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Protein Kinase A-Dependent Phosphorylation Modulates DNA-Binding Activity of Hepatocyte Nuclear Factor 4

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Hepatocyte nuclear factor 4 (HNF4), a liver-enriched transcription factor of the nuclear receptor superfamily, is critical for development and liver-specific gene expression. Here, we demonstrate that its DNA-binding activity is modulated posttranslationally by phosphorylation in vivo, ex vivo, and in vitro. In vivo, HNF4 DNA-binding activity is reduced by fasting and by inducers of intracellular cyclic AMP (cAMP) accumulation. A consensus protein kinase A (PKA) phosphorylation site located within the A box of its DNA-binding domain has been identified, and its role in phosphorylation-dependent inhibition of HNF4 DNA-binding activity has been investigated. Mutants of HNF4 in which two potentially phosphorylatable serines have been replaced by either neutral or charged amino acids were able to bind DNA in vitro with affinity similar to that of the wild-type protein. However, phosphorylation by PKA strongly repressed the binding affinity of the wild-type factor but not that of HNF4 mutants. Accordingly, in transfection assays, expression vectors for the mutated HNF4 proteins activated transcription more efficiently than that for the wild-type protein when cotransfected with the PKA catalytic subunit expression vector. Therefore, HNF4 is a direct target of PKA which might be involved in the transcriptional inhibition of liver genes by cAMP inducers.

The liver-enriched transcription factor hepatocyte nuclear factor 4 (HNF4) (54) is involved in close association with other factors in hormonal and dietary control of liver and intestine genes. In addition, HNF4 has a potential role as a developmental regulator that has been conserved during evolution from invertebrates to vertebrates. HNF4 expression is restricted to the liver, kidney, and intestine (60) and, in *Drosophila melanogaster*, to the malpighian tubules (61). HNF4 exists as various isoforms. At least five different cDNAs are generated by differential splicing from a single gene, the HNF4α gene, in the amino- and carboxy-terminal regions; in all isoforms the DNA-binding domain remains unchanged (6, 14, 20, 33, 53, 54). Recently, novel human and *Xenopus* HNF4 isoforms (HNF4γ and HNF4β, respectively) have been identified and shown to be derived from two distinct and differentially expressed genes (14, 24). HNF4 is expressed very early during embryo development and has been found to be a crucial positive-acting factor for the expression of the HNF1 gene (55), placing HNF4 at the top of a transactivator hierarchy in hepatic cells (23, 36). Disruption of the HNF4α gene led to early embryonic death due to malfunction of the yolk sac (7).

HNF4 is a member of the nuclear receptor superfamily that binds to DNA at direct repeats separated by one nucleotide (DR1). This transactivator preexists as very stable homodimers in solution, and no HNF4-specific ligand has been identified so far (27). Therefore, HNF4 is classified as an orphan nuclear receptor, characterized by two zinc finger DNA-binding motifs, a large conserved hydrophobic domain containing the dimerization, and a putative ligand-binding domain (54). Structural studies indicate that the first HNF4 finger (P-box region) is unrelated to that in other nuclear receptors and suggest that HNF4 could define a new subclass in the nuclear receptor family (27). Activation domains have been determined and are located in the hypervariable amino-terminal A/B region and in the highly conserved carboxy-terminal E region (17). Recent studies revealed that HNF4 activates transcription by facilitating assembly of the preinitiation complex intermediate via direct physical interactions with TFIIB (42).

In the control regions of many genes expressed in the liver, binding sites for HNF4 have often been found in the vicinity of other binding sites for liver-enriched factors (C/EBP [44], HNF1 [13, 34], and HNF3 [19]), ubiquitous factors (NF-Y [26] and CREB [48]), or nuclear receptors (GR [18]). These various associations constitute regulation units for the liver-restricted genes encoding apolipoproteins, coagulation factors, serum proteins, and cytochrome P450s and those involved in the metabolism of fatty acids, amino acids, and glucose (reviewed in reference 53). Many binding sites initially found to bind HNF4 are also recognized by other orphan members of the nuclear receptor superfamily present in hepatocytes and nonhepatic cells, such as ARP1 and COUP-TF; overexpression of these generally antagonizes HNF4-mediated transcriptional activation (5, 15, 32, 38, 45).

L-type pyruvate kinase (L-PK) gene expression is controlled at a transcriptional level positively by carbohydrates in the presence of insulin and negatively by glucagon via cyclic AMP (cAMP) (47, 56, 57). This regulation has been extensively studied ex vivo and in vivo, and our laboratory and others have demonstrated that a short sequence (L4L3) from bp −183 to −121 upstream of the transcription start site is sufficient to confer the hormonal and dietary regulation on the L-PK promoter (1, 8, 40, 56). The transcriptional response to carbohydrates and hormones has been ascribed to the L4 element (bp −183 to −150), which binds the upstream stimulatory factor (USF) (13), termed GRE for glucose response element, functioning in close cooperation with the contiguous L3 element binding HNF4 (1, 40, 56). Interestingly, the L4L3 sequence, required for activation of the L-PK gene by glucose plus insulin, is also needed for its cAMP-mediated transcriptional inhibition (1, 8, 40, 56). The functional cooperation between the L4 and L3 elements in basal and induced activity of the L-PK

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promoter has been confirmed by H. Towle's group (41). In addition, we recently demonstrated that L-PK expression in adult liver and pancreas parallels the occupation of the L3/HNF4 binding site, as mapped by in vivo footprinting experiments (46). As the in vivo repressive effect of glucagon and cAMP on the L-PK gene is very rapid (taking less than 15 min [57]) and does not require protein synthesis, it was interesting to evaluate the DNA-binding activity and phosphorylation status of USF and HNF4 under various hormonal and nutritional conditions.

We have previously indicated that USF-binding activity is not dependent on the nutritional and hormonal conditions (13). Since HNF4 also plays an important role as an accessory factor of the glucose response element (GRE), we have studied the DNA-binding properties and phosphorylation state of HNF4 under various metabolic conditions. We demonstrate in this paper that the in vivo phosphorylation of HNF4 depends on the diet; it is decreased by a carbohydrate-rich diet and is increased by fasting or in refed animals given glucagon or isoproterenol and phosphodiesterase inhibitors. In vitro phosphorylation of HNF4 by cAMP-dependent protein kinase A (PKA) occurs in the A-box region and decreases DNA-binding activity. In cultured cells, this results in decreased HNF4-dependent transactivation.

MATERIALS AND METHODS

Plasmid constructions. All plasmids were constructed by standard cloning procedures. HNF4 cDNA was obtained by PCR amplification with pf7 (54) as a template with outside primers containing engineered *Bam*HI 5′ and *Eco*RI 3′ sites. Site-directed mutagenesis of the HNF4 sequence was performed by fusion of a two-part PCR amplification with the previous outside primers and internal primers containing, for neutral substitution, the engineered *Ngo*MI site (GCC GGC [Ala Gly]), resulting in the mutant termed HNF4 Ala, or, for acidic substitution, the *Xho*I site (CTC GAG [Leu Glu]), resulting in the HNF4 Glu mutant. All mutations and ligation junctions were confirmed by sequencing.

Recombinant protein production. Recombinant proteins were expressed in *Escherichia coli* by inserting the HNF4 constructs into the *Bam*HI and *Eco*RI sites of the T7-based expression vector pRSET (Invitrogen) to produce an N-terminal fusion with six histidine residues. Recombinant HNF4 proteins were produced in BL21(DE3)pLysS cells upon IPTG (isopropyl-β-D-galactopyranoside) induction and purified by nickel affinity chromatography according to the manufacturer's instructions (Novagen). Prior to use in gel shift assays, the recombinant proteins were denaturated in 6 M guanidinium-HCl and dialyzed against 20 mM HEPES (pH 7.6)–60 mM KCl–1 mM EDTA–1 mM dithiothreitol (DTT)–10% (vol/vol) glycerol in the presence of 1 mM $ZnCl_2$ to allow protein refolding and activity recovery.

