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Nuclear Factor 1 Family Members Interact with Hepatocyte Nuclear Factor 1α to Synergistically Activate L-type Pyruvate Kinase Gene Transcription*

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Shin-ichi Satoh[‡], Takashi Noaki[‡], Tatsuya Ishigure[‡], Shigehiro Osada[§], Masayoshi Imagawa[§], Naoyuki Miura[¶], Kazuya Yamada[‖], and Tamio Noguchi[‡1]

From the [‡]Department of Applied Molecular Biosciences, Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa-ku, Nagoya 464-8601, Japan, [§]Department of Molecular Biology, Graduate School of Pharmaceutical Sciences, Nagoya City University, Mizuho-ku, Nagoya 467-8603, Japan, [¶]Department of Biochemistry, Hamamatsu University School of Medicine, Hamamatsu, Shizuoka 431-3192, Japan, and [‖]Department of Biochemistry, Faculty of Medical Sciences, University of Fukui, Matsuoka, Fukui 910-1193, Japan

Transcription of hepatic *L-type pyruvate kinase* (*L-PK*) gene is cell type-specific and is under the control of various nutritional conditions. The *L-PK* gene contains multiple *cis*-regulatory elements located within a 170-bp upstream region necessary for these regulations. These elements can synergistically stimulate *L-PK* gene transcription, although their mechanisms are largely unknown. Because nuclear factor (NF) 1 family members bind to specific *cis*-regulatory elements known as L-IIA and L-IIB and hepatocyte nuclear factor (HNF) 1α binds to the adjacent element L-I, we examined the functional and physical interactions between these two transcription factors. Reporter gene assay showed that these two factors synergistically activated the *L-PK* promoter containing the 5′-flanking region up to −189. Although two NF1-binding sites are required for the maximum synergistic effect of NF1 family members with HNF1α, significant functional interaction between the two factors was observed in the *L-PK* promoter containing two mutated NF1-binding sites and also in the promoter containing only the HNF1α-binding site, raising the possibility that NF1 proteins function as HNF1α co-activators. Chromatin immunoprecipitation assay revealed that both NF1 proteins and HNF1α bound to the promoter region of the *L-PK* gene *in vivo*. *In vitro* binding assay confirmed that NF1 proteins directly interacted mainly with the homeodomain of HNF1α via their DNA-binding domains. This interaction enhanced HNF1α binding to the L-I element and was also observed in rat liver by co-immunoprecipitation assay. Thus, we conclude that cooperative interaction between NF1 family members and HNF1α plays an important role in hepatic *L-PK* transcription.

Pyruvate kinase (ATP, pyruvate 2-*O*-phosphotransferase; PK)[2] is an important regulatory enzyme in the glycolytic pathway. Four PK isozymes are known to exist in mammals and are designated as L, R, M_1, and M_2 types (1). They are produced from two genes, *PKL* and *PKM*, by alternative use of two promoters and mutually exclusive alternative splicing, respectively (2–4). The L-type isozyme (L-PK) is expressed in a tissue-specific manner; this form is expressed primarily in hepatic parenchymal cells and is also present in pancreatic β cells, kidney, and small intestine (1). Hepatic *L-PK* gene expression is also transcriptionally controlled positively by glucose in the presence of insulin and negatively by polyunsaturated fatty acids or glucagon via cyclic AMP (5–8). These regulations of the *L-PK* gene transcription are mediated via the multiple *cis*-regulatory elements located within a 170-bp upstream region from the transcription start site, as shown in Fig. 1 (8–10). We have named these elements L-I (−94 to −76), L-II (−149 to −126), and L-III (−170 to −150) (9). In addition, another group has identified a weak negative element (−116 to −99) between L-I and L-II (10). Although this element has been named L2, we will refer to this as L-IIA and L-II as L-IIB hereafter. Although L-IIB is considered to be a regulatory element responsible for polyunsaturated fatty acid suppression of *L-PK* gene transcription (8), L-III itself possesses carbohydrate responsiveness and is thus termed a carbohydrate response element (11, 12). In the natural promoter context, however, L-III alone does not confer carbohydrate responsiveness to the promoter, and synergistic interaction between L-IIB and L-III is crucial for carbohydrate responsiveness (13, 14). Therefore, L-IIB also functions as an accessory element for L-III. We have observed functional interactions between L-I and L-IIB, L-IIB and L-III, but not L-I and L-III (9). The inclusion of all three elements oriented in the same direction showed the maximum synergistic effect, indicating that these elements function as a unit. This unit possesses cell type specificity as well as carbohydrate responsiveness *in vivo* (15, 16). Thus, the transcription factors that bind to these elements may interact with each other, and these interactions may be crucial to the further understanding of *L-PK* gene transcriptional regulation.

It has been shown that hepatocyte nuclear factor (HNF) 1α binds to L-I (9, 10) and that nuclear factor (NF) 1 family members bind to L-IIA (10). Although several transcription factors are reported to interact with the L-III element (10, 17–19), recent studies revealed that carbohydrate response element-binding protein (ChREBP) is an important physiological factor (20–23). ChREBP is reported to bind to L-III as a heterodimer with Max-like protein X. It has been widely accepted that HNF4α is involved in transcriptional activation of the *L-PK* gene, because reporter gene assays and binding assays showed that HNF4α stimulates the *L-PK* promoter activity by binding to the L-IIB element (10, 13, 24). However, we suspected an alternative mechanism, because NF1 family members also bind to the L-IIB element as well as the accessory element in the S_{14}

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[1] To whom correspondence should be addressed: Dept. of Applied Molecular Biosciences, Graduate School of Bioagricultural Sciences, Nagoya University, Furo-cho, Chikusa-ku, Nagoya 464-8601, Japan. Tel.: 81-52-789-4121; Fax: 81-52-789-4121; E-mail: tnoguchi@agr.nagoya-u.ac.jp.

[2] The abbreviations used are: PK, pyruvate kinase; HNF, hepatocyte nuclear factor; NF, nuclear factor; ChREBP, carbohydrate response element-binding protein; GST, glutathione *S*-transferase; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation; RSV, Rous sarcoma virus.

