histidine residues) which were fused to the amino terminus for facilitated purification. We also similarly generated carboxy-terminal deletion derivatives of HNF-4 (Fig. 1A and B, lanes 3 to 5): HNF-4ΔC1, which lacks 66 C-terminal residues (67); HNF-4ΔC2, which lacks 110 C-terminal residues spanning the proline-rich tail; and HNF-4ΔC3, which essentially contains only the putative DNA binding domain of HNF-4 and the residues amino terminal to it.

To examine any potential ligand and/or auxiliary factor dependence (75) of HNF-4 for binding to its cognate elements, affinity-purified HNF-4 and its derivatives were analyzed for binding to site A by EMSA. Figure 2A shows that bacterially expressed HNF-4 bound efficiently to site A, revealing a major DNA complex (lane 1). This complex was specific, since it could be inhibited in the presence of an excess of site A oligonucleotide (lane 2) but not by oligonucleotides lacking an HNF-4 cognate element (data not shown). Furthermore, the complex formed by HNF-4 on site A could be supershifted by an HNF-4-specific antibody (67). These results indicated that the DNA binding ability of HNF-4 did not require a ligand or auxiliary factors.

We also examined, by EMSA, the ability of the bacterially

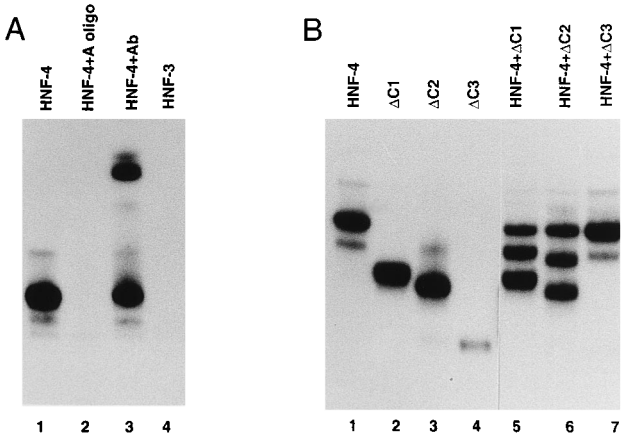

FIG. 2. DNA binding characteristics of recombinant HNF-4. (A) Specificity and ligand independence of HNF-4 interaction with apoAI site A. For EMSA, an end-labelled site A oligonucleotide (oligo) probe was incubated (30 min on ice) with 25 ng of recombinant HNF-4 preparation (lanes 1 to 3) or an equivalent amount of recombinant HNF-3β as the control (lane 4). Additions included 25 ng of unlabelled site A oligonucleotide (lane 2) and an anti-HNF-4 antiserum (Ab [lane 3]). (B) Homodimeric binding of HNF-4 to site A. Full-length HNF-4 (lanes 1 and 5 to 7) and truncated mutants HNF-4ΔC1 (lanes 2 and 5), HNF-4ΔC2 (lanes 3 and 6), and HNF-4ΔC3 (lanes 4 and 7) were incubated with site A (as described for panel A) for EMSA either individually (lanes 1 to 4) or in the indicated combination (lanes 5 to 7).

expressed HNF-4 carboxy-terminal deletion mutants to interact with site A. Figure 2B shows that a given amount of HNF-4ΔC1 and HNF-4ΔC2 yielded a band approximately equivalent in intensity to that of the full-length protein. However, HNF-4ΔC3 displayed a weaker band, consistent with the loss of large stretches of hydrophobic amino acids believed to contribute to nuclear receptor dimerization and DNA binding (23, 70). These results were corroborated by saturation binding analysis of these proteins, which suggested that the binding affinity of HNF-4ΔC3 is ca. 10-fold weaker than that of wild-type HNF-4 or that of HNF-4ΔC1 and HNF-4ΔC2 (data not shown). In view of the unusual geometry of site A, in which three hexanucleotide half-sites for nuclear receptor binding are arrayed in tandem (39), we also determined the higher-order configuration of HNF-4 in the site A complex. For this purpose, equimolar amounts of full-length HNF-4 and each of the deletion derivatives were incubated with site A, and the resulting complexes were analyzed by EMSA. As expected, HNF-4ΔC1 and HNF-4ΔC2, but not HNF-4ΔC3, heteromerized with the full-length protein, yielding complexes of intermediate mobility. Furthermore, the appearance of a single complex with intermediate mobility strongly argued for the homodimeric binding of HNF-4 to site A, in agreement with other reports (19, 33, 67). This also indicated that despite the potential for a trimeric complex, HNF-4 occupies site A as a dimer.