Animal nutritional and hormonal treatments. Three-month-old Wistar rats were subjected to different previously described nutritional and hormonal treatments to study L-PK expression in the rat liver (56). Animals were fasted for 48 h and then separated into three groups. The first group was then fasted for an additional 16-h period (fasted conditions). The second group was refed during this 16-h period with a high-carbohydrate diet (refed conditions). The third group was also refed with the high-carbohydrate diet and subjected to different treatments throughout the refeeding period. To increase signal transduction via the cAMP pathway in group 3, glucagon (500 μg/kg), 3-isobutyl-1-methylxanthine (IBMX) (40 μmol/kg), isoproterenol hydrochloride (5 mg/kg), and IBMX plus isoproterenol were injected intraperitoneally for an additional 3-h period (cAMP repression conditions), and then the animals were sacrificed.

Nuclear extract preparation and EMSA. Nuclear extracts were prepared from rat liver according to the procedure of Gorski et al. (16) in the presence of protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, 1% [vol/vol] aprotinin, 25 mM benzamidine, 1 μg of leupeptin per μl, 1 μg of antipain per μl, 1 μg of chymotrypsin inhibitor per μl, 2 mM Na_3VO_4, 50 mM NaF, 40 mM β-glycerophosphate, and 25 mM pyrophosphate).

Electrophoretic mobility shift assays (EMSA) were performed as previously described (58) at 4°C in binding buffer containing 1 to 5 μg of rat liver nuclear extracts with 5 μg of poly(dI-dC) or 20 to 40 ng of recombinant HNF4 proteins with 100 ng of poly(dI-dC) and 2.5 μg of bovine serum albumin in the presence of 0.1 to 0.5 ng of kinase-treated 5′-end-labeled double-stranded probe. For competition assays, 10 ng of MLP probe or 2.5 to 20 ng of L3 probe was used as a specific competitor, and for supershift experiments, anti-HNF4 antibodies (kindly provided by F. Sladek) were included in the binding reactions. DNA-binding complexes separated on nondenaturating polyacrylamide gels were quantified by using a PhosphorImager (Molecular Dynamics) or a densitometer device (Shimadzu).

The sequences of L4L3, HNF4, USF, HNF1, HNF3 and NF-Y oligonucleotides were as follows: L4L3 L-PK site (bp −172 to −121), ATGGGCGCACG GGGCACTCCCGTGGTTCCTGGACTCTGGCCCCCAGTGTACA; HNF4 rat L-PK/L3 site (bp −147 to −126), TCGATCCTGGACTCTGGCCCCCAGT; HNF4 rat phosphoenolpyruvate carboxykinase (PEPCK) site (bp −452 to −432), GGCCCACGGCCAAAGGTCATGACCG; HNF4 rat tyrosine aminotransferase (TAT) site (bp −3601 to −3582), CGCTGCTGCTCTTTGATCTG TAGGC; USF MLP adenovirus 2 site (bp −70 to −43), AGGTGTAGGCCAC GTGACCGGGTGGTTCC; HNF1 rat L-PK/L1 site (bp −96 to −68), CTAGC TGGTTATACTTTAACCAGGACTCA; HNF3 rat transthyretine (bp −111 to −90), GTTGACTAAGTCAATAATCAGA; and NF-Y rat albumin (bp −93 to −65), GGGGTAGGAACCAATGAAATGAAAGGTTA.

Ex vivo phosphorylation and immunoprecipitation of HNF4. HepG2 cells were labeled for 2 h with 1 mCi of [^{32}P]orthophosphate (Amersham) per ml in phosphate-free Dulbecco's modified Eagle's medium (Gibco BRL) containing 15 μg of the tyrosine kinase inhibitor genistein per ml. Where mentioned, cells were treated with PKA inducers (1 mM 8-bromo-cAMP, 0.1 mM 8-CPT-cAMP, 50 μM forskolin, and 1 mM IBMX) for 15 min before being harvested. At the end of the labeling period, cells were washed with phosphate-buffered saline containing 1% (wt/vol) sodium pyrophosphate and lysed in ice-cold radioimmunoprecipitation assay (RIPA) buffer (20 mM Tris HCl [pH 8], 1% [vol/vol] Nonidet P-40, 0.5% [wt/vol] deoxycholate, 0.1% [wt/vol] sodium dodecyl sulfate [SDS], 1 mM phenylmethylsulfonyl fluoride, 1% [vol/vol] aprotinin, 25 mM benzamidine, 2 mM Na_3VO_4, 50 mM NaF, 40 mM β-glycerophosphate, 25 mM pyrophosphate, and Complete protease inhibitor cocktail [Boehringer Mannheim]) containing 0.42 M NaCl. Cells were disrupted by multiple congelation-decongelation steps and by passing the lysate several times through a 22-gauge needle. Cells debris were centrifuged, and the supernatant diluted with 3 volumes of RIPA buffer without NaCl was precleared with whole rabbit serum adsorbed on protein A-Sepharose beads (Pharmacia) and subsequently subjected to immunoprecipitation with a saturating amount of anti-HNF4 (a generous gift from F. Ringeisen and M. Yaniv) or nonimmune serum overnight at 4°C. The beads were washed several times with RIPA buffer containing 150 mM NaCl, and the immunocomplexes were eluted in Laemmli buffer (125 mM Tris HCl [pH 6.8], 4% [wt/vol] SDS, 20% [vol/vol] glycerol, 0.004% [wt/vol] bromophenol blue, 10% [vol/vol] 2-mercaptoethanol), resolved by 10% (wt/vol) polyacrylamide gel electrophoresis (PAGE), and autoradiographed. Labeled proteins were quantified by using a PhosphorImager (Molecular Dynamics), and the relative amount of radioactivity was measured by volume integration with the ImageQuant software.

In vitro treatment with PKA and PP1. In vitro phosphatase treatments were carried out with 2 U of recombinant protein phosphatase 1 (PP1) in a reaction buffer containing 50 mM Tris HCl (pH 7), 0.1 mM EDTA, 5 mM DTT, 0.2 mM $MnCl_2$, and 5 mM caffeine in the presence of 2.5 μg of nuclear extracts for 30 min at 30°C according to the manufacturer's instructions (New England Biolabs). In vitro phosphorylation by PKA was performed with 100 U of the recombinant PKA catalytic subunit (Promega) in a reaction buffer containing 20 mM HEPES (pH 8), 1.2 mM EDTA, 60 mM KCl, 1 mM DTT, 5 mM $MgCl_2$, and 1 mM ATP in the presence of 2.5 μg of nuclear extracts or 40 ng of recombinant proteins mixed with 2.5 μg of bovine serum albumin for 30 min at 30°C. Specific inhibition of PKA activity was performed by adding 10 μg of the peptide inhibitor PKI (Promega) in the reaction buffer.

Western blot analysis. Proteins separated by SDS–12% (wt/vol) PAGE were transferred to nitrocellulose membranes in a Bio-Rad apparatus according to the manufacturer's instructions. After blocking of nonspecific protein binding sites for 1 h at room temperature in TBST (50 mM Tris HCl [pH 8], 150 mM NaCl, 0.05% [vol/vol] Tween 20) containing 5% (wt/vol) nonfat dry milk, the blots were incubated with affinity-purified anti-USF2/ZIP (58) or anti-HNF4 (a generous gift from J. M. Tian) at a 1:500 dilution or anti-HNF1 antibodies (kindly provided by T. Chouard and M. Yaniv) at a 1:1,000 dilution for 2 h at room temperature. Following three washes of 15 min each in TBST, the secondary antibody, peroxidase-conjugated swine anti-rabbit (Dako), was added at a 1:2,000 dilution and left for 45 min at room temperature. The blots were washed again as above described, and peroxidase activity was detected by autoradiography with the ECL enhanced chemiluminescence system (Amersham).

Expression vectors and reporter constructs. For expression in mammalian cells, the different HNF4 cDNAs were cloned into a eukaryotic expression vector driven by the cytomegalovirus immediate-early promoter region. The expression vector pSV PKA Cα, coding for the catalytic Cα subunit of PKA, and the reporter gene pSom/CAT, which contains the rat somatostatin cAMP-responsive element (CRE) cloned immediately 5′ to the thymidine kinase promoter (10), were gifts from P. Sassone-Corsi. The chloramphenicol acetyltransferase (CAT) reporter plasmid $(L3)_4$-54PK/CAT, which consists of four tandemly repeated L3 boxes fused to the minimal promoter −54PK/CAT (1), was made by ligating multimerized L3 oligonucleotide into the blunt-ended *Bam*HI restriction site of the −54PK/CAT plasmid.