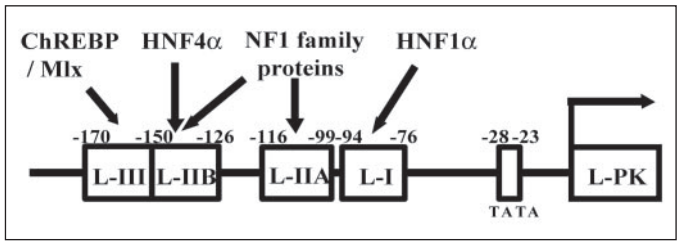

FIGURE 1. **A schematic diagram of multiple *cis*-regulatory elements of the rat *L-PK* gene.** Transcription factors interacting with these elements are shown. *Mlx,* Max-like protein X.

gene, whereas HNF4α does not bind to this element in the S_{14} gene (25). The S_{14} accessory element located in the adjacent region to carbohydrate response element is involved in carbohydrate responsiveness of the S_{14} gene and therefore corresponds to L-IIB in the *L-PK* gene (14). In addition, expression of *HNF4α* and *L-PK* genes in the liver are regulated by hormones and diet in opposite ways (26, 27). However, functional significance of NF1 family members in the *L-PK* gene regulation has never been clarified. We assumed that NF1 family proteins play important roles in transcriptional regulation of the *L-PK* gene by interactions with HNF1α and ChREBP.

HNF1α (also called LF-B1) is a homeodomain-containing transcription factor expressed in liver, kidney, pancreatic β cells, and small intestine (28, 29). HNF1α is implicated in hepatocyte differentiation and is required for liver-specific expression of several genes, including albumin, β-fibrinogen, and other genes for homeostasis and metabolisms of glucose, lipids, steroids, and amino acids. HNF1α consists of 628 amino acids and contains three functional domains, an N-terminal dimerization domain, a DNA-binding domain (a Pit1-Oct1-Unc86-like domain and an atypical homeodomain), and a C-terminal transactivation domain.

NF1 family proteins are ubiquitously expressed and regulate, not only ubiquitously expressed genes, but also hormonally and developmentally regulated genes (30–32). There are four *NF1* genes in mammals, *NF1-A, -B, -C,* and *-X*, from which numerous NF1 isoforms are generated through alternative splicing or promoter usage. NF1 proteins are highly conserved in the N-terminal DNA-binding/dimerization domains, whereas the C termini contain diverse transactivation/repression domains.

In this study, we focused our research on the role of NF1 family members in transcriptional regulation of the *L-PK* gene. We examined functional and physical interaction between HNF1α and NF1 family proteins that bind to adjacent regions of the *L-PK* promoter and found that cooperative interaction between HNF1α and NF1 family proteins plays an important role in *L-PK* transcription in the liver.

EXPERIMENTAL PROCEDURES

Materials—The dye terminator cycle sequencing kit was obtained from PE Applied Biosystems. The dual luciferase reporter gene assay kit, pGL3-Basic, pRL-SV40, and the T7 TNT quick-coupled transcription/translation system were purchased from Promega. The Qiagen plasmid and Qiaex gel extraction kits and pQE-30 were purchased from Qiagen. Horseradish peroxidase-conjugated secondary antibodies, ECL plus reagent, Hyperfilm, pGEX vectors, protein A-Sepharose, glutathione-Sepharose, [γ-^{32}P]ATP (111 TBq/mmol), and [^{35}S]methionine (43.17 TBq/mmol) were obtained from Amersham Biosciences. Mutan-Super Express kit, T4 polynucleotide kinase and restriction endonucleases were purchased from Takara. Plasmid pSPORT 1 and Wako PURE system, an *in vitro* transcription/translation system, were obtained from Invitrogen and Wako, respectively.

Animals and Treatment—6–8-week-old male Sprague-Dawley and Wister rats were used. To examine the effect of a high carbohydrate diet, rats were starved for 24 or 48 h and were then refed with a high carbohydrate diet containing 61% dextrin and 20% sucrose (5). All animal experiments were approved by the Animal Experiment Committee, Nagoya University, and rats were treated in accordance with the guidelines.

Plasmids—Reporter plasmids pLluc189 and pLluc62 were generated by ligating promoter fragments of the *L-PK* gene isolated from pLcat189 and pLcat 62 (9) into pGL3-Basic vector, respectively. Reporter plasmid p(LI)$_2$luc62 was produced by insertion of two copies of L-I oligonucleotide (−94 to −76) into the XbaI site of pLluc62. HNF1α expression plasmid pRSV-HNF1α was generously provided by Drs. P. Monaci and A. Nicosia (Instituto di Ricerche di Biologia Moleculare, Rome, Italy). To construct pRSV-NF1 expression plasmids, cDNAs of NF1 family members were isolated from corresponding cDNA clones in pBluescript (31) using appropriate restriction enzymes and ligated into pGM4 vector (25) containing the Rous sarcoma virus (RSV) long terminal repeat as a promoter. NF1-C cDNA was isolated from the above cDNA clone by digestion with SalI and XhoI and ligated into the SalI/XhoI sites of pSPORT 1. The resultant plasmid was then digested with SalI and HindIII, and the cDNA was isolated and inserted into the SalI/HindIII sites of pQE-30 to produce pQE-NF1-C. HNF1α expression plasmid in *Escherichia coli*, pT7-B1, was described previously (9). Plasmids expressing glutathione S-transferase (GST) fusion proteins of NF1 family members or HNF1α were constructed as follows. cDNA fragments encoding complete coding sequences of NF1 family members were isolated from the above cDNA clones by digestion with SalI/NotI and were then inserted into the SalI/NotI sites of pGEX-5X-1. GST fusion plasmids of pGST-NF1-A (10–244), pGST-NF1-B (11–239), pGST-NF1-C (10–246), and pGST-NF1-X (10–245) were described previously (31). The following oligonucleotides were used as primers for PCR to amplify the DNA fragments of HNF1α: HNF1α-4Bam/S, 5′-CGGGATCCAAGTTGAGCCAGCTGCAGAC-3′; HNF1α-286Eco/AS, 5′-CGGAATTCTATACGTGTCCATGGCCAG-3′; HNF1α-318Bam/S, 5′-CGGGATCCGGTGTGCGGTATGGACAGTC-3′; HNF1α-616Eco/AS, 5′-CGGAATTCTCTCGATGACACCGTGGTTG-3′; HNF1α-149Eco/AS, 5′-CCGGAATTCTGTTGAGGTGCTGGGACAG-3′; HNF1α-149Bam/S, 5′-CGCGGATCCAACAAGGGCACCCCCATGAAG-3′; HNF1α-196Eco/AS, 5′-CCGGAATTCTGGTTGGCAGCTCATCACCTG-3′; HNF1α-195Bam/S, 5′-CGCGGATCCCCAACCAAAAAGGGGCGGAG-3′; and HNF1α-286Eco/AS, 5′-CGGAATTCTATACGTGTCCATGGCCAG-3′. Fragments HNF1α-(4–616), HNF1α-(4–286), HNF1α-(318–616), HNF1α-(4–149), HNF1α-(149–196), and HNF1α-(195–286) were obtained by PCR with the HNF1α-4Bam/S and HNF1α-616Eco/AS, HNF1α-4Bam/S and HNF1α-286Eco/AS, HNF1α-318Bam/S and HNF1α-616Eco/AS, HNF1α-4Bam/S and HNF1α-149Eco/AS, HNF1α-149Bam/S and HNF1α-196Eco/AS, and HNF1α-195Bam/S and HNF1α-286Eco/AS primers, respectively, using pRSV-HNF1α as a template. These amplified PCR products were digested by BamHI/EcoRI and then ligated into the BamHI/EcoRI sites of the pGEX-4T2 vector. The sequences of all of the plasmids were confirmed by DNA sequencing.