Ligand-independent activation of transcription by HNF-4 in vitro. To examine the transcriptional activity of recombinant HNF-4 in vitro, we monitored the response to HNF-4 of two core promoters of divergent intrinsic strength under the control of site A (see Materials and Methods) in template constructs containing a G-free cassette reporter: the template A_{4x}MLΔ53 contained four copies of site A upstream of the strong adenovirus ML core promoter (50), and the template A_{2x}AI contained two copies of site A upstream of the relatively weak apoAI core promoter elements which constitute the natural target for the apoAI liver-specific enhancer (62). The control template (ML200) consisted of a shorter G-free cassette under the control of the ML promoter. As a source of transcription factors, we employed nuclear extracts from HeLa cells, a cell type that does not express HNF-4 but in which site A-mediated stimulation of transcription by exogenous HNF-4 has been observed (data not shown). We have previously shown that while these extracts contain the orphan receptors ARP-1 and/or COUP-TF1, which also bind to site A, the endogenous factors do not interfere with the basal transcription activity of the templates described above in the in vitro transcription assay (50).

When supplemented with recombinant HNF-4, the basal transcription levels from both test templates (A_{4x}MLΔ53 [Fig. 3A] and A_{2x}AI [Fig. 3B]) were stimulated in a dose-dependent manner. Transcriptional enhancement of up to 50- and 20-fold were noted on each of the templates A_{2x}AI and A_{4x}MLΔ53, respectively. At lower HNF-4 concentrations, transcription levels from the control template were minimally affected, indicating the site A dependence of the transcription-stimulatory activity of recombinant HNF-4 (lanes 1 and 2 in Fig. 3A and B). Only at the highest HNF-4 concentration tested, was nonspecific stimulation of transcription observed (lanes 3 and 4 in Fig. 3A and lane 4 in Fig. 3B). An equivalent amount of a control preparation from *E. coli*, not expressing HNF-4, had no effect (data not shown). These results indicated that HNF-4-dependent transcriptional activation can be simulated in a cell-free system.

The recapitulation of HNF-4 function in a cell-free transcription system supplemented with a bacterially derived preparation of recombinant receptor strongly raised the possibility that this orphan receptor does not require a ligand to function. To exclude the possibility that the crude extract in which HNF-4 activity was assayed contained trace amounts of a potential "intracrine" ligand (55), we also employed extensively dialyzed chromatographic fractions derived from the extract to reconstitute basal and HNF-4-stimulated transcription from the A_{4x}MLΔ53 template (Fig. 4A). Full stimulatory activity of HNF-4 was retained under these assay conditions. Furthermore, no effect of a variety of possible candidate compounds (e.g., 17-β-estradiol, all-*trans*-retinoic acid, and 9-*cis*-retinoic acid) added to the HNF-4-dependent in vitro transcription