Cell transfection and CAT assays. COS cells were cultured at 37°C in Dulbecco's modified Eagle's medium supplemented with 5% (vol/vol) fetal calf serum and antibiotics. COS cells were transfected by the calcium phosphate precipitation method with 500 ng of the various HNF4 expression vectors and 5 μg of the CAT reporter plasmid in the presence of 0.5 to 1.5 μg of the catalytic subunit of PKA. Cells were harvested 30 h after transfection, and cellular lysates were prepared for CAT assays by using 50 μg of protein extracts as previously

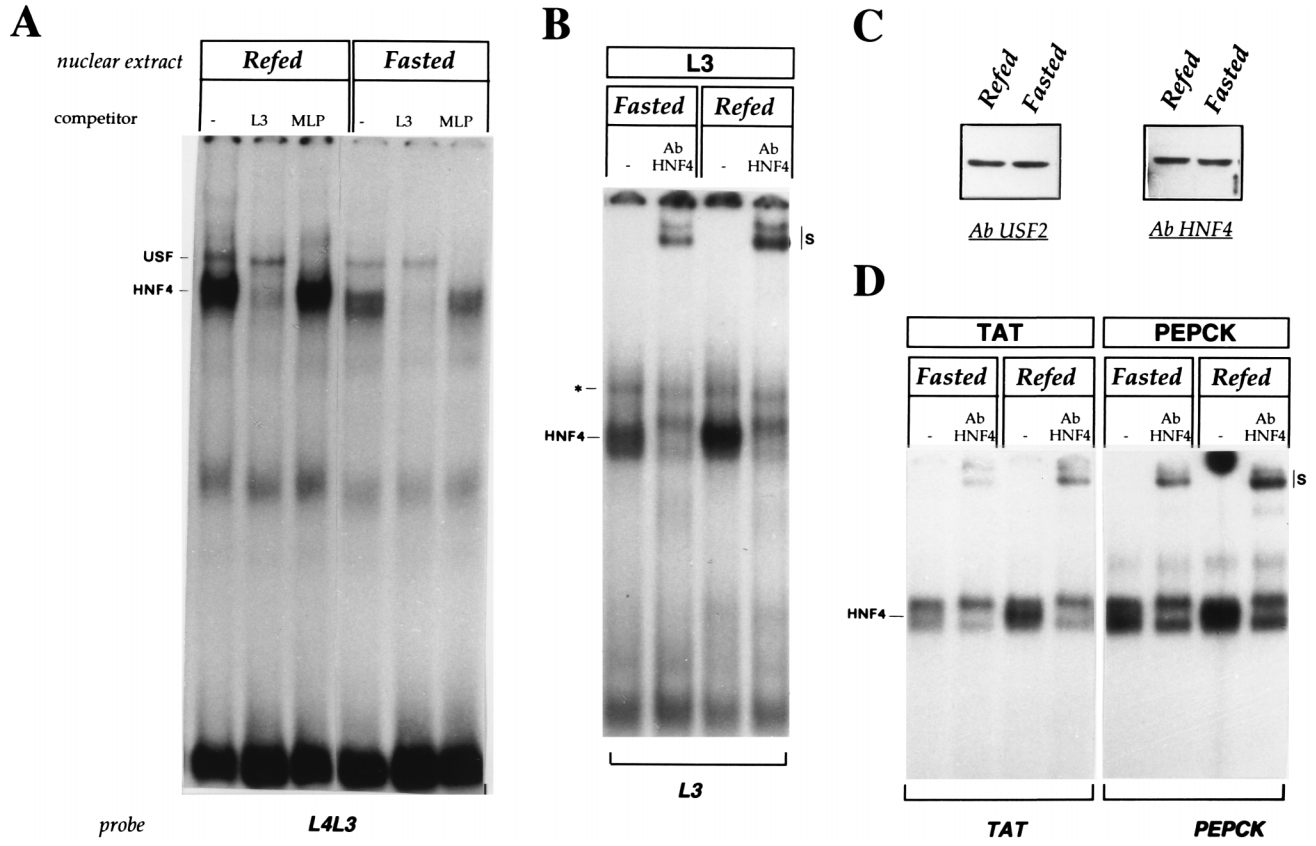

FIG. 1. HNF4 DNA-binding activity in liver nuclear extracts from refed and fasted animals. (A) EMSA were performed by using the L4L3 DNA probe from the L-PK promoter in the presence of 2.5 μg of rat liver nuclear extracts from refed or fasted animals. Ten nanograms of unlabeled L3 or MLP oligonucleotide was added in competition experiments (lanes L3 and MLP). The positions of HNF4 and USF complexes are indicated. (B) EMSA were performed as described above by using the L3 DNA probe from the L-PK promoter, and HNF4-containing complexes were supershifted by using anti-HNF4 antibodies (lanes Ab HNF4). The positions of the HNF4 DNA-binding complex and the supershifted HNF4 DNA-binding complexes (S) are indicated. The asterisk indicates nonspecific protein complexes. (C) HNF4 and USF2 proteins present in fasted and refed nuclear extracts were visualized by Western blot analysis with polyclonal specific anti-HNF4 (Ab HNF4) and anti-USF2 (Ab USF2) antibodies. (D) EMSA were performed as described above by using the HNF4 binding sites of the TAT and PEPCK gene promoters, and HNF4-containing complexes were supershifted by using anti-HNF4 antibodies (lanes Ab HNF4). The positions of the HNF4 DNA-binding complex and the supershifted HNF4 DNA-binding complexes (S) are indicated.

described (1). CAT activities were calculated as the percentage of the acetylated form of chloramphenicol versus the nonacetylated substrate, and results are expressed as a ratio of the percentage of KSV2CAT activity. Each experiment was repeated two to four times.

RESULTS

HNF4 DNA-binding activity is modulated by diet. An end-labeled DNA fragment spanning the L4L3 region of the L-PK promoter (bp −183 to −120) was incubated with crude liver nuclear extracts isolated from refed and fasted rats (Fig. 1A). The proteins interacting with the L4L3 region of the L-PK promoter have been identified previously as the liver-enriched transcription factor HNF4, binding the L3 site, and the ubiquitous heterodimeric transcription factors USF1/USF2, binding the L4 site (13). The intense, faster-migrating, retarded complex visualized in refed nuclear extracts is assigned to HNF4, while the weak, more slowly migrating, retarded complex corresponds to USF transcription factors. Competition with either unlabeled competitor oligonucleotide L3 (HNF4 binding site of the L-PK promoter) or MLP (consensus binding site for USF proteins) demonstrated the specificity of the interactions. Surprisingly, when nuclear extracts from fasted rats were used, the pattern of the fast retarded bands was modified. The intensity of the HNF4-containing complex was dramatically decreased compared to that of the USF-containing complex, which is used as an internal control. Further experiments will be conducted with the L3 probe (HNF4 binding site from the L-PK promoter), which allows a better resolution in the DNA-binding complexes. Depletion of HNF4 DNA-binding complexes by using anti-HNF4 antibodies results in supershifted complexes (Fig. 1B, lanes Ab HNF4). The resulting faint bands were attributed to HNF4 isobinders (13), possibly COUP-TF or ARP1, as they were competed for by the L3 oligonucleotide (Fig. 1A, lanes L3, and data not shown) but not by anti-HNF4 antibodies (Fig. 1B, lanes Ab HNF4). The presence of USF and HNF4 proteins in the fasted and refed nuclear extracts was checked by Western blot analysis with specific antibodies (Fig. 1C). The amounts of USF and HNF4 proteins were similar in the refed and fasted nuclear extracts tested. Therefore, the decreased signal intensity observed in fasted nuclear extracts for the HNF4 complex was clearly due to a loss of DNA-binding activity and not to a decrease in protein concentration. Similar results were obtained with other DNA probes with different affinities for HNF4, the HNF4 binding sites of the TAT and PEPCK gene promoters (Fig. 1D), indicating that the decreased binding activity is independent of the DNA-binding motif.