Mutagenesis—Site-directed mutagenesis was performed using the Mutan-Super Express kit according to the manufacturer's instructions. The mutations were introduced into pLluc189 using the following oligonucleotides: mL-IIA, −127 5′-GTACAAGGCTTCAGTACTCAAGAGAGATGC-3′ −98; mL-IIB, −156 5′-CCCGTGGTTCCTAGTACTTGCCAGCCAGTGTACAAG-3′ −121 (the mutated nucleotides are underlined). All mutations were confirmed by DNA sequencing.

TABLE ONE

Nucleotide sequences of oligonucleotides used in EMSAs

Mutated nucleotides are underlined.

Oligonucleotides	Nucleotide sequences
L-I	−94 5′-CTAGCTGGTTATACTTTAACCAG-3′ −76 3′-GACCAATATGAAATTGGTCCTAG-5′
L-IIA	−123 5′-GATCAAGGCTTCCGTTGGCAAGAGAGATGCT-3′ −97 3′-TTCCGAAGGCAACCGTTCTCTCTACGACTAG-5′
mL-IIA	−123 5′-GATCAAGGCTTCAGTACTCAAGAGAGATGCT-3′ −97 3′-TTCCGAAGTCATGAGTTCTCTCTACGACTAG-5′
mL-IIA′	−12 5′-GTACAAGGCTTCAGTACTCAAGAGAGATGC-3′ −98 3′-CATGTTCCGAAGTCATGAGTTC T CTCTACGCTAG-5′
L-IIB	−149 5′-GATCTTCCTGGACTCTGGCCCCCAGTGT-3′ −126 3′-AAGGACCTGAGACCGGGGGTCACACTAG-5′
mL-IIB	−149 5′-GATCTTCCTAGTACTTGCCAGCCAGTGT-3′ −126 3′-AAGGATCATGAACGGTCGGTCACACTAG-5′
L-A	−187 5′-CTAGCGATCCAGCAGCATGGGCGCCACGGGGCACTCCCGTGGTTC-3′ −147 3′-GCTAGGTCGTCGTACCC GCGTGGCCCCGTGAGGGCACCAAGGTAC-5′
L-B	−98 5′-GATCCTAGCTGGTTATACTTTAACCAGGACTCATCTCATCT-3′ −62 3′-GATCGACCAATATGAAATTGGTCCTGAGTAGAGTAGACTAG-5′
NF1-Ade	5′-GATCTTTTGGCTTGAAGCCAATATGAG-3′ 3′-AAAACCGAACTTCGGTTATACTCCTAG-5′

Reporter Gene Assays—HeLa cells were co-transfected with 600 ng of reporter plasmid, 10 ng of pRL-SV40, and the indicated amount of RSV expression vectors (Figs. 2, 4, and 5). The total amount of DNA was adjusted by adding empty vector, when required. All plasmids used for transfection were prepared using the Qiagen plasmid Midi kit. The transfection conditions and luciferase reporter assays have been described previously (33).

Electrophoretic Mobility Shift Assays (EMSAs)—Nuclear extracts were prepared from rat liver and HeLa cells as described previously (25, 33). Rat liver nuclear extracts were heated at 60 °C for 5 min and centrifuged at 15,000 revolutions/min. The resultant supernatant was used for EMSAs. HNF1α and NF1-C were synthesized *in vitro* from pT7-B1 using the T7 TNT quick-coupled transcription/translation system and from pT7-NF1-C using the Wako PURE system, respectively, and both were also used for EMSAs. The oligonucleotides used in EMSAs are listed in TABLE ONE. Double-stranded oligonucleotides were 5′-end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. 10 μg of nuclear extract were incubated for 30 min at room temperature with the ^{32}P-labeled probe in a reaction mixture containing 12.5 mM HEPES-KOH (pH 7.9), 10% glycerol, 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, and 1 μg of poly(dI-dC). When *in vitro* synthesized HNF1α and NF1-C were used for EMSAs, the reaction mixture contained 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.5 mM dithiothreitol, 10% glycerol, 0.05% Nonidet P-40, 1 μg of sonicated single-stranded DNA, and the ^{32}P-labeled probe. Competition experiments included a 200-fold molar excess of unlabeled competitor oligonucleotides. Supershift assays were carried out by preincubating the nuclear extract with anti-NF1 antibody or anti-HNF1α antibody (34) for 30 min at room temperature before the addition of labeled oligonucleotide. Anti-M_1-PK antibody (35) was used as a control. Polyclonal anti-NF1 antibody 8199 was kindly provided by Dr. Naoko Tanese (New York University School of Medicine, New York, NY). After the binding reaction, the mixture was subjected to electrophoresis on a 4% polyacrylamide gel in 0.5× Tris borate-EDTA buffer at 200 V for 1 h. The gel was dried and analyzed by a FUJIX BAS-2500 image analyzing system.

Chromatin Immunoprecipitation (ChIP) Assays—Rat hepatocytes were obtained from male Sprague-Dawley rats by the collagenase perfusion method (36). ChIP assays were performed essentially as previously described (37). Hepatocytes were treated with 1% formaldehyde at 37 °C for 15 min. The cross-linking reaction was stopped by the addition of glycine to a final concentration of 0.125 M. After being washed with cold phosphate-buffered saline, the cells were suspended in lysis buffer containing 50 mM Tris-HCl (pH 8.1), 1% SDS and 10 mM EDTA. The lysate was sonicated to generate DNA fragments with a length of <1,000 bp. After removal of cell debris by centrifugation, the supernatant was diluted 10-fold with buffer containing 16.7 mM Tris-HCl (pH 8.0), 1.1% Triton X-100, 1.2 mM EDTA, 167 mM NaCl and then precleared for 1 h with protein A-Sepharose in the presence of normal rabbit IgG and 2 μg of sonicated denatured DNA to reduce the nonspecific background. The supernatant was incubated with normal rabbit IgG, anti-NF1 antibody or anti-HNF1α antibody, or without any antibody overnight at 4 °C and then incubated with protein A-Sepharose for 2 h at 4 °C. The reaction mixture was centrifuged, and protein A-Sepharose was washed extensively. DNA was eluted from the precipitate, reverse cross-linked by incubating at 65 °C for 6 h, and then treated by proteinase K. The purified DNA was dissolved in water and analyzed by PCR using primer sets for the promoter region (−246 to −23, 5′-TACAGACCTGATCTGAGCCTTTG-3′ and 5′-TTTATACTGCGCCTTTGTCAGTG-3′) and exon 12 (5′-GCTCTGGCTATACCAACATCATGC-3′ and 5′-CGGATTGAGACAGGGATATACAAC-3′) of the *L-PK* gene. The input sample contained 1/250 of the supernatant used for immunoprecipitation. PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining.

