Members of the nuclear factor 1 family and hepatocyte nuclear factor 4 bind to overlapping sequences of the L-II element on the rat pyruvate kinase L gene promoter and regulate its expression

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The L-II element (–149 to –126 bp) in the enhancer unit of the rat pyruvate kinase L (PKL) gene is required for cell-type-specific transcription and induction by carbohydrates. This element was found to bind multiple nuclear proteins with different heat stabilities. A heat-labile factor was shown to be hepatocyte nuclear factor (HNF) 4 by the electrophoretic mobility-shift assay (EMSA) using various competitor DNAs and anti-HNF4 serum. A heat-stable factor was purified from rat liver nuclear extract and was resolved as two protein bands migrating at about 33 kDa on SDS/polyacrylamide gels. Peptide sequence analysis revealed that these proteins were nuclear factor (NF) 1-L and NF1/Red1. The heat-stable factor was also identified as a member of the NF1 family by using various competitor DNAs and anti-NF1 serum in an EMSA. In addition, we found that a factor bound to the accessory site of the rat *S*₁₄ gene, which is necessary for carbohydrate responsiveness of this gene, was also a member of the NF1 family, raising the possibility that the NF1

family is involved in the carbohydrate regulation of gene transcription by interactions with other proteins. The NF1 family members and HNF4 interacted with overlapping sequences of the L-II element, wherein the 5' half-site was more critical for NF1 binding, and the 3' site was more important for HNF4 binding. Co-transfection of a vector expressing either NF1-L or NF1/Red1 repressed the transcription of the PKL enhancer unit–chloramphenicol acetyltransferase (CAT) fusion gene in HepG2 cells, whereas co-transfection of a vector expressing HNF4 activated the transcription of the same reporter gene. Furthermore NF1 family members antagonized the effect of HNF4 on PKL enhancer unit–CAT fusion gene expression when both expression plasmids were co-transfected. We conclude that NF1 family members and HNF4 regulate transcription of the PKL gene in an opposing manner by binding overlapping sequences of the L-II element.

INTRODUCTION

Pyruvate kinase (PK, ATP–pyruvate O²-phosphotransferase, EC 2.7.1.40), a key glycolytic enzyme, has four isoenzymes in mammals, L, R, M₁ and M₂ types [1–3]. The L-type isoenzyme is expressed in liver, kidney, small intestine and pancreatic β -cells, and the R-type is expressed in erythrocytes [1–4]. Both R- and L-type isoenzymes are encoded by the PKL gene [5]. The rat gene is about 9.3 kb in length and consists of 12 exons and 11 introns. The first (R) and second (L) exons encode the 5'-terminal sequences specific to the R- and L-types respectively, whereas the remaining exons are common to the two types. Therefore the PKL gene produces the L- and R-type isoenzymes by using distinct tissue-specific promoters [5]. Transcription of the PKL gene in primary cultured hepatocytes is regulated by multiple *cis*-acting elements within the 170 bp upstream region of exon L, which are named L-I (–94 to –76 bp), L-II (–149 to –125 bp) and L-III (–170 to –150 bp) [6]. These elements synergistically stimulate transcription from heterologous as well as homologous promoters when oriented in the same direction, indicating that they function as an enhancer unit [6]. This unit works in an orientation- and position-independent manner and shows cell-type specificity. Using transgenic mice harbouring a fusion gene containing the enhancer unit described above linked

to the chloramphenicol acetyltransferase (CAT) gene, we and others demonstrated that this unit was responsible for the transcriptional stimulation of the PKL gene by dietary glucose and fructose as well as the tissue-specific expression of this gene [7–11]. Electrophoretic mobility-shift assays (EMSAs) have shown that hepatocyte nuclear factor (HNF) 1 binds to L-I and unknown factor(s) bind to L-II and L-III [6]. On the other hand, Vaulont et al. [12] reported that HNF1 binds to the L-I, liver factor-A1/hepatocyte nuclear factor 4 (HNF4) to the L-II, and adenovirus major late transcription factor/upstream stimulating factor (USF) to the L-III.