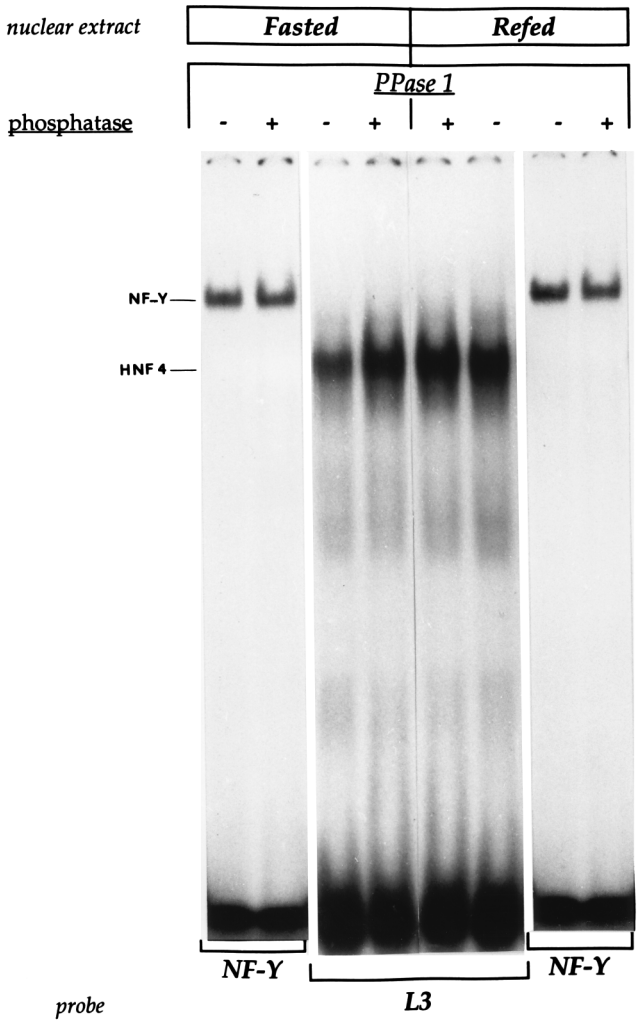

FIG. 2. Effects of PP1 treatment on HNF4 DNA-binding activity in the liver. Liver nuclear extracts from fasted and refed animals (2.5 μg) were treated with 2 U of PP1 and subjected to gel shift assays with the L3 and NF-Y DNA probes. The positions of the HNF4 and NF-Y complexes are indicated.

HNF4 DNA-binding activity is restored in fasted nuclear extracts after phosphatase treatment. Nuclear extracts from refed and fasted animals were incubated with the threonine/serine protein phosphatase PP1, and gel shift assays were performed with the HNF4 binding site of the L-PK promoter (Fig. 2). The NF-Y probe (rat albumin CAAT box) was used in parallel to control the quality of nuclear extracts after phosphatase treatment. After PP1 treatment, HNF4 DNA-binding activity was recovered in liver nuclear extracts from fasted rats; it became similar to that in nuclear extracts from refed rats either subjected to the PP1 treatment or not. These results indicate that phosphorylation of the HNF4 protein on threonine or serine residues induced under fasted conditions decreases its DNA-binding activity.

In vivo HNF4 DNA-binding activity is sensitive to agents stimulating intracellular cAMP accumulation. We then investigated whether the decrease in HNF4 binding activity observed in fasted rats could be mimicked in carbohydrate-fed rats by agents that increase the cAMP concentration in hepatocytes. In fact, treating refed animals with glucagon, IBMX, isoproterenol, or IBMX plus isoproterenol markedly reduced HNF4 DNA-binding activity (Fig. 3A, left). This reduction was specific to HNF4 and was not accompanied by a similar modification in the binding activities of other transcription factors, whether liver specific (HNF1 and HNF3) or ubiquitous (USF and NF-Y) (Fig. 3A, right). Western blot analysis confirmed the presence of similar levels of HNF1, USF2, and HNF4 proteins in all nuclear extracts tested (Fig. 3B), indicating that the decrease in intensity of DNA complexes was related to a loss of DNA-binding activity and not of protein levels.

Effects of PKA inducers on ex vivo HNF4 phosphorylation. We next determined whether PKA was able to phosphorylate HNF4 ex vivo in intact mammalian cells. Human hepatoma HepG2 cells were metabolically labeled with [^{32}P]orthophosphate and treated for 15 min with PKA inducers (cAMP analogs, IBMX, and forskolin). Cellular extracts were immunoprecipitated with anti-HNF4 or nonimmune serum, and the labeled proteins fractionated by SDS-PAGE were visualized by autoradiography. The identity of the phosphoproteins as HNF4 was confirmed by their appropriate molecular mass (54 kDa) and by comigration with HNF4 protein from HepG2

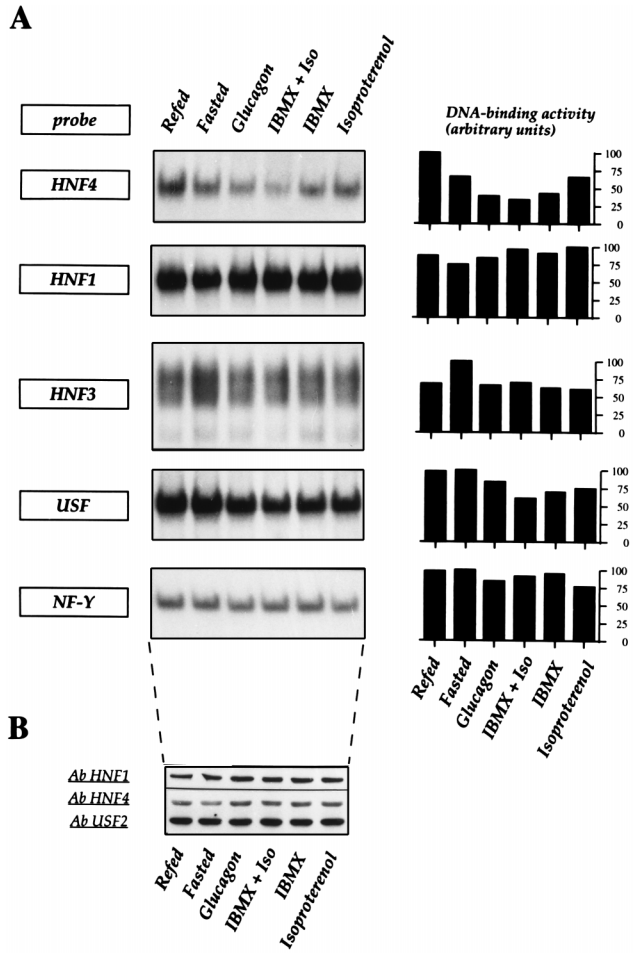

FIG. 3. Effects of inducers of intracellular cAMP accumulation on HNF4 DNA-binding activity. (A) Liver nuclear extracts from fasted and refed rats and refed rats treated with glucagon (500 μg/kg), IBMX (40 μmol/kg), isoproterenol (Iso) (5 mg/kg), and IBMX plus isoproterenol were incubated with various DNA probes binding either liver-specific (HNF4, HNF1, and HNF3) or ubiquitous (USF and NF-Y) factors. The DNA-binding activity was quantified and plotted as a percentage of the highest activity measured, as shown on the right. (B) Western blot analysis was performed with anti-HNF1, anti-HNF4, and anti-USF2 antibodies.