Western Blotting—The proteins were separated by electrophoresis using 9 or 10% SDS-polyacrylamide gels, followed by transfer to membranes. The membranes were incubated for 1 h at room temperature with the antibodies indicated in Figs. 8 and 9 and then washed three times with phosphate-buffered saline containing 0.1% Tween 20 before being probed with horseradish peroxidase-conjugated second antibodies. Blots were developed using ECL plus reagent and exposed to Hyperfilm, according to the manufacturer's protocols.

GST Pull-down Assays—^{35}S-labeled HNF1α was synthesized from pT7-B1 using the T7 TNT quick-coupled transcription/translation system in the presence of [^{35}S]methionine according to the manufacturer's protocol. Histidine-tagged NF1-C was expressed from pQE-NF1-C in *E. coli*. Preparation of GST fusion proteins containing NF1 family members or HNF1α and pull-down analysis were carried out as described previously (33). Finally, samples were analyzed by SDS-polyacrylamide gel electrophoresis followed by autoradiography or Western blotting.

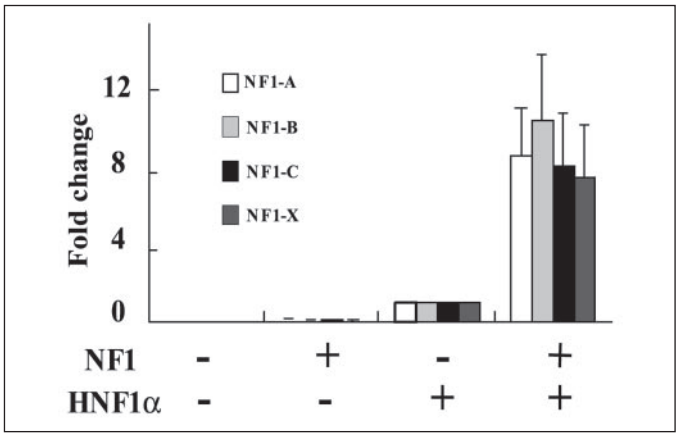

FIGURE 2. **Synergistic effects of NF1 family members and HNF1α on the *L-PK* promoter.** HeLa cells were co-transfected with 600 ng of pLluc189, 10 ng of pRL-SV40, and 100 ng of expression vectors shown. The luciferase activity is expressed relative to cells transfected with HNF1α vector alone. Data are the mean ± S.D. of at least three transfection experiments.

Co-immunoprecipitation—Rat liver nuclei were isolated as described previously (25), and nuclear extracts were prepared with immunoprecipitation buffer (40 mM Tris-HCl, pH 7.8, 0.3% Nonidet P-40, and 150 mM NaCl). The nuclear proteins (13.5 mg) were first precleared by incubation with 60 μl of a 50% protein A-Sepharose slurry containing normal rabbit IgG for 1 h at 4 °C and then incubated with either normal rabbit IgG or anti-NF1 antibody overnight at 4 °C. Immune complexes were collected by incubation with 30 μl of a 50% protein A-Sepharose slurry for 1 h followed by centrifugation. Immunoprecipitates were washed five times with immunoprecipitation buffer and subjected to Western blotting with anti-HNF1α antibody.

RESULTS

HNF1α and NF1 Family Members Synergistically Activate L-PK Promoter Activity—To examine the functional interaction between NF1 family members and HNF1α, we used a non-hepatic cell line, HeLa, for the reporter gene assays, because this cell line does not express endogenous HNF1α. The reporter gene used was pLluc189, containing the 5′-flanking region of the *L-PK* gene up to −189. This region contains L-I, -IIA and -IIB, and L-III elements that are bound by HNF1α, NFI family members and ChREBP, respectively. Although co-transfection with NF1 family members resulted in only slight increases in the reporter activity (NF1-A, 1.7 ± 0.26; NF1-B, 1.6 ± 0.12; NF1-C, 5.5 ± 2.3; NF1-X, 2.8 ± 0.71), co-transfection with HNF1α expression vector caused ~50-fold enhancement in the activity when compared with cells that were not co-transfected with either transcription factor (Fig. 2). Co-transfection of both HNF1α and NF1 expression vectors resulted in an additional 8–10-fold increase in the HNF1α-mediated activation of the *L-PK* promoter. This synergistic effect was observed in all NF1 isoforms. These results indicate that NF1 family proteins weakly activated *L-PK* transcription in the absence of HNF1α but greatly enhanced the activity through functional interaction with HNF1α.