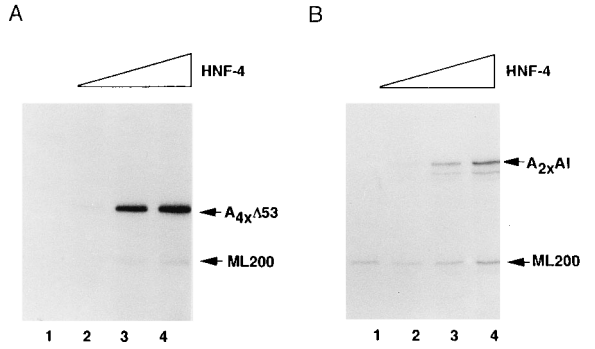

FIG. 3. Activation of transcription by HNF-4 in vitro. (A) In vitro transcription reaction mixtures employing G-free templates and HeLa cell nuclear extract contained 100 ng of A_{4x}MLΔ53 and, as control, 20 ng of ML200. Increasing amounts of recombinant HNF-4 were added to the reaction mixtures: lane 1, 0; lane 2, 25 ng; lane 3, 100 ng; and lane 4, 250 ng. Reaction mixtures were assembled on ice, and incubation was at 30°C for 1 h. ^{32}P-labelled RNA products were analyzed by denaturing PAGE. The autoradiogram was densitometrically scanned to quantitate band intensities. For each lane, the intensities of the A_{4x}MLΔ53 transcript were normalized against those of the control template ML200. Fold activation (text) is the ratio of normalized A_{4x}MLΔ53 transcript intensities in HNF-4-containing reaction mixtures to that in reaction mixtures without HNF-4. (B) In vitro transcription reactions were performed exactly as described for panel A, except that A_{2x}AI was used as the test template. Band intensities were quantitated as described for panel A to measure fold activation.

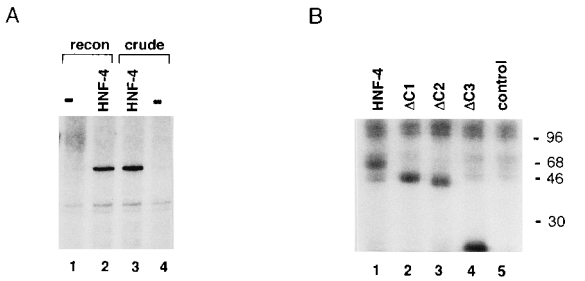

FIG. 4. Ligand-independent transcription activation by HNF-4. (A) Transcription activity was reconstituted (recon [lanes 1 and 2]) with TFIIA (2 μg [MonoS fraction]), the phosphocellulose P11–0.5 M KCl fraction (6 μg), and the phosphocellulose P11–0.85 M KCl fraction (2.5 μg). (Each fraction was extensively dialyzed [see Materials and Methods]). Transcription in lanes 3 and 4 (crude) was carried out with crude HeLa nuclear extracts (10 μg). Two hundred fifty nanograms of test template A_{4x}MLΔ53 and 25 ng of control template ML200 were incubated with no additions (lanes 1 and 4) or with 100 ng of recombinant HNF-4 (lanes 2 and 3). Reaction mixtures were processed as described in the legend to Fig. 3. (B) Phosphorylation of HNF-4 and its derivatives in HeLa nuclear extracts. A total of 100 ng (each) of full-length HNF-4 (lane 1), HNF-4ΔC1 (lane 2), HNF-4ΔC2 (lane 3), and HNF-4ΔC3 (lane 4) was incubated (30 min at 30°C) with 2.5 μg of total HeLa nuclear extract in transcription buffer containing no additional nucleotide triphosphates except for 1 μCi (at 3,000 Ci/mmol) of [γ^{32}-P]ATP per 20 μl of reaction mixture. Lane 5 received no recombinant factor. Laemmli sample buffer was added to terminate the reactions, which were analyzed by autoradiography after SDS-PAGE. Numbers on the right mark the migration of molecular mass markers (kilodaltons).

system was evident (data not shown). We conclude that transactivation by HNF-4 does not depend upon a small diffusible intracellular molecule. Considering that HNF-4 activates transcription in nearly all cell-types tested (including *S. cerevisiae*) (18), this is unlikely to reflect assay-specific relaxation of constraints normally relieved by a putative ligand, as has been invoked to explain in vitro activation of ligand-independent transcription by the estrogen receptor (16).

An alternative possibility is that HNF-4 activity could be potentiated by covalent modification of the bacterially expressed protein in the in vitro transcription system. As a first step toward addressing this possibility, we examined whether HNF-4 underwent phosphorylation in the presence of a HeLa cell nuclear extract (2, 42). For this purpose, recombinant HNF-4 was incubated with the extract in the presence of [γ-^{32}P]ATP. Figure 4B (lane 1) shows that in addition to labelling of endogenous extract proteins, a substantial amount of label was transferred to HNF-4. Labelling of HNF-4 was reduced in the presence of excess *S*-γ-ATP, a nonhydrolyzable analog of ATP (not shown). These results suggested that HNF-4 could be phosphorylated. Examination of the phosphorylation properties of the HNF-4 deletion mutants HNF-4ΔC1, HNF-4ΔC2, and HNF-4ΔC3, all of which could be labelled in this assay, further revealed that phosphorylation sites are clustered toward the amino-terminal end of the molecule (lanes 2 to 4). Although the precise sites of phosphorylation on HNF-4 have not yet been determined, potential serine/threonine (both protein kinase A and protein kinase C) sites are present throughout the HNF-4 molecule (35, 67). Alternatively, tyrosine phosphorylation sites (56) could be utilized in a manner analogous to that of the estrogen receptor (1).