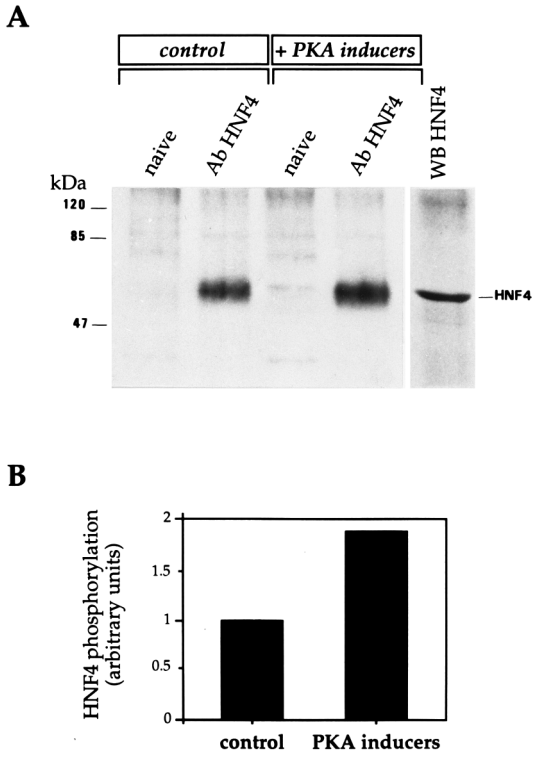

FIG. 4. Ex vivo stimulation of HNF4 phosphorylation by PKA inducers. (A) HepG2 cells were metabolically labeled with [^{32}P]orthophosphate and incubated for 15 min with PKA inducers before harvesting. Cellular extracts were immunoprecipitated with anti-HNF4 (Ab HNF4) or nonimmune (naive) serum, and labeled proteins were quantified with a PhosphorImager. The immunoprecipitated HNF4 protein comigrates with HNF4 revealed by Western blotting from HepG2 nuclear extracts, which is presented on a separate lane as a marker (WB HNF4). Molecular masses are shown at the left. (B) The quantification of labeled endogenous HNF4 proteins was monitored with a PhosphorImager and analyzed with ImageQuant (Molecular Dynamics). The radioactivity of the HNF4 bands in lanes Ab HNF4 in panel A was standardized by the radioactivity of an anonymous band independent of the treatment with PKA inducers, present in the same extract treated with nonimmune serum (naive).

nuclear extracts revealed by Western blotting on a separate lane (Fig. 4A, lane 5). HNF4 is known to be a phosphoprotein (29, 35) and, indeed, is labeled in the absence of activation of the cAMP signaling pathway (Fig. 4A, lane 2). However, PKA stimulation resulted in a 1.8-fold increase in the level of HNF4 phosphorylation (Fig. 4).

In vitro phosphorylation by PKA modulates HNF4 DNA-binding activity. In order to determine whether PKA-dependent phosphorylation could modulate the binding affinity of HNF4 from liver nuclear extracts or be purified as a recombinant protein in *E. coli*, we performed in vitro phosphorylation with the catalytic subunit of PKA in the presence or absence of the PKA inhibitor PKI. Figure 5 shows that in vitro PKA-dependent phosphorylation of recombinant HNF4 or HNF4 present in nuclear extracts from refed rats inhibits DNA binding as shown by gel shift assays (lanes PKA). The PKA-specific inhibitor PKI abolished the inhibition of DNA binding by PKA (Fig. 5, lanes PKA+inh.), demonstrating the specificity of the action of this kinase. A similar treatment by PKA of HNF4 present in nuclear extracts from fasted rats only slightly decreased HNF4 DNA-binding activity, indicating that this factor was already partially phophorylated in the livers of fasted rats. As already mentioned, the faint retarded band preserved after phosphorylation may be ascribed to HNF4 isobinders, the DNA binding of which is not altered by PKA-dependent phosphorylation.

The HNF4 protein exhibits a consensus PKA phosphorylation site (131-RRSS-134) (31) in a region located near its DNA-binding domain (Fig. 6). This region is downstream of the two zinc finger motifs and within the A-box region, which has been shown to be important in determining the binding specificity of another orphan receptor, NGFI-B (59). Indeed, recent studies have shown that phosphorylation of a specific serine residue located within the A box by PKA or an NGF-dependent protein kinase interferes with its binding to DNA in vitro or in vivo (21, 22). Alignment of the NGFI-B and HNF4 A-box sequences shows that the serine phosphorylated in NGFI-B and the putative PKA-dependent phosphorylation site in HNF4 are located at the same distance from the zinc finger motif. Furthermore, the amino acid sequence of the A box, including the consensus phosphorylation site, is entirely conserved among HNF4 proteins of different species (Fig. 6). These observations are consistent with a direct effect of PKA-dependent phosphorylation on HNF4 DNA-binding activity. To examine the role of the putative HNF4 phosphorylation site in the action of PKA, mutated HNF4 proteins were created by inserting neutral or acidic residues in place of Ser133 and Ser134 (Fig. 7A). First, the DNA-binding affinities of the mutated and wild-type HNF4 proteins were evaluated by competition in gel shift assays with the L3 oligonucleotide as a probe. No difference in binding affinity was observed, suggesting that these mutations do not interfere with DNA binding (Fig. 7B). Second, DNA-binding activity after PKA-dependent phosphorylation was monitored by gel shift assays with the L3 probe. The phosphorylated wild-type HNF4 protein showed a large decrease in DNA-binding activity compared to its unphosphorylated form, whereas the two mutated HNF4 proteins were little affected by PKA treatment (Fig. 7C and D). Addition of the specific PKA inhibitor PKI in the phosphorylation reaction abolished the effect of phosphorylation on wild-type HNF4. Thus, these results indicate that the consensus PKA-dependent phosphorylation site located in the A box plays a crucial role in modulating the HNF4 DNA-binding activity and suggest that activation of the cAMP pathway may be involved in the transcriptional modulation of HNF4 by hormones and nutrients.

Ex vivo PKA-dependent phosphorylation modulates HNF4 transcriptional activity. In order to evaluate whether ex vivo phosphorylation within the A box by PKA modifies the transactivation properties of HNF4, we performed transient-transfection assays with an HNF4-responsive CAT reporter construct consisting of four tandemly repeated L3 sites in front of the minimum −54PK promoter. Wild-type or mutated HNF4 expression vectors were cotransfected with increasing amounts of the expression vector for the catalytic subunit of PKA. These experiments were performed with COS cells, which are devoid of endogenous HNF4 and in which the basal activity of the minimum −54PK promoter is not significantly modulated by PKA (data not shown). In the absence of the PKA expression vector, cotransfection of either wild-type or mutated HNF4 expression vectors with the $(L3)_4$-54PK/CAT reporter gene resulted in a similar stimulation of CAT activity compared to that with the control (Fig. 8A). In contrast, in the presence of increasing amounts of the PKA expression vector, the transcriptional activity of wild-type HNF4 progressively decreased relative to that of either of the mutants (Fig. 8B). The efficiency of the PKA catalytic subunit was monitored by using a reporter gene linked to the CRE from the somatostatin gene; as shown in Fig. 8C, this reporter gene was very efficiently stimulated by cotransfection with the PKA expression vector.