Both L-IIA and L-IIB Are Crucial for Maximum Synergistic Effect of NF1 Family Members—Previous studies suggested that NF1 family proteins bind to both L-IIA and L-IIB (10, 25). To determine which site is necessary for synergistic interaction between NF1 proteins and HNF1α, we introduced mutations into both sites of pLluc189 independently or simultaneously. As demonstrated by EMSAs, we confirmed that NF1 proteins bound to both L-IIA and L-IIB elements (Fig. 3). However, NF1 proteins failed to recognize mutated oligonucleotides of both L-IIA and L-IIB. We used NF1-C expression plasmid as a representative of NF1 family members for transfection experiments. When NF1-C alone was transfected as an expression plasmid, the promoter activity of the wild type reporter was only slightly stimulated (Fig. 4). Therefore, it was difficult to evaluate the effect of mutations on the activation of pLluc189 by NF1-C alone. However, when the pLluc189 reporter plasmid containing either mutated sequences of L-IIA or L-IIB was transfected with NF1-C and HNF1α expression plasmids into HeLa cells, synergistic activation by NF1-C and HNF1α was decreased by 44 or 35%, respectively. Interestingly, both mutations, which caused a further decrease in the activity ($p < 0.05$ compared with mL-IIA or mL-IIB), did not completely eliminate the synergistic effect of NF1-C with HNF1α. Thus, although both NF1-binding sites are required for the maximum synergistic effect of NF1 family members with HNF1α, NF1 proteins may be able to functionally interact with HNF1α without binding to promoter DNA to some extent. Alternatively, the reporter plasmid may contain additional unknown NF1-binding elements. The first possibility was further examined using $p(LI)_2$luc62 as a reporter. This plasmid contains two copies of the L-I element (HNF1α-binding site) of the *L-PK* gene but not the NF1-binding site. As shown in Fig. 5, this reporter was activated by HNF1α but not by NF1-C. However, co-transfection of HNF1α and NF1-C expression plasmids resulted in a further marked increase in activity, and this activation of NF1-C was dose-dependent. On the other hand, the activity of pLluc62, which contains neither the NF1- nor HNF1α-binding sites, was not stimulated by transfection with HNF1α alone or HNF1α and NF1-C (data not shown). These results confirmed that synergistic effects between HNF1α and NF1 proteins do not necessarily require NF1 binding to the *L-PK* promoter. The second possibility was examined by EMSAs using rat liver nuclear extracts. The formation of complexes of labeled L-IIB was competitively inhibited by the addition of a 200-fold molar excess of unlabeled L-IIB and NF1-Ade but not by mL-IIB (Fig. 3). The addition of unlabeled L-A, mL-IIA′, and L-B oligonucleotides, which correspond to the regions between −187 and −147, −127 and −98, and −98 and −62, respectively, also failed to affect the formation of the complexes (data not shown). Thus, it is unlikely that the reporter plasmid pLluc189 contains other NF1-binding elements besides L-IIA and L-IIB.

NF1 Family Members and HNF1α Actually Associate with L-PK Promoter in Hepatocytes—To examine whether NF1 proteins and HNF1α associate with *L-PK* promoter *in vivo* under physiological conditions, hepatocytes were isolated from rats starved for 48 h or refed with a high carbohydrate diet and used for ChIP assays. A specific primer set for PCR was used to produce a 224-bp DNA fragment of the *L-PK* gene promoter region (−246 to −23) (4). This region included L-I, L-IIA, L-IIB, and L-III sites. Another primer set was used to amplify a 219-bp region in exon 12 of the *L-PK* gene as a control. A PCR product specific to the *L-PK* promoter region was obtained from immunoprecipitates with anti-HNF1α antibody or anti-NF1 antibody, and this product was observed in both the starved and refed states (Fig. 6). In contrast, there was no amplified band when DNA samples were obtained in the absence of antibody or with normal rabbit IgG or when primers specific for exon 12 of the *L-PK* gene were used for PCR. These results indicate that the *L-PK* promoter is occupied by HNF1α and NF1 family members *in vivo* under physiological conditions and that this binding is not under nutritional control.

NF1 Family Members Directly Bind to Mainly Homeodomain of HNF1α through Its N-terminal DNA-binding Domain—Our reporter gene assays indicate that HNF1α and NF1 family proteins cooperatively stimulate *L-PK* gene transcription. To examine physical interaction between HNF1α and NF1 family proteins, we carried out GST pull-down assays. ^{35}S-labeled HNF1α was synthesized by an *in vitro* tran-

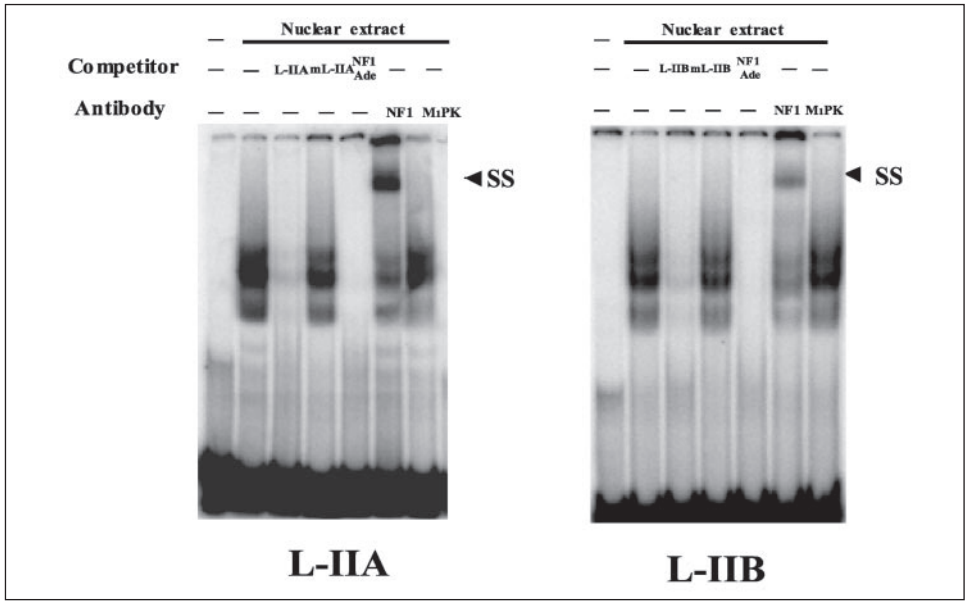

FIGURE 3. **EMSAs of NF1 family members.** End-labeled L-IIA (*left*) or L-IIB (*right*) oligonucleotides were incubated with 10 μg of rat liver nuclear extract. Unlabeled oligonucleotides were added as competitors at 200-fold molar excess where indicated. The nuclear extract was incubated with anti-NF1 antibody or anti-HNF1α antibody where indicated. *SS*, supershifted complex.