Expression of the PKL gene is regulated by nutritional and hormonal factors [13–21]. The rate of transcription of the PKL gene is decreased by starvation and increased by refeeding with a high-glucose or -fructose diet in rat liver. The effects of these carbohydrates require the presence of insulin and appear to be mediated through a common metabolite. These effects are antagonized by glucagon and polyunsaturated fatty acid [15,22]. A carbohydrate-response element (ChoRE) was identified within the L-III element [23–26]. Moreover, synergistic interaction between the L-II and L-III elements is critical for carbohydrate responsiveness as well as cell type-specific expression [6,24,26]. Transcription of the rat *S*₁₄ gene is also stimulated by carbohydrates through a ChoRE that is comprised of sequences

Abbreviations used: CAT, chloramphenicol acetyltransferase; ChoRE, carbohydrate-response element; EMSA, electrophoretic mobility-shift assay; HNF, hepatocyte nuclear factor; L-II/IBP, L-II-binding protein; NF, nuclear factor; PK, pyruvate kinase; USF, upstream stimulating factor.

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very similar to the L-III element [27,28]. A full response of this gene to glucose requires both a ChoRE and an adjacent accessory site, which has a sequence reminiscent of the L-II element [29]. Interestingly, an accessory factor interacts with the accessory site of the S_{14} gene and also binds to the L-II element [29]. In addition, the L-II element is reported to be responsive to polyunsaturated fatty acids [30]. Thus the identification and characterization of the L-II- and L-III-binding proteins is very important for the elucidation of the molecular mechanisms underlying the transcriptional activity of the PKL gene as well as regulation by carbohydrates and polyunsaturated fatty acids.

As a first step, we report the purification of a heat-stable L-II-binding protein (L-IIBP). Determination of its partial amino acid sequences reveals identity with NF1/Red1 and NF1-L, members of the nuclear factor (NF) 1 family. These transcription factors and HNF4 bind specifically to overlapping sequences of the L-II element and regulate transcription of the PKL gene in an opposing manner. In addition, we show that the NF1 family also binds to the accessory site of the S_{14} gene.

MATERIALS AND METHODS

Materials

[γ - 32 P]ATP (3000 Ci/mmol) was purchased from Du Pont/New England Nuclear, streptavidin-agarose from Gibco-BRL, poly(dA-dT) from Boehringer-Mannheim and biotinamidite from Applied Biosystems. A molecular-mass marker and a two-dimensional silver stain kit were obtained from Daiichi Pure Chemicals. The rainbow marker and 1-deoxy-[*dichloroacetyl*- 14 C]chloramphenicol (60 mCi/mmol) were from Amersham Life Science. Restriction endonucleases and other DNA-modifying enzymes were obtained from Takara, Toyobo and New England Biolabs. DEAE-Sepharose Fast Flow, heparin-Sepharose CL-6B, HiLoad Sephacryl S-100HR and glutathione-Sepharose were products of Pharmacia-LKB Biotechnology. *Achromobacter* protease I, fetal calf serum and Pica Gene, a luciferase assay kit, were purchased from Wako Pure Chemicals, Irvine Science Japan and Toyo Ink respectively.

Oligonucleotides

Oligonucleotides used in this study are listed in Table 1. They were synthesized with a 380A DNA synthesizer (Applied Biosystems). These oligonucleotides were annealed with their complementary oligonucleotides to produce double-stranded DNA as described previously [6]. For affinity chromatography, the antisense strand of the L-II oligonucleotide was 5'-biotinylated using a biotinamidite reagent in the same synthesizer.

Table 1 Oligonucleotide sequences used in this study

Bases mutated are indicated by underlining.