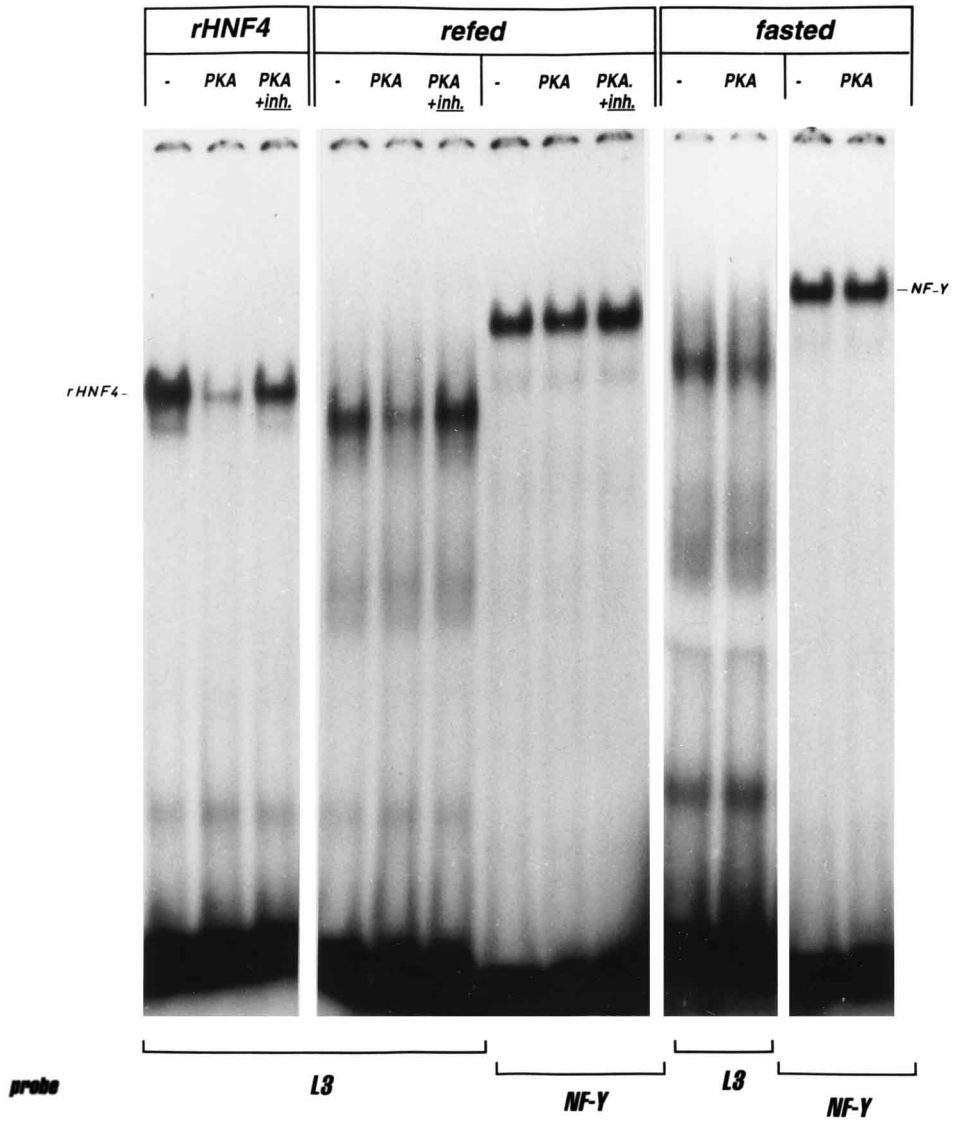

FIG. 5. Effect of in vitro phosphorylation by PKA of recombinant HNF4 or liver nuclear extracts on HNF4 DNA-binding activity. Forty nanograms of recombinant HNF4 (rHNF4) or 2.5 μg of nuclear extracts from fed and fasted rats was incubated with 100 U of PKA catalytic subunit, and then the HNF4 DNA-binding activity was checked by EMSA with the L3 and NF-Y probes (lanes PKA). The PKA inhibitor was added to the phosphorylation reaction to test the specificity of the PKA action (lanes PKA+inh.).

Therefore, the PKA inhibitory effect clearly seems to depend on the presence of the PKA-specific phosphorylation site of the A box.

DISCUSSION

Posttranslational modification of transcription factors by phosphorylation provides a rapid cellular response to environmental cues. For instance, it has been shown that some steroid receptors can be regulated by phosphorylation in the absence of a ligand (11). Previous studies have also suggested that tyrosine and also serine/threonine phosphorylation were required for DNA-binding activity and consequently for transactivation efficiency of the orphan nuclear receptor HNF4 in cultured mammalian cells (29, 35). We now show that HNF4 DNA-binding activity can be regulated, in vivo, ex vivo, and in vitro, by PKA-dependent phosphorylation. The PKA catalytic subunit has been identified in the nucleus and is able to phosphorylate numerous transcription factors, thus modulating positively or negatively their *trans*-acting properties. It appears that HNF4 can now be added to the list of factors whose activity is inhibited by PKA. A PKA-dependent phosphorylation site (131-RRSS-134) lies within a region of HNF4 referred to as the A box, located downstream of the two zinc finger motifs. The A box was originally defined as a sequence of the NGFI-B factor which interacts with adenine residues at the 5′ end of the core recognition motif (59). This A box region has also been shown to be important in DNA recognition of the human thyroid hormone receptor β (37) and, very recently, to be required for high-affinity DNA binding of HNF4 (28). Interestingly, the three-dimensional structure of RXR-TR heterodimers has revealed that the basic amino acids within the A box also form protein-DNA contacts in the minor groove and play an important role in stabilizing DNA binding (49).

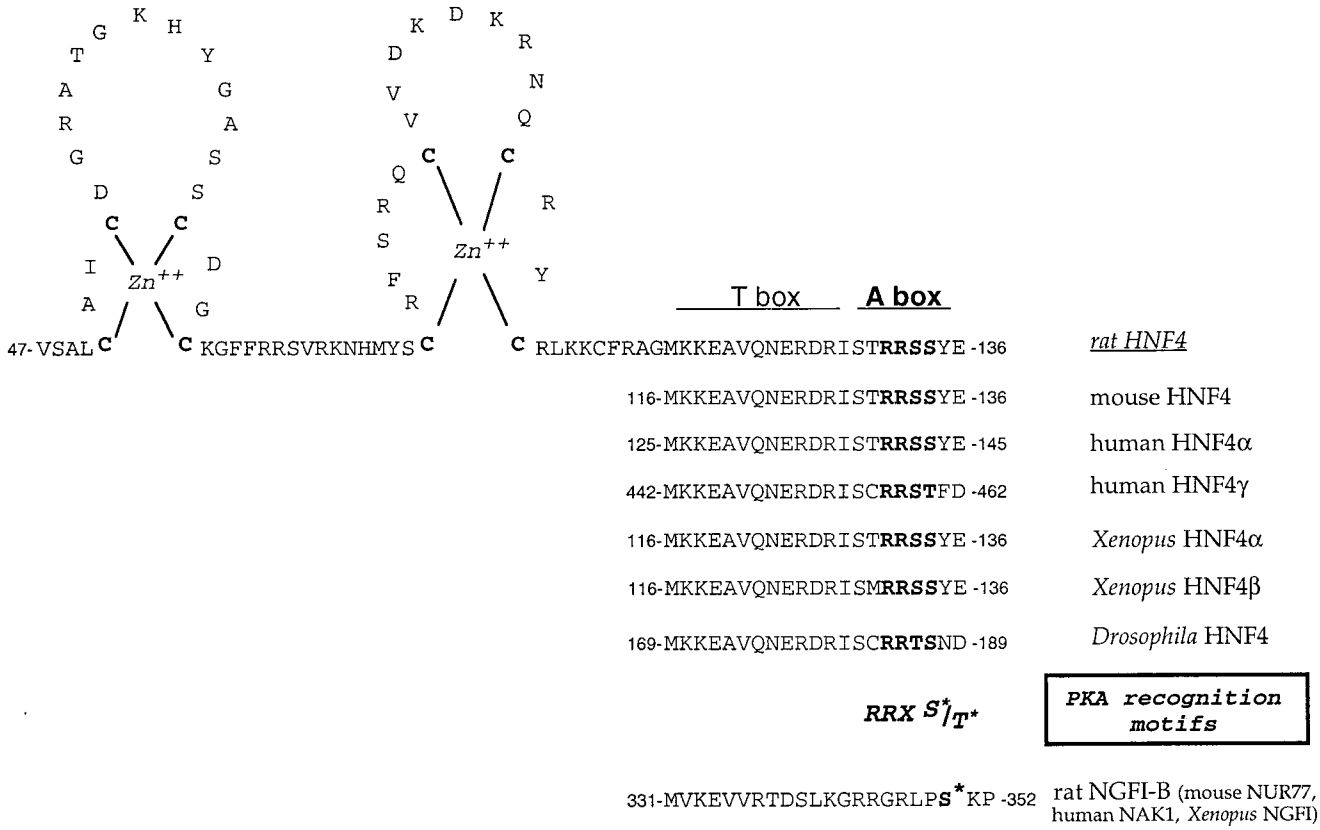

FIG. 6. Rat HNF4 DNA-binding domain. The two zinc finger motifs are shown with the downstream T and A boxes. HNF4 amino acid sequences from different species are aligned with that of the NGFI-B proteins. Numbers indicate amino acids in rat HNF4 (54), mouse HNF4 (20), human HNF4α (6), human HNF4γ (14), *Xenopus* HNF4α (23), *Xenopus* HNF4β (24), *Drosophila* HNF4 (61), and rat NGFI-B (21). The serine residue phosphorylated in the NGFI-B protein is indicated by an asterisk. Potential PKA phosphorylation sites within the HNF4 proteins are in boldface and are compared to the consensus PKA recognition motifs (31).