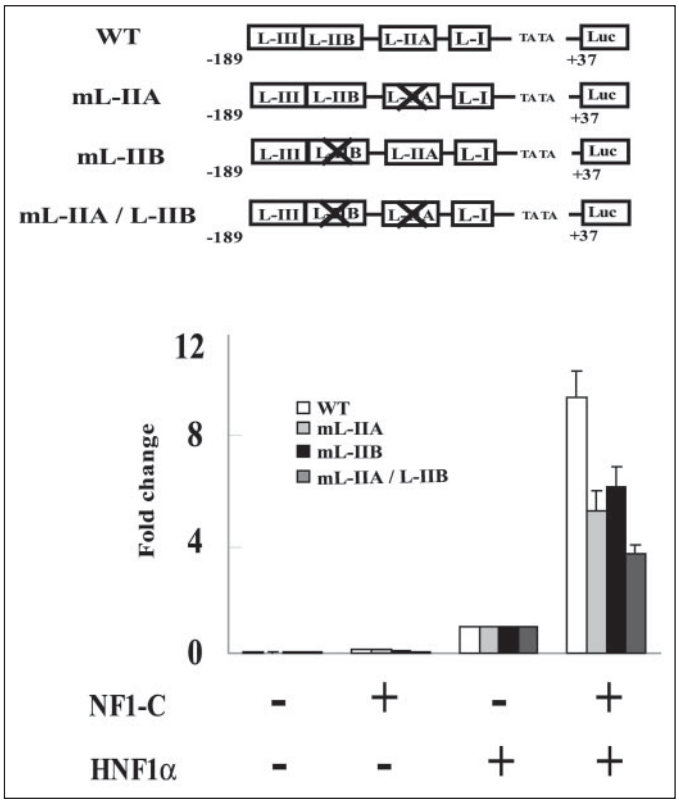

FIGURE 4. **Effects of mutation at L-IIA and L-IIB on the synergistic activation of the *L-PK* promoter by NF1-C and HNF1α.** The *upper panel* shows schematic diagrams of the *L-PK* promoter constructs. The *cis*-acting elements are indicated by *boxes*, and *boxes* containing mutated sequences are shown by *crosses* (X). HeLa cells were co-transfected with 600 ng of each reporter plasmid, 10 ng of pRL-SV40, and 100 ng of expression vectors shown. The luciferase (*Luc*) activity is expressed relative to cells transfected with HNF1α vector alone. Data are the mean ± S.D. of at least three transfection experiments.

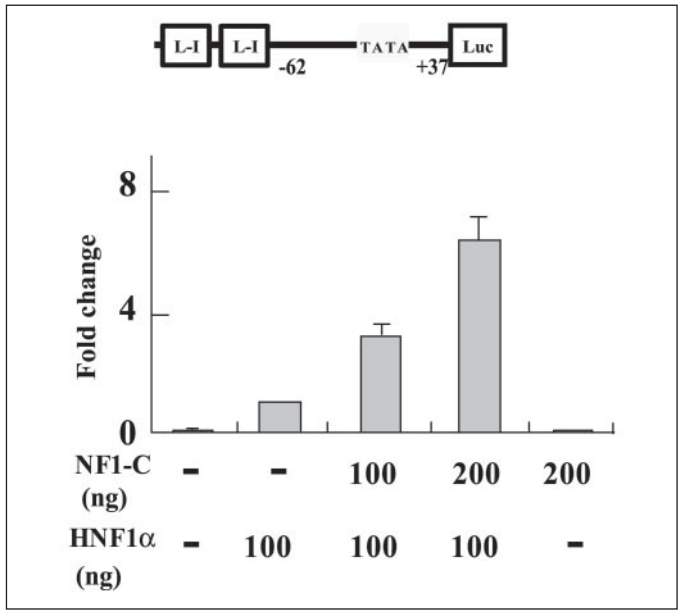

FIGURE 5. **NF1-C enhanced the HNF1α-dependent activation of transcription.** The *upper panel* shows a schematic diagram of the promoter construct. HeLa cells were co-transfected with 600 ng of p(LI)2luc62, 10 ng of pRL-SV40, and the indicated amount of expression vectors. The luciferase (*Luc*) activity is expressed relative to cells transfected with HNF1α alone. Data are the mean ± S.D. of at least three transfection experiments.

scription/translation system and incubated with GST, GST-NF1-C, or GST-NF1-X immobilized to glutathione-Sepharose beads. As shown in Fig. 7*A*, ^{35}S-labeled HNF1α interacted with GST-NF1-C and GST-NF1-X but not with GST alone. These data clearly demonstrate direct interaction between HNF1α and NF1-C or NF1-X. Because all of the NF1 family proteins can enhance HNF1α-mediated activation of the *L-PK* promoter and N-terminal DNA-binding regions are highly conserved among NF1 family members, we hypothesized that these regions are responsible for physical binding to HNF1α. To examine this hypothesis, GST fusion proteins with N-terminal regions of NF1 family proteins were prepared and incubated with ^{35}S-HNF1α. As shown in Fig. 7*B*, the N-terminal regions of all NF1 family members specifically interacted with HNF1α. Therefore, we conclude that the N-terminal DNA-binding regions of NF1 family members are involved in direct interaction with HNF1α.

We also performed GST pull-down assays to identify the region of HNF1α required for interaction with NF1 family proteins. Histidine-tagged NF1-C expressed in *E. coli* was purified by affinity chromatography and incubated with GST or GST fusion proteins containing various

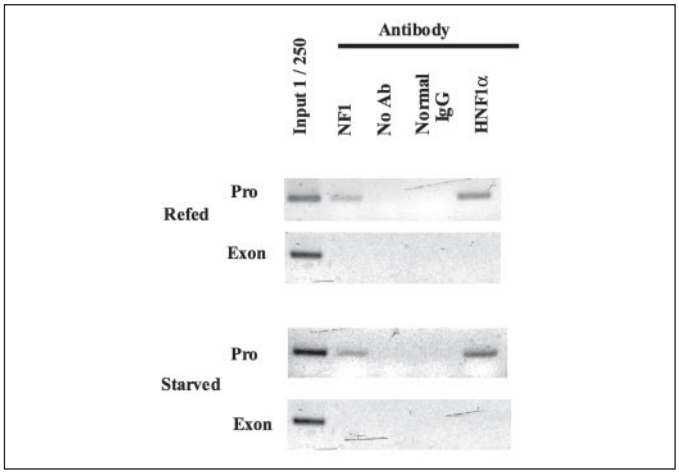

FIGURE 6. **ChIP assay of NF1 and HNF1α binding to the *L-PK* promoter in the liver.** Rats were starved for 48 h or refed with a high carbohydrate diet. Hepatocytes were isolated from the liver and subjected to formaldehyde cross-linking followed by immunoprecipitation with anti-NF1 antibody or anti-HNF1α antibody. Precipitated DNA was analyzed by PCR with a primer set specific for the promoter or exon 12 of the *L-PK* gene. Input corresponds to 0.4% of the amount of DNA used in the assay. *Ab*, antibody; *Pro*, promoter region.

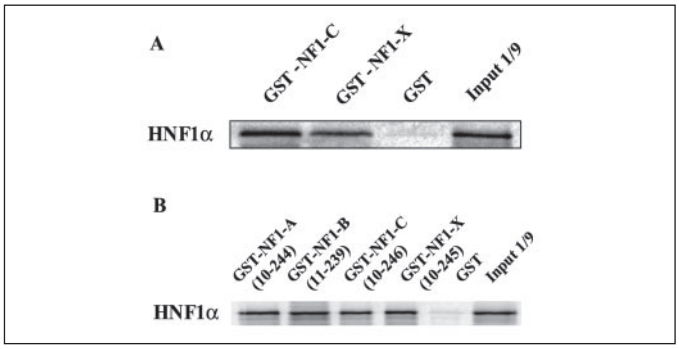

FIGURE 7. **DNA-binding domains of NF1 family members interact with HNF1α.** GST pull-down experiments were performed with ^{35}S-labeled HNF1α and GST or GST fusion proteins containing the full-length of NF1-C, NF1-X (*A*) or the indicated regions of the NF1 family members (*B*). Input corresponds to 1/9 the amount of the labeled protein used in the assay.