Identification of an HNF-4 transactivation domain. To map the activation domain(s) of HNF-4, we evaluated the transactivation potential of the various HNF-4 deletion mutants on the transcription of the template A_{4x}MLΔ53 in vitro (Fig. 5A). Wild-type HNF-4 and HNF-4ΔC1 displayed nearly identical activities (circa 20-fold over the basal level [lanes 2 and 3]). On the other hand, the transactivation activity of HNF-4ΔC2, which retained normal DNA binding affinity for site A, was dramatically reduced to ca. twofold over the basal level. Similarly, when HNF-4ΔC3, its amount normalized for DNA binding activity equivalent to that of other HNF-4 variants (see above), was included in the reaction mixture, nearly basal levels of transcription from the test template were observed. These results identify a segment between amino residues 345 and 389 on the HNF-4 molecule (67) as being responsible for the transactivation potential of HNF-4. This region includes a domain nearly identical in sequence to the AF-2 region of

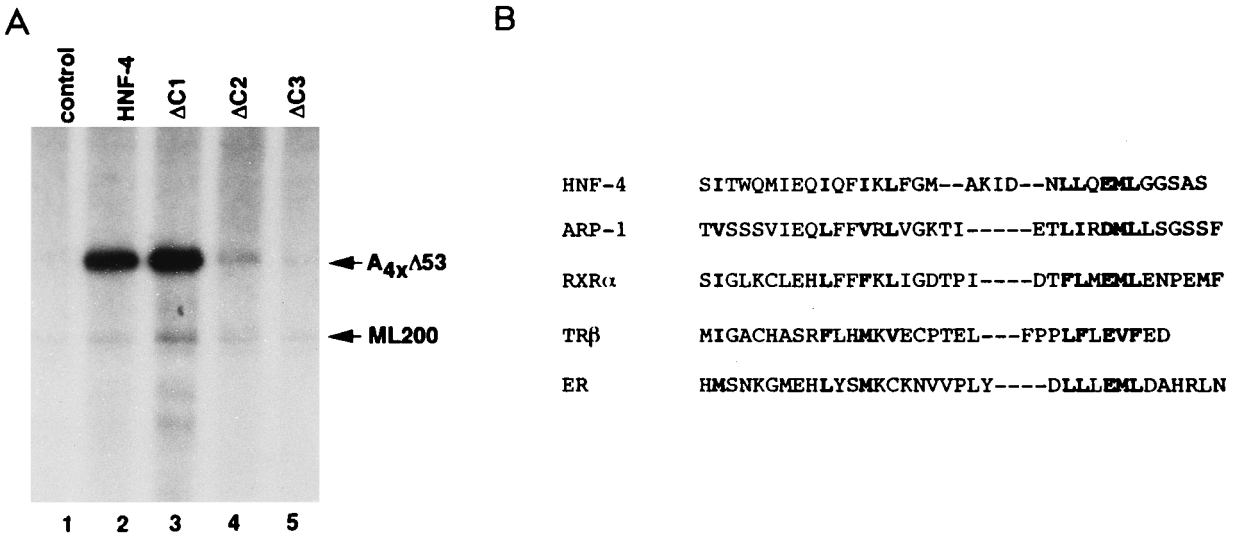

FIG. 5. Localization of an HNF-4 activation domain. (A) Each of the indicated bacterially expressed proteins (100 ng) was incubated with 100 ng of test template A_{4x}MLΔ53 and 25 ng of ML200 as a control in a HeLa nuclear extract-based transcription assay as described in the legend to Fig. 3. Lanes: 1, no addition; 2, wild-type HNF-4; 3, HNF-4ΔC1; 4, HNF-4ΔC2; 5, HNF-4ΔC3. Band intensities were quantitated as described for Fig. 3A to measure fold activation. (B) Comparison of sequences of putative AF-2-like domains of HNF-4 (67), ARP-1 (50), retinoid X receptor (RXRα [15]), thyroid receptor β (TRβ [4, 15]), and the estrogen receptor (ER [12, 15]). Gaps have been included to maximize alignment.