Element	Sequence	Position
L-II	GATCTTCCTGGACTCTGGCCCCAGTGT	— 149 to — 126 rat PKL gene
mL-IIA	GATCTTCCT <u>TT</u> ACTCTGGCCCCAGTGT	
mL-IIC	GATCTTCCTGGACTCTGTTTGCCAGTGT	
NF1-Ade	GATCTTTTGGCTTGAAGCCAATATGAG	+ 22 to + 44 adenovirus origin of replication
AR2	GATCCCGAGCCAGTGAGCTTAGCCC	— 131 to — 108 human α_1 -antitrypsin gene
S14-ac	GATCGCAAGGGAACACTCTGTTTGCCAGTCT	— 1467 to — 1440 rat S_{14} gene

Isolation of nuclei and preparation of nuclear extract from rat liver

Liver nuclei were prepared from 6-week-old male Sprague-Dawley rats by the method of Hagenbuehle and Wellauer [31] with minor modifications. All operations were carried out at 4 °C. Livers were homogenized in a glass Dounce homogenizer in 5 vol. of buffer A (15 mM Hepes/KOH, pH 7.8, 60 mM KCl, 15 mM NaCl, 14 mM 2-mercaptoethanol, 0.15 mM spermine, 0.5 mM spermidine, 1 mM PMSF and 0.2 % Nonidet P40) containing 0.3 M sucrose. The homogenate was layered on top of a cushion of 0.9 M sucrose in buffer A and centrifuged at 2500 *g* for 10 min at 4 °C. The precipitated crude nuclei were resuspended in buffer A containing 0.9 M sucrose and re-centrifuged. The pellet was resuspended in buffer A and centrifuged at 2500 *g* for 10 min at 4 °C.

Preparation of nuclear extract from pure nuclei was performed by the method of Frain et al. [32] with minor modifications. Briefly, pure nuclei were resuspended in 5 vol. of buffer B (20 mM Hepes/NaOH, pH 7.9, 420 mM NaCl, 1.5 mM MgCl₂, 0.1 mM EDTA, 1 mM dithiothreitol, 1 mM PMSF and 10 % glycerol) using a Dounce homogenizer, gently stirred with a magnetic stirrer on ice for 45 min, and then centrifuged at 27000 *g* for 15 min. A 0.01 vol. of 10 % Nonidet P40 was added to the supernatant, gently stirred on ice for 10 min and centrifuged at 27000 *g* for 5 min. The clear supernatant was dialysed twice for 2 h against 50 vol. of buffer C (25 mM Tris/HCl, pH 8.0, 1 mM EDTA, 5 mM MgCl₂, 0.1 % Nonidet P40, 1 mM dithiothreitol and 10 % glycerol) containing 0.1 M KCl. The dialysate was centrifuged at 27000 *g* for 10 min at 4 °C to remove the insoluble materials. The protein concentration of the supernatant, designated crude nuclear extract, was determined using a protein assay (Bio-Rad Laboratories).

Purification of heat-stable L-IIBP

All operations were carried out at 4 °C. L-IIBP activity was monitored by EMSA (see below) with a 32 P-end-labelled L-II oligonucleotide. Crude nuclear extract was prepared from 312 rat livers and applied to a DEAE-Sepharose Fast Flow column equilibrated with buffer C containing 0.1 M KCl. The column was washed with buffer C containing 0.3 M KCl to remove the contaminated DNA. The flow-through fractions were pooled, and 0.33 g/ml of (NH₄)₂SO₄ was added and the mixture was stirred for 30 min on ice. After centrifugation at 10000 *g* for 10 min at 4 °C, the precipitate was resuspended in buffer C containing 0.1 M KCl and dialysed against the same buffer. The dialysate was then applied to a heparin-Sepharose CL-6B column equilibrated with buffer C containing 0.1 M KCl, washed with 3 bed volumes of the same buffer and eluted in a stepwise fashion from 0.3 to 0.6 M KCl in buffer C. The active fractions eluted with 0.6 M KCl in buffer C were pooled, precipitated with (NH₄)₂SO₄ and dialysed against 0.2 M KCl in buffer C. The dialysate was subjected to HiLoad Sephacryl S-100HR column chromatography. The active fractions were pooled, precipitated with (NH₄)₂SO₄ and dialysed against 0.1 M KCl in buffer C. The dialysate was then incubated at 60 °C for 20 min and the denatured proteins were removed by centrifugation.