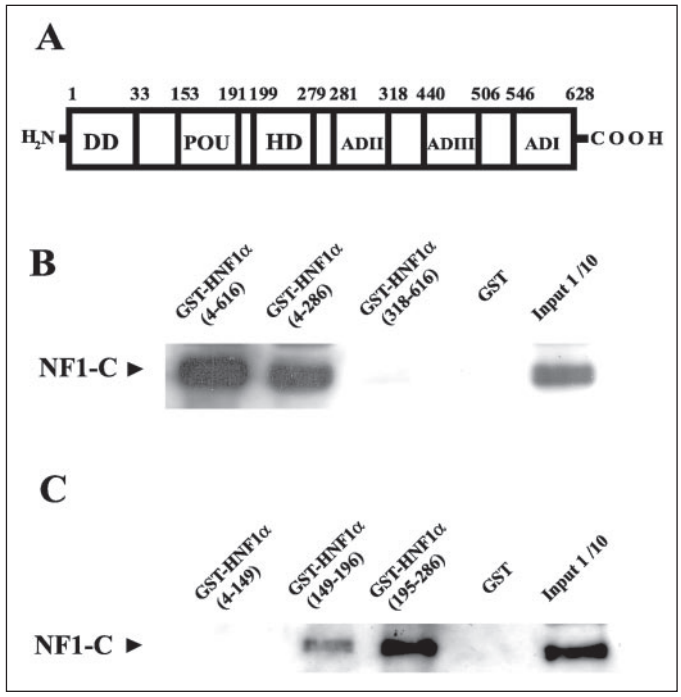

FIGURE 8. **Identification of the region of HNF1α interacted with NF1-C.** *A*, a scheme of the HNF1α structure with the various domains is shown. *DD*, dimerization domain; *POU*, Pit1-Oct1-Unc86-like domain; *HD*, homeodomain; *AD*, activation domain. *B* and *C*, bacterially expressed NF1C was incubated with GST or GST fusion proteins containing the indicated region of HNF1α, and bound NF1-C was analyzed by Western blotting. Input corresponds to 10% of the amount of NF1-C used in the assay.

regions of HNF1α. Bound NF1-C was analyzed by Western blotting. Purified NF1-C showed two bands on SDS-polyacrylamide gel. This may be due to the limited proteolysis that occurred during extraction and purification of NF1-C. The results indicated that NF1-C interacted with GST-HNF1α-(4–286) as well as GST-HNF1α-(4–616) but not with GST-HNF1α-(318–616) and GST alone (Fig. 8*B*). Because the N-terminal region of HNF1α consists of a dimerization domain, Pit1-Oct1-Unc86-like domain, and homeodomain (Fig. 8*A*), we examined these regions individually. As shown in Fig. 8*C*, NF1-C bound to GST-HNF1α-(149–196) and GST-HNF1α-(195–286) but not to GST-HNF1α-(4–149) and GST alone. GST-HNF1α-(195–286) showed much higher affinity to NF1-C than GST-HNF1α-(149–196). Thus, we conclude that NF1 family proteins bind directly to the homeodomain of HNF1α via their DNA-binding domains.

NF1 Family Members Directly Associate with HNF1α in Vivo—To assess the physical interaction between HNF1α and NF1 family proteins in rat liver, we performed co-immunoprecipitation assay with the anti-NF1 antibody. The liver nuclear extracts were prepared from rats refed with a high carbohydrate diet for 16 h and were immunoprecipitated with anti-NF1 antibody or normal rabbit IgG. Immunoprecipitates were then analyzed by Western blotting using an anti-HNF1α antibody. As shown in Fig. 9, HNF1α was detected in the immunoprecipitates from rat liver nuclear extracts by the anti-NF1 antibody but not those by normal IgG. The same results were obtained when the liver nuclear extracts were obtained from rats starved for 24 h (data not shown). These results indicate that NF1 family members interact with HNF1α in rat liver and that this interaction is not under the control of nutritional conditions.

Binding of HNF1α to the L-PK Promoter Is Enhanced by Interaction with NF1 Family Members—DNA binding affinity of HNF1α may be increased by interaction with NF1 family members. To examine this hypothesis, we first performed EMSAs with nuclear extracts from HeLa cells transfected with HNF1α and/or NF1-C expression vectors. When labeled L-I oligonucleotide containing the HNF1α-binding site was used as a probe, no specific complexes were detected with nuclear extracts from HeLa cells transfected with NF1-C or empty vector (Fig. 10*A*). However, transfection of HNF1α resulted in formation of the complexes that were competed by the addition of anti-HNF1α antibody. Co-transfection of HNF1α and NF1-C caused increases in intensities of these bands that were dependent on the amount of NF1-C transfected. A supershifted band was observed by the addition of anti-NF1 antibody to nuclear extracts. These results suggest that the DNA complexes contain both NF1-C and HNF1α and that DNA binding of HNF1α is stimulated by physical interaction with NF1-C. This was further confirmed by EMSAs using HNF1α and NF1-C that were synthesized in the *in vitro* transcription/translation system (Fig. 10*B*). We confirmed synthesis of both factors by Western blotting (data not shown). A DNA-protein complex was observed by incubation with HNF1α but not by NF1-C. The intensity of the complex was enhanced by the addition of increased NF1-C. This complex was reduced by the addition of a 200-fold molar excess of unlabeled L-I oligonucleotide or anti-HNF1α antibody and supershifted by the addition of anti-NF1 antibody. Thus, we conclude that NF1 family members enhance binding of HNF1α to the *L-PK* promoter through their direct interactions.

DISCUSSION

Transcription factors not only bind to the cognate DNA sequences but also interact with one another to regulate gene transcription. In addition, these factors recruit non-DNA-binding proteins such as co-activators and co-repressors. They, in turn, form a ternary complex, interact with basic transcription machinery, and confer transcriptional activity to the gene promoter. Here we demonstrated for the first time that NF1 family members play an important role in the transcriptional activation of the *L-PK* gene by interacting with HNF1α *in vivo* under physiological conditions as follows. First, both NF1 family proteins and HNF1α bound to the promoter region containing L-I, L-IIA, L-IIB, and L-III of the *L-PK* gene in liver, as shown by ChIP assays. Second, NF1 family members directly interacted with HNF1α in liver as shown by co-immunoprecipitation assays, which resulted in increased binding of HNF1α to the L-I element and cooperative activation of the *L-PK* gene transcription. Although four family members of the *NF1* gene are expressed in the liver (30, 31), all of them can be involved in cooperative interaction with HNF1α through their DNA-binding domains, which are highly conserved among NF1 family members.