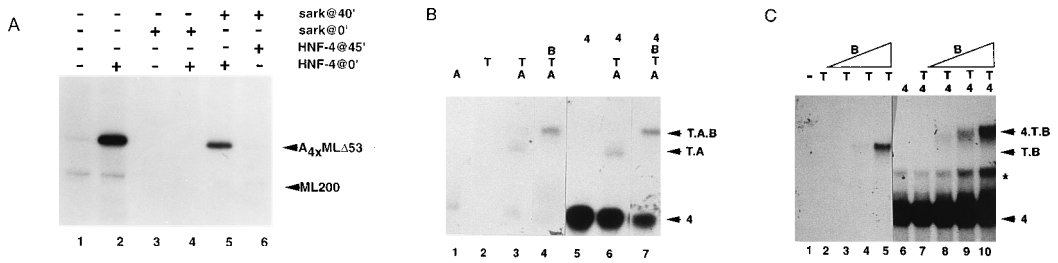

FIG. 6. Facilitation of preinitiation complex formation by HNF-4. (A) Transcription reaction mixtures (HeLa nuclear extract) containing 100 ng of test template A_{4x}MLΔ53 and 25 ng of ML200 as a control, but no nucleotide triphosphate, were assembled on ice. HNF-4 was added at time zero (0′ [i.e., the time at which incubation at 30°C was started]) (lanes 2, 4, and 5) or at 45 min (45′) into the incubation (lane 6). Sarkosyl (sark [0.025%]) was added at time zero (lanes 3 and 4) or at 40 min (40′ [lanes 5 and 6]). After nucleotide triphosphate addition (at 45 min), incubation was continued for an additional 30 min. Samples were processed as described in the legend to Fig. 3A. (B) For EMSA, a DNA fragment probe (A-TATA) containing site A upstream of the apoAI TATA element was incubated with the following factors for 30 min at 30°C: TFIIA (A [MonoS fraction; 1 μg]), lanes 1, 3, 4, 6, and 7; TBP (T [10 ng]), lanes 2, 3, 4, 6, and 7; TFIIB (B [20 ng]), lanes 4 and 7; and HNF-4ΔC1 (4 [50 ng]), lanes 5 to 7. Arrows indicate the presumptive complexes thus formed. (C) EMSA reactions were performed as described for panel B. HNF-4ΔC1 (4 [50 ng]), lanes 6 to 10; TBP (T [10 ng]), lanes 2 to 5 and 7 to 10; TFIIB (B), 50 ng for lanes 3 and 8, 100 ng for lanes 4 and 9, and 250 ng for lanes 5 and 10]. Arrows indicate presumptive complexes. The asterisk indicates a nonspecific band.

other nuclear receptors (4, 12, 15), which is predicted to form a helix-loop-helix structure (Fig. 5B).

The role or indeed the existence in HNF-4 of a putative AF-1 domain remains unclear. (Note the relatively small amino-terminal A/B region in HNF-4.). Since the HNF-4 derivatives HNF-4ΔC2 and HNF-4ΔC3 were not active in our assay and since an HNF-4 mutant lacking the AF-2 region was unable to activate transcription from the erythropoetin enhancer in HeLa cells (19), an amino-terminal HNF-4 activation domain, if present, is not sufficient for activation. The ability of individual HNF-4 segments to effect autonomous transcription activation has not been tested. An alternative explanation remains that the HNF-4 A region might contain a promoter or cell-type-specific AF-1, as has been suggested for some steroid receptors (24, 70).