Negative regulation of DNA binding by phosphorylation has already been demonstrated for other members of the nuclear receptor superfamily. For instance, phosphorylation of the TRα2 ligand-binding domain acts at a distance in reducing binding affinity, probably by changing the overall conformation of the protein (30). In contrast, NGFI-B phosphorylation occurs precisely within the A-box region, so that the reduction of DNA binding is likely to be due to a local effect. Indeed, when phosphorylation sites are located either within the DNA-binding domain or nearby, phosphorylation could interfere with DNA binding by electrostatic repulsion between phosphate groups on the protein and phosphates on the DNA or could inhibit contacts between the protein and the DNA by steric hindrance. In an attempt to mimic phosphorylation in the HNF4 molecule, glutamic acid was substituted for serine at position 134, and the relative affinity of this mutant was determined. In fact, the DNA-binding activity of this mutated protein was indistinguishable from that of the wild-type HNF4 protein, which indicated that the presence of a negative charge within the A box is not sufficient to interfere with the ability of HNF4 to bind DNA.

Replacing the serine residues of the PKA-specific phosphorylation site with either acidic or neutral amino acids had exactly the same effects: both mutant HNF4 proteins had the same affinity for their cognate DNA element as unphosphorylated wild-type HNF4 but were insensitive to the PKA action, indicating that addition of the phosphate group could act more by steric hindrance and/or conformational modification than by electrostatic repulsion. Whatever the mechanism by which phosphorylation of the A box regulates binding affinity of HNF4, this phenomenon appears to be essential for the in vivo function of HNF4 in different species. Indeed, the phosphorylation site is conserved in all known HNF4 sequences from invertebrates to vertebrates. The recently cloned human HNF4γ isoform (14), with a tissue distribution distinct from that of HNF4α, shared with the latter a striking conservation of this PKA phosphorylation site within its A box (conservative amino acid substitution Ser to Thr at position 134). The same interspecies conservation of these phosphorylation sites is found in the NGFI-B family members, as shown in Fig. 5. In addition to the inhibitory phosphorylation by PKA of a site in the A box, HNF4 has been reported to require phosphorylation on undetermined tyrosine residues and also on serine/threonine residues in order to be active (29, 35). Therefore, HNF4 could integrate different types of extra- and intracellular cues mediated by tyrosine kinases or phosphatases and by cAMP-dependent protein kinases or serine phosphatases such as PP1 or PP2a and thus could ensure the fine tuning of various genes involved in the control of metabolism. Consistent with this hypothesis, it has been recently reported that in vivo HNF4 DNA-binding activity in the liver is reduced by dietary protein restriction (43). Recently, it has been also reported that in response to the acute phase, the DNA-binding activity of HNF4 is modulated by posttranslational modification upon injury-induced phosphorylation (2).

However, the observation that cAMP-dependent regulatory mechanisms can be associated with HNF4 inactivation appears to be in contradiction with the presence of HNF4 binding sites

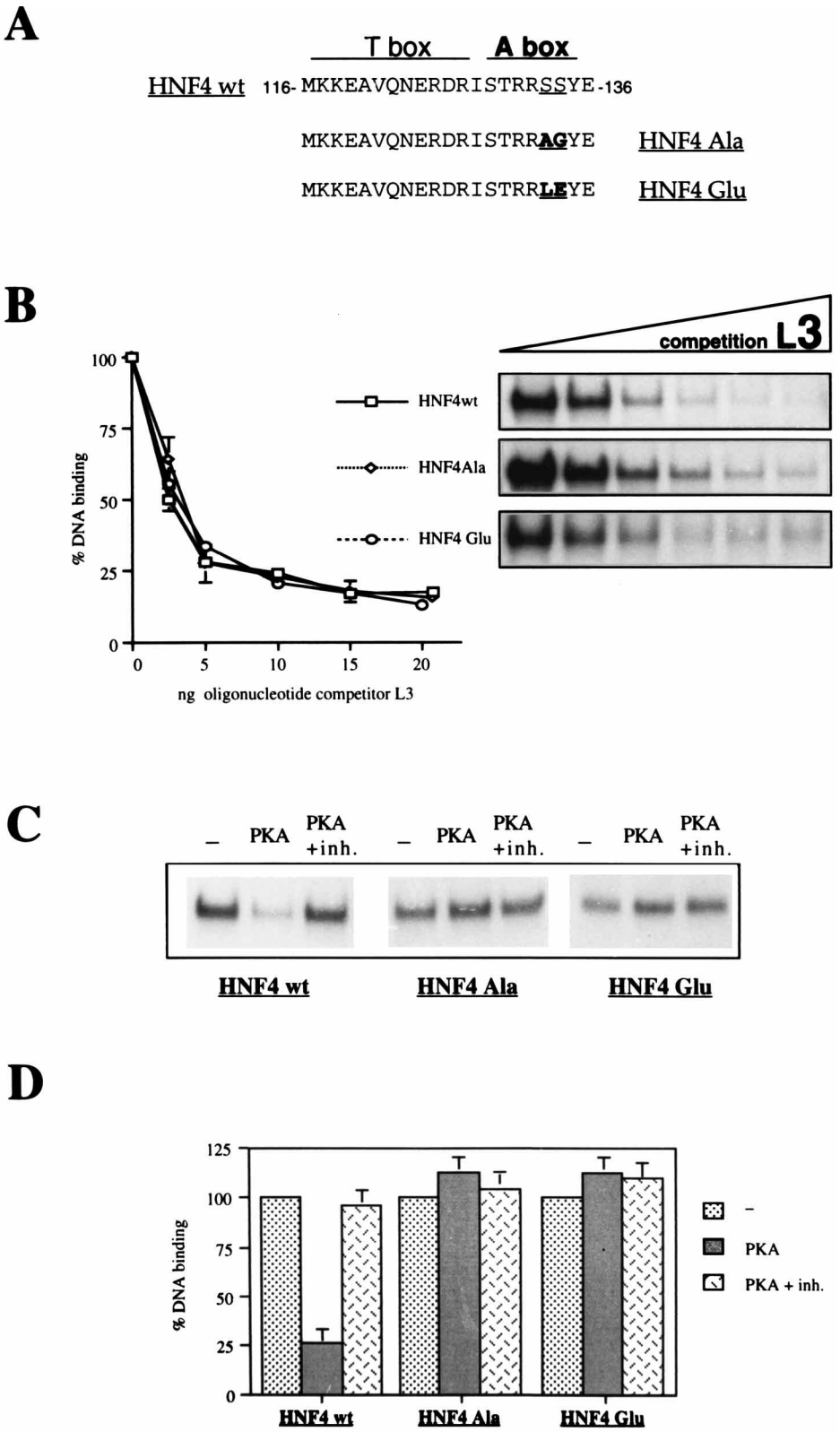

FIG. 7. DNA-binding activities of wild-type and mutated HNF4 proteins after in vitro PKA phosphorylation. (A) Sequences of the mutated HNF4 proteins at positions 133 and 134. Serine residues were changed to either neutral or acidic residues, and mutant proteins were termed HNF4 Ala and HNF4 Glu, respectively. Mutated amino acids are in boldface. wt, wild type. (B) Comparison of DNA-binding affinities of the wild-type and mutated HNF4 proteins. The relative DNA-binding affinity was evaluated by competition in gel shift assays with increasing amounts of the unlabeled L3 oligonucleotide as shown on the right. The percentage of residual DNA binding is plotted on the left. Error bars indicate standard deviations. (C) In vitro phosphorylation by PKA does not interfere with DNA-binding activity of the mutated HNF4 proteins. DNA binding of PKA-phosphorylated HNF4 proteins was checked by gel shift assay with the L3 DNA probe (lanes PKA), and the specificity of PKA phosphorylation was confirmed by addition of the PKA inhibitor in the phosphorylation buffer (lanes PKA+inh.). (D) Quantification of the HNF4 DNA-binding activity upon in vitro PKA phosphorylation.