Although maximum synergistic effect between NF1 family members and HNF1α on the *L-PK* gene required binding of NF1 family proteins to both the L-IIA and L-IIB elements, significant functional interaction was still observed, even in the absence of NF1 binding to the two elements because of their mutations. It is unlikely that the reporter plasmid pLluc189 contains additional NF1-binding elements, because EMSAs showed that no other sequences than L-IIA and L-IIB in the *L-PK* promoter region between −62 and −187 interacted with NF1 proteins and because another reporter plasmid of pLluc62 containing the promoter region up to −62 did not respond to NF1-C with or without HNF1α. In addition, it has been reported that no other nuclear protein-binding sequences than the four elements described above were observed in the promoter region up to −183 (10). Moreover, NF1 proteins activated the promoter containing only the HNF1α-binding site. Thus, we consider the interesting possibility that NF1 family members function as co-activators of HNF1α, although this issue must be further addressed in future studies. There are co-activators with histone acetyltransferase activity that can physically interact with HNF1α (38, 39). They include p300/cAMP-response element-binding protein, p300/cAMP-response element-binding protein-associated factor, receptor-associated co-activator 3, and steroid receptor co-activator-1. We examined whether p300 is involved in the synergistic transcriptional effect of NF1 proteins and HNF1α on the *L-PK* gene using the reporter gene assay. The results indicated that p300 did not enhance NF1- and HNF1α-mediated activation of the *L-PK* promoter.[3] The reason for this is unclear, because p300 has been reported to interact with the activation domain of HNF1α (39). However, it is possible that other co-activators are involved in cooperative activation of the *L-PK* gene. In addition, it has been reported that HNF4α functions as a co-activator of HNF1α and cooperates with p300 to achieve the highest HNF1α-mediated transcription (40). However, its physiological significance remains to be determined.

Although the *L-PK* promoter is under the control of nutritional conditions, occupancy of NF1 proteins and HNF1α on the *L-PK* promoter was not altered by nutritional manipulations. Physical interaction between the two transcription factors was also observed in starved and refed states, suggesting that this interaction is also not regulated by dietary carbohydrate. Thus, ChREBP/Max-like protein X binding to the L-III element of the *L-PK* promoter is critical for carbohydrate regulation of the *L-PK* gene as reported (20–23). Nevertheless, cooperative interaction between NF1 proteins and HNF1α plays an important role in carbohydrate regulation as well as cell type-specific transcription of the *L-PK* gene in liver, because maximum transcriptional activity of this gene is achieved through functional interactions among all *cis*-acting elements. Furthermore, it is possible that NF1 family members are also involved in the transcriptional regulation of the *L-PK* gene by interacting with ChREBP. However, further studies are required to solve this problem.

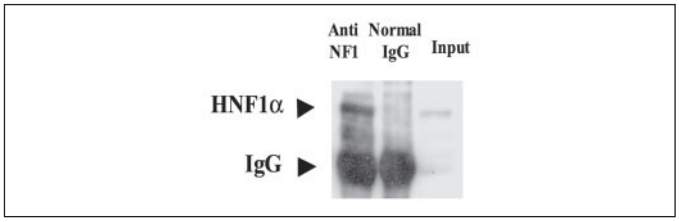

FIGURE 9. **Co-immunoprecipitation analysis of NF1 family members and HNF1α in rat liver.** Rats were refed with a high carbohydrate diet for 16 h. The liver nuclear extracts were prepared and subjected to immunoprecipitation with anti-NF1 antibody or normal rabbit IgG. The precipitates were then analyzed by immunoblotting with anti-HNF1α antibody. Input corresponds to 0.23% of the nuclear extracts used in the assay.

[3] S. Satoh and T. Noguchi, unpublished results.

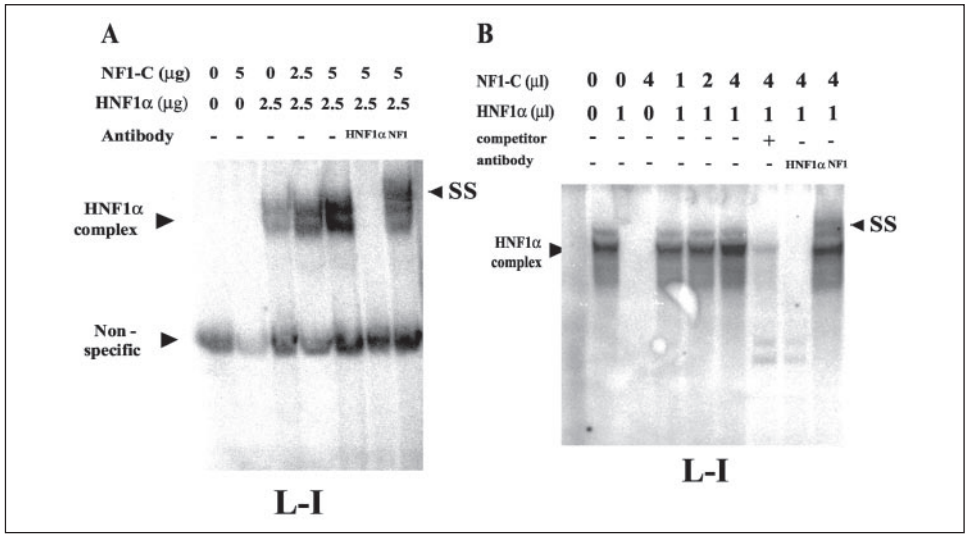

FIGURE 10. **NF1-C enhances HNF1α binding to the L-I element.** *A*, HeLa cells were transfected with the indicated amounts of pRSV-HNF1α and/or pRSV-NF1-C. Nuclear extracts were prepared from cells after 48 h and incubated with anti-HNF1α antibody or anti-NF1 antibody where indicated. EMSAs were performed using ^{32}P-labeled L-I oligonucleotide as a probe. *B*, HNF1α and NF1-C were synthesized using an *in vitro* transcription/translation system. The indicated amounts of products were used for EMSAs as described under "Experimental Procedures." The total amount of reticulocyte lysates was adjusted by adding unprogrammed lysates. Oligonucleotide was added as a competitor at 200-fold molar excess where indicated. *SS*, supershifted complex.

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