HNF-4 stimulates transcription by facilitating PIC formation. Transcription is a multistep process. The primary event of the cycle entails assembly of RNA polymerase II and its associated basal transcription machinery (consisting minimally of TFIIA, TFIIB, TFIID, TFIIE, TFIIF, and TFIIH) on core promoter elements (58, 69) to form the PIC. Subsequently, the promoter contacts are relinquished and the RNA polymerase begins elongating the nascent RNA chains. Finally, upon termination and release from the template, the polymerase is free to recycle. To examine which of these potentially rate-limiting steps is targeted by HNF-4, we employed the detergent Sarkosyl in the in vitro assay to dissect the transcription cycle into events that occur prior to or subsequent to PIC formation (27) (Fig. 6A). When Sarkosyl was included in the reaction mixture at initial times, basal transcription from both templates A_{4x}MLΔ53 and ML200 (lane 3) and HNF-4-activated transcription from A_{4x}MLΔ53 (lane 4) were completely inhibited. When Sarkosyl was added after the PIC had been allowed to form in the absence of nucleotide triphosphates, only a slight diminution of transcription from the templates was observed, possibly due to restriction of the RNA polymerase to a single round of transcription (lane 5). Similarly, no effect of Sarkosyl on HNF-4 transactivation of the A_{4x}MLΔ53 template was evident in reactions in which HNF-4 was present from initial times (lane 5). In contrast, upon introduction of HNF-4 after PIC formation and Sarkosyl addition, an increase in the transcriptional activity of A_{4x}MLΔ53 could no longer be elicited (lane 6). These results imply that HNF-4 function is dependent upon its availability prior to PIC formation at the target promoter. This further suggests that HNF-4 acts by increasing the number of (stable) PIC complexes at the promoter and not by stimulating subsequent steps of the process (e.g., elongation and recycling).

To ascertain which step in the PIC assembly pathway could be regulated by HNF-4, we determined its effects on the assembly of the basal factors TBP, an integral component of TFIID, and TFIIB on the apoAI core promoter. For the EMSA, a DNA fragment (designated A-TATA) containing the TATA box from the apoAI gene promoter immediately downstream of site A was used as a probe (Fig. 6B). In this analysis, to better resolve the basal complex from the HNF-4 complex, we used HNF-4ΔC1, whose behavior is identical to that of the wild type (Fig. 5). We performed these experiments in both the presence and absence of TFIIA, which stabilizes TBP interactions with the promoter. Consistent with previous reports (7, 47), no binding of recombinant TBP (T) alone to the probe could be detected under the conditions selected (lane 2). In the presence of a partially purified preparation of TFIIA, a band corresponding to the T.A promoter complex was clearly observed (Fig. 6B, lane 3). Recombinant TFIIB, which has no DNA binding activity of its own (51), quantitatively supershifted the T.A complex to a discrete higher-order complex (T.A.B) (Fig. 6B, lane 4). When HNF-4 was included in the binding reaction mixture, the T.A and T.A.B complexes were essentially unaffected (Fig. 6B, lanes 6 and 7).

In the absence of TFIIA, formation of a T.B complex which was barely detectable (Fig. 6C, lane 3) at lower TFIIB concentrations became significant with increasing amounts of TFIIB (lanes 4 and 5). However, in the presence of HNF-4, formation of a slowly migrating novel band was appreciably stimulated (lanes 8 to 10). The dependence of this band on the presence of three polypeptides (viz. HNF-4, TBP, and TFIIB), each a highly purified bacterially expressed recombinant product, plus the dose dependence on TFIIB strongly argue that this band corresponds to a tripartite DNA complex of HNF-4, TBP, and TFIIB (4.T.B). Thus, the T.B complex in the presence of HNF-4 (as 4.T.B) is detectable at the lowest TFIIB concentration tested (compare lanes 3 and 8) and is significantly stronger at higher TFIIB concentrations than the corresponding T.B complex obtained in the absence of HNF-4 (compare lanes 5 and 9 and 6 and 10). This result shows that HNF-4 facilitates PIC formation by stabilizing and/or enhancing the nascent T.B complex. We further interpret the failure to observe an effect in the presence of TFIIA (Fig. 6B) as resulting from the inherent stability of the T.A.B complex (48).

Direct interaction of HNF-4 with the basal transcription factor TFIIB. In the next series of experiments, we determined