Finally, L-IIBP was purified by L-II oligonucleotide-specific affinity chromatography by the method of Hagenbuehle and Wellauer [31] with minor modifications. The heated fraction was added to 2.67 μ g/ml poly(dA-dT) and stood at room temperature for 15 min. Then it was mixed with 133 μ g of biotinylated double-stranded L-II oligonucleotide at room temperature for 30 min. After the binding reaction, streptavidin-agarose (20 μ l/ μ g of DNA) was added. A streptavidin-biotin coupling

reaction was carried out at room temperature for 30 min with gentle rotating. The mixture was centrifuged at 2000 *g* for 10 s, washed thoroughly with buffer C containing 0.1 M KCl and eluted with 3 vol. of buffer C containing 1 M KCl. The eluate was diluted to 0.1 M KCl in buffer C, and 86.4 μ g of biotinylated double-stranded L-II oligonucleotide was added; the eluate was then subjected to a second DNA-affinity chromatography. The active fractions were pooled and subjected to SDS/PAGE on a 10% or a 10/20% gradient gel. Separated proteins were visualized by silver stain.

Peptide sequencing of heat-stable L-IIBP

Purified protein was precipitated with 10% trichloroacetic acid, resuspended in 0.5 M Tris/HCl, pH 8.0, containing 4 M urea and digested with 0.2 μ g of *Achromobacter* protease I at 37 °C for 16 h. The reaction mixture was subjected to reverse-phase HPLC using a C₁₈ column. Peptide peaks were recovered and subjected to peptide sequence analysis (Applied Biosystems; 473A).

EMSA

The indicated amount of nuclear extract was incubated for 30 min with a ³²P-labelled oligonucleotide (0.1 ng) and 1 μ g of poly(dA-dT) in buffer C. In the case of the affinity-purified fraction, the reaction was carried out under the same conditions except for the presence of 10 μ g of BSA and the absence of poly(dA-dT). Competition experiments included a 100- or a 200-fold molar excess of unlabelled competitor DNAs. For the heat-stability experiment, nuclear extract was heated at 60 °C for 5 min before being added to the binding reaction. Supershift assays were carried out by preincubating the nuclear extract with 1 μ l of anti-NF1 serum (partially purified on a Protein A-Sepharose column), anti-HNF4 serum or a preimmune serum for 30 min at room temperature before the addition of the probe. The anti-NF1 serum, which recognizes the DNA-binding domain of NF1 family proteins, was a gift from Dr. Naoko Tanese (New York University, New York, NY, U.S.A.). After the binding reaction, the mixture was subjected to PAGE (6% gel) in 45 mM Tris/45 mM boric acid/1 mM EDTA at 200 V for 60 min, and the gel was dried and exposed to Kodak XAR film.

Plasmids

Reporter plasmids pLcat62' and p(L-III-L-II-L-I)Lcat62' were described previously [6]. An *EcoRI*–*Bam*HI fragment containing the coding region of rat HNF4 was isolated from pBS-HNF4CL4 [33], which was kindly provided by Dr. Takashi Osumi (Himeji Institute of Technology, Hyogo, Japan). This fragment was blunt-ended with Klenow fragment and inserted into the *Hinc*II site of pUC18. A *Hind*III–*Bam*HI fragment from this plasmid was inserted into the *Hind*III–*Bam*HI sites of pGM4, which contained the Rous sarcoma virus long terminal repeat as a promoter and the simian virus 40 polyadenylation site, to produce expression plasmid pRSV-HNF4. Expression plasmid pRSV-NFL21 was constructed by insertion of the rat NF1-L cDNA into pGM4. The plasmids pGM4 and pRSV-NFL21 were kindly provided by Dr. Paolo Monaci, Dr. Alfredo Nicosia and Dr. Riccardo Cortese (Istituto di Ricerche di Biologia Molecolare 'P. Angeletti', Rome, Italy) (P. Monaci, A. Nicosia and R. Cortese, unpublished work). To construct the expression plasmid pRSV-NF1/Red1, the entire coding sequence of hamster NF1/Red1 was amplified by PCR using pNF1/Red1 [34] (obtained from the American Type Culture Collection) as a template. The sense and antisense