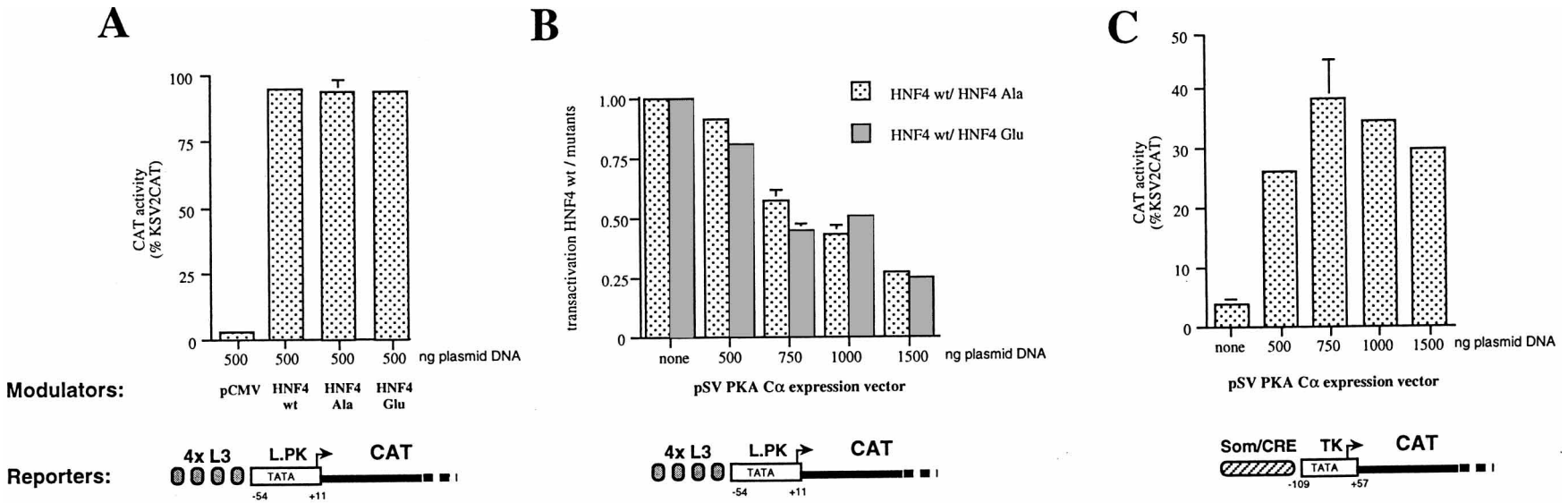

FIG. 8. Influence of PKA-dependent phosphorylation of wild-type and mutated HNF4 proteins on their transactivation activities. (A) COS cells were cotransfected with 5 μg of the reporter construct $(L3)_4$-54PK/CAT and 500 ng of the wild-type (HNF4 wt) or mutated (HNF4 Ala and HNF4 Glu) pCMV-HNF4 expression vectors or the empty pCMV expression vector (pCMV). Results are expressed as percentages of KSV2CAT activity in arbitrary units. Error bars indicate standard deviations. (B) COS cells were cotransfected with 5 μg of the reporter construct $(L3)_4$-54PK/CAT, 500 ng of one of the three HNF4 expression vectors, and increasing amounts of the expression vector for the catalytic subunit of PKA. To take into account the possible nonspecific influence of PKA on the transcriptional machinery, CAT activities have been normalized within each experiment to the activity observed without PKA transfection, and results are reported as the ratio of CAT activity in the presence of wild-type HNF4 to CAT activity in the presence of mutated HNF4 for each amount of PKA expression vector. (C) The activity of the transfected PKA catalytic subunit was monitored by cotransfection with 5 μg of the pSom/CAT reporter gene, which contains the rat somatostatin CRE cloned immediately 5′ to the thymidine kinase promoter. Activity with increasing amounts of PKA expression vector was compared to that with the unstimulated reporter construct. Results are expressed as percentages of KSV2CAT activity in arbitrary units.

in the regulatory regions of genes positively regulated by cAMP, for instance, the TAT and PEPCK genes. Indeed, the hormone-regulated expression of the TAT gene is mediated by two regions, containing the glucocorticoid response element at −2.5 kb and the CRE at −3.6 kb, which specifically interact with the liver-enriched transcription factors HNF3 and HNF4, respectively (48). It has been demonstrated that HNF4 synergizes with the proteins binding the CRE to confer cAMP responsiveness. How can these findings be reconciled with our data? We can imagine that, in vivo, HNF4 isobinders such as COUP-TF could also cooperate with CREB bound to a contiguous CRE and thus participate in the transcriptional activation by cAMP. Recently, both HNF4 and COUP-TF have been shown to be required for induction of PEPCK gene transcription by glucocorticoids (18, 50). Although antagonistic effects have been described for these two transcription factors, for instance, in the L-PK promoter (13), they are rather functionally redundant in the PEPCK promoter context. Therefore, on PKA-dependent phosphorylation and due to its decreased affinity for its cognate element, HNF4 could be replaced by COUP-TF or ARP1, which could cooperate in the positive response of the PEPCK gene to glucocorticoids and of the TAT gene to cAMP. However, HNF4 isobinders such as COUP-TF and ARP1 generally act as negative regulators of transactivation by HNF4 through direct competition for DNA binding (5, 15, 32, 38, 45), the presence of strong repressor domains, and perhaps interaction with TFIIB in a nonproductive conformation (39).

In the pancreas, where HNF4 is absent, the L-PK gene is inactive. However, in vivo footprinting data indicate that the L-PK promoter is in an open chromatin conformation and the HNF1 and NF1 sites are occupied, most likely by these factors which are synthesized in this tissue. The L3 box is also occupied, although the footprint is slightly different from that observed in the liver. Most likely, this L3 element is occupied by COUP-TF in the pancreas; this factor represents the only L3 binding activity found by gel shift assays (46). These data indicate that HNF4 binding seems to be indispensable for expression of the L-PK gene and cannot be replaced by isobinders of the COUP-TF type. In addition, comparing footprinting results for the pancreas with those for the liver suggests that binding of HNF4 could be required for a proper formation of the L4-binding complex, i.e., of the glucose response complex. This hypothesis is consistent with the fact that mutations in both the L3 and L4 boxes of the L-PK promoter are able to abolish glucose responsiveness (1, 41).

Therefore, we hypothesize that PKA-dependent phosphorylation of HNF4, upon glucagon secretion in vivo, could lead to a partial replacement on the L3 box of HNF4 by COUP-TF, which is unable to cooperate in building a functional glucose response complex on the L4 box and thus results in the loss of glucose-dependent activation and transcriptional inhibition. Of course, this hypothesis does not rule out the possibility that cAMP-dependent mechanisms may also act at another level, for instance, directly on a partner of USF in the glucose-response complex. In support of this, HNF4 does not seem to be always present in the glucose-responsive regions of other carbohydrate-regulated genes which are also negatively regulated by cAMP; for instance, no HNF4 binding site exists in the proximity of the so-called carbohydrate response element of the *Spot 14* gene (52).

Moreover, in those genes depending on HNF4, regulation through this factor is unlikely to be limited to direct phosphorylation-dephosphorylation. Indeed, other members of the nuclear receptor superfamily are known to be regulated by interaction with various corepressors or coactivators (reviewed in reference 25). For instance, among the negative regulators, calreticulin (3, 9, 51) and TRUP (4) interact specifically with DNA- or ligand-binding domains of the retinoic acid, thyroid hormone, and glucocorticoid receptors. Calreticulin expression is positively modulated by cAMP (12), which is therefore able to inhibit the action of some nuclear receptors by indirect mechanisms. Members of the nuclear receptor superfamily are especially well suited to participate in the tuning of tightly regulated genes such as those involved in metabolism, which integrate a variety of signals from outside and inside cells. Even an orphan receptor such as HNF4, whose ligand is not yet characterized or does not exist, can be regulated by different kinases, phosphatases and, most likely, protein interactions. This factor has been shown to play an important role in the regulation of various metabolic genes in the liver. We show in this paper that HNF4 is a direct target of cAMP-dependent protein kinase, in vitro, ex vivo, and in vivo. PKA-dependent phosphorylation of HNF4 decreases its affinity for its cognate binding elements, which could play an important role in the cAMP-dependent transcriptional inhibition of liver-specific genes, and in particular of genes activated by carbohydrates (47, 57).

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