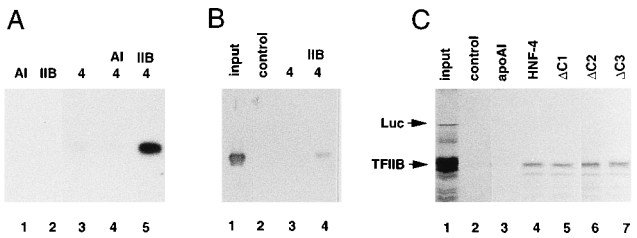

FIG. 7. Direct interaction of HNF-4 with basal transcription factor TFIIB. (A) Stabilization of HNF-4 DNA binding activity by TFIIB. EMSA reaction mixtures contained the A-TATA probe incubated (30 min at 30°C) with the following factors: 5 ng of HNF-4 (4 [lanes 3 to 5]), 100 ng of recombinant apoAI (AI [lanes 1 and 4]), and 100 ng of TFIIB (IIB [lanes 2 and 5]). (B) Recruitment of TFIIB to the HNF-4–DNA complex. Biotinylated A-TATA fragment (50 ng per reaction mixture) was immobilized to M-280 streptavidin Dynabeads (5 μl) and incubated (30 min on ice) with 0.5 μg of bovine serum albumin (BSA [lane 2]) or 0.5 μg of HNF-4 (lanes 3 and 4) in binding buffer (Materials and Methods). After being washed, 100 ng of TFIIB was added to reaction mixtures in lanes 2 and 4. After incubation (30 min at 30°C), the beads were washed four times in the same buffer supplemented with 0.01% Nonidet P-40. The beads were resuspended in Laemmli sample buffer and analyzed by Western blotting with an anti-TFIIB antiserum (51). Twenty-five percent of the input TFIIB is shown in lane 1. (C) Template-independent interaction of TFIIB with full-length and truncated HNF-4. A total of 2.5 μg each of BSA (lane 2), recombinant apoAI (lane 3), HNF-4 (lane 4), HNF-4ΔC1 (lane 5), HNF-4ΔC2 (lane 6), and HNF-4ΔC3 (lane 7) was incubated with Ni^{2+}-nitrilotriacetic acid-agarose. After washes to remove unadsorbed material, the resin was incubated with 5 μl of ^{35}S-labelled TFIIB. The resin was washed extensively and resuspended in Laemmli sample buffer. The samples were resolved by SDS-PAGE and autoradiographed. Twenty-five percent of the input TFIIB is shown in lane 1. Luc, luciferase.

whether HNF-4 facilitated T.B complex formation through TFIIB. First, by EMSA, we observed that at a relatively low concentration of HNF-4, which resulted in a diminished yield of the HNF-4 DNA complex (Fig. 7A, lane 3), TFIIB stabilized HNF-4 interaction with site A (lane 5). This phenomenon was specific, since the equivalent amount of control recombinant apoAI, which is not known to function as a transcription factor, was unable to enhance HNF-4 DNA binding activity (lane 4). However, since TFIIB did not affect the mobility of the HNF-4 DNA complex, it was unlikely that it was present in the complex. One interpretation of these data is that a putative TFIIB–HNF-4 complex forms more avidly than an HNF-4 complex but that the TFIIB component being loosely bound does not survive in the EMSA complex. Additionally, whether this effect is peculiar to the unmodified recombinant HNF-4 protein employed in our study remains to be determined.

Second, with an assay that did not involve electrophoretic separation of complexes (which could mask potential HNF-4–TFIIB interactions in the assay shown in Fig. 7A), we could show binding of TFIIB to site A-bound HNF-4 (Fig. 7B). To this end, the A-TATA probe was immobilized on magnetic beads and saturated with HNF-4. After removal of unbound protein, binding of TFIIB to the complex was assessed by monitoring (by Western [immunoblot] analysis) the TFIIB content of the material retained on the beads after extensive washing. The results showed that TFIIB could be recruited to the promoter if HNF-4 was prebound to site A (lane 4) but not in its absence (lane 2).

Finally, direct DNA-independent physical interactions between HNF-4 and TFIIB were demonstrated, as has been reported for many activators (45, 69), including other nuclear receptors (3, 17, 31, 46, 50, 70). For this purpose, we monitored retention of radioactively labelled TFIIB on HNF-4 that had been immobilized on a Ni^{2+}-agarose matrix via the polyhistine tag at the amino terminus (Fig. 7C). To further enable us to map the regions on HNF-4 that participate in this interaction, we included the carboxy-terminal deletion mutants of HNF-4 in our analysis. Additionally, as a control, we employed an equivalent amount of recombinant histidine-tagged preparation of apoAI. Retention of TFIIB on apoAI-containing resin was negligible. However, a significant amount of TFIIB was retained on resin containing wild-type HNF-4 as well as the deletion mutants, including HNF-4ΔC3 (lanes 5 to 7). The specificity of this interaction was further evident from the lack of retention of labelled luciferase, which was also included in the binding reaction mixture. These data map a TFIIB interaction domain of HNF-4 to the amino-terminal half of the molecule. Consistent with this observation, each of the various C-terminal mutants of HNF-4 responded to TFIIB-mediated stimulation of their DNA binding activity (data not shown). However, the possibility that there is an additional carboxy-terminal TFIIB interaction domain, as has been documented for the thyroid receptor (3), cannot be ruled out.