primers contained *Hind*III and *Bam*HI sites at their 5' ends respectively. The resultant product was inserted into the *Hind*III–*Bam*HI sites of pGM4 after digestion with *Hind*III and *Bam*HI. Plasmid pactL, a luciferase reporter vector containing the β -actin enhancer/promoter, was used as an internal control to normalize for variations in transfection efficiency [35]. All plasmids were prepared by CsCl-density-gradient centrifugation to ensure that they had a closed circular form. The sequences of all of the constructed plasmids were confirmed by dideoxy sequencing.

Cell culture and DNA transfections

HepG2 cells, a human hepatoma cell line, were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37 °C in a 5% CO₂ incubator. Then 5 μ g of reporter CAT plasmid, 1 μ g of pactL and various amounts of pRSV-HNF4, pRSV-NF1/Red1 or pRSV-NFL21 were transfected into HepG2 cells by the calcium phosphate method [36]. The amount of total expression plasmid was adjusted by the addition of pGM4. The medium was changed 24 h after transfection.

Luciferase and CAT assays

The cells were harvested for determination of luciferase and CAT activities 48 h after transfection. Luciferase and CAT assays were carried out as described previously [35]. The amounts of acetylated and unacetylated [¹⁴C]chloramphenicol were determined by a Fujix bio-image analyser BAS2000 (Fuji Photo Film Co. Ltd) to determine the percentage conversion of the acetylated form. All transfections were performed at least three times.

Production of anti-HNF4 serum

The plasmid pGST-HNF4 that contains the rat HNF4 cDNA [37] was kindly provided by Dr. Frances M. Sladek (University of California, Riverside, CA, U.S.A.) and used to transform the *Escherichia coli* BL21(DE3) strain. Expression of the GST-HNF4 fusion protein was induced by 1 mM isopropyl thio- β -D-galactoside for 4 h and the fusion protein was purified from extracts of *E. coli* using glutathione-Sepharose beads. The relative purity of the protein was analysed by SDS/PAGE followed by staining with Coomassie Brilliant Blue. The purified protein was used to raise antiserum in New Zealand White rabbits.

RESULTS

Multiple proteins bind to the L-II element

The L-II oligonucleotide probe produced at least three protein–DNA complexes with rat liver nuclear extract in an EMSA using a 6% polyacrylamide (acrylamide/bisacrylamide = 19:1) gel, suggesting that the L-II region is recognized by multiple proteins (Figure 1A). In our previous study, in which a 4% polyacrylamide (acrylamide/bisacrylamide = 15:0.4) gel was used [6], these bands were indistinguishable. We tested whether the proteins that bind to the L-II oligonucleotide could be distinguished by their sensitivity to heat-treatment. A slowly migrating band disappeared after heating the rat liver extract at 60 °C for 5 min, whereas two fast migrating bands were resistant to this treatment (Figure 1A) and were detectable even after incubation at 90 °C for 5 min (results not shown). Together, these results indicate that there are at least two factors bound to the L-II oligonucleotide. Since HNF4 has

Figure 1 EMSA of L-IIBP and HNF4

End-labelled L-II (A) or AR2 (B) oligonucleotides were incubated with 5 or 10 μ g of rat liver nuclear extract respectively. In the case of AR2 oligonucleotide, the mixture contained a 200-fold molar excess of the competitor DNAs shown at the top. The heat-treated nuclear extract was incubated at 60 °C for 5 min. Preimmune serum (Preim) or an antiserum directed against HNF4 or NF1 was added to the binding reaction as indicated. The arrows on the left and right indicate the positions of the protein–L-II or protein–AR2 complexes and supershifted complexes with the antibodies respectively.