These results thus identify TFIIB as a component of the basal transcription machinery which is targeted by HNF-4 and, together with data presented in Fig. 4, suggest that the HNF-4 interaction with TFIIB and transcription activation per se are distinct activities (see Discussion).

DISCUSSION

We have shown that HNF-4 activates transcription by facilitating PIC formation via a mechanism that involves direct physical interactions with TFIIB, a key component of the PIC (7, 41, 58). The following observations suggest that the physical interaction between HNF-4 and TFIIB has important consequences for the nascent activated transcription complex. First, TFIIB is efficiently recruited to the promoter by DNA-bound HNF-4 in the absence of TBP (Fig. 7B). Second, TFIIB potentially stabilizes HNF-4's interaction with site A (Fig. 7A). While this may simply be a side effect of more consequential interactions elsewhere, the phenomenon has also been documented for other transcription factors, such as COUP-TF1 (71) and the herpesvirus protein ICP-4 (68). It most likely arises from favorable conformational changes within the activator. Third, efficient recruitment of TBP to the HNF-4-bound promoter (in the absence of TFIIA) is stimulated by TFIIB (Fig. 6C). Furthermore, cooperativity in the formation of a tripartite complex consisting of the synthetic activator GAL4-AH, TBP, and TFIIB has been reported (11). This finding, together with TFIIB-dependent stabilization of TBP binding to the TATA box and the activator-induced conformational changes in TFIIB (30, 57), argues that the activator (HNF-4), TBP, and TFIIB constitute a functional unit. The mutual stabilizing effects of these factors may indeed reflect alternative assembly pathways (58, 60). Thus, TFIIB can potentially enter the complex via either the activator or TBP or, most likely, through cooperative interactions with both. Viewed in this way, TFIIB is an integral component of the activator complex as much as it is of the basal machinery. This blurs the boundary between the two complexes and is in contradistinction to models of activated transcription which ascribe a passive role to components of the basal machinery (69).

Although we have demonstrated that HNF-4 stimulates TBP-TFIIB promoter complex formation, our results further underscore that the role of HNF-4 in the activated transcription complex is not limited to this step, since HNF-4 mutants HNF-4ΔC2 and HNF-4ΔC3 that retain the ability to interact with TFIIB were inactive in our functional assay. Transcription activation by HNF-4 in vitro (this study) and in HeLa cells (19) strongly depends upon a carboxy-terminal AF-2 domain. Therefore, a two-step HNF-4-mediated activation pathway can

be proposed. The initial step entails formation of an interdigitated complex spanning activator target sites and core promoter sequences via joint interactions with TFIIB, as discussed above. In this AF-2-independent step, an interface for TFIIB interaction could be furnished by residues represented in HNF-4ΔC3, the smallest derivative tested. Subsequently, the AF-2 domain of HNF-4 could facilitate entry of other components, including RNA polymerase II (in association with TFIIF), TFIIE, and TFIIH, that enter the complex downstream of TFIIB. In view of the fact that TBP is invariably found in the context of holo-TFIID in tight association with various TBP-associated factors (TAFs) which are contacted by many activators (9) and of the possibility that many basal factors are associated with RNA polymerase II holoenzyme (36, 37), the proposed interactions may not necessarily unfold sequentially. Rather, multiple interdependent protein-protein interactions could result in cooperative formation of the fully activated transcription complex. Either way, distinct intramolecular activities residing in HNF-4 would synergize to facilitate PIC assembly, in agreement with the suggestion that eukaryotic activators function during multiple steps of this process (11). Given the involvement of hTAF30 in transcriptional activation by the estrogen receptor (32) and the strong homologies in the AF-2 domains of HNF-4 and the estrogen receptor (15), a role for hTAF30 is also likely, as is the involvement of novel coactivators (44). Future studies will therefore focus on the influence of HNF-4 on the downstream events mediated by these factors.

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