been reported to interact with the L-II element [25,26], we performed a supershift experiment with anti-HNF4 serum (Figure 1A). Incubation of liver nuclear extract with the anti-HNF4 serum resulted in a supershifted band and a decreased abundance of the slowly migrating band. However, no supershifted complex was formed with a preimmune serum or when nuclear extract was heated at 60 °C for 5 min. To confirm this result, the AR2 oligonucleotide from the human α_1 -antitrypsin gene was used as a probe in an EMSA. This oligonucleotide contains a well-characterized HNF4-binding site [38]. As shown in Figure 1(B), a single protein–DNA complex was formed after incubation with liver nuclear extract, but not with the extract that was heated at 60 °C for 5 min. The formation of this complex was prevented by the addition of a 200-fold molar excess of unlabelled L-II as well as AR2, but not by unrelated oligonucleotides such as NF1-Ade and S14-ac. Incubation with the anti-HNF4 serum produced a supershifted band. When the labelled L-II oligonucleotide was incubated with the unheated extract, only the slowly migrating band was competed for by the

Figure 2 Competition analysis of L-IIBP by EMSA

An end-labelled L-II oligonucleotide was incubated with 5 μ g of rat liver nuclear extract with (B) or without (A) heat-treatment at 60 °C for 5 min. The competitor DNAs shown at the top were used at the indicated fold molar excess. The arrows on the left indicate the positions of the protein–L-II complexes.

addition of unlabelled AR2 oligonucleotide (Figure 2A). Thus we conclude that the heat-labile slowly migrating protein–DNA complex contains HNF4.

The heat-stable L-IIBP belongs to the NF1 protein family

We purified the heat-stable L-IIBP from 1740 mg of crude nuclear extract which was prepared from 312 male Sprague–Dawley rat livers (see the Materials and methods section). Purified heat-stable L-IIBP was subjected to electrophoresis on an SDS/10% polyacrylamide gel and was visualized as two bands migrating at about 33 kDa by silver staining (Figure 3A). However, since proteins smaller than 30 kDa were not detectable on this gel, we re-evaluated the purity of the preparation using a 10/20% gradient gel (Figure 3B). No contaminating protein below 30 kDa was found. We estimated from Figure 3(A) that the total amount of each protein purified was about 20 μ g. In accordance with the protein-staining pattern, the L-II-binding activity of the purified heat-stable protein was resolved as two bands using the EMSA (Figure 3C). These bands were competed for by a 200-fold molar excess of the unlabelled L-II oligonucleotide.

In order to determine amino acid sequences of the heat-stable

Figure 3 Analysis of the purified heat-stable L-IIBP

Purified heat-stable L-IIBP was separated by SDS/PAGE on a 10% (A) or 10/20% gradient (B) gel and the gel was developed with silver stain. M indicates molecular-mass markers. The arrows indicate the positions of purified L-IIBP. (C) The purified protein was analysed by EMSA using the L-II oligonucleotide as a probe in the presence or absence of a 200-fold molar excess of unlabelled L-II. The arrows indicate the positions of the L-IIBP complexes.

L-IIBP, the purified protein was precipitated with trichloroacetic acid and digested with lysyl endopeptidase, which cleaves the peptide bond on the C-terminal side of lysine residues. The resulting peptides were separated by reverse-phase HPLC and seven peptides were sequenced. The amino acid sequences of six peptides were very similar to those of NF1 family member proteins and the remaining peptide (peptide 7) was unknown (Table 2). Four NF1 family proteins have been identified in mammals, including CTF/NF1, NF1-L, NF1/Red1 and NF1/X [34,39,40]. The amino acid sequences derived from peptides 1, 2 and 3 were identical with conserved regions of all of the characterized NF1 proteins. Peptides 4 and 5 contained sequences

identical with rat NF1-L. The sequence of peptide 6 was identical with that of hamster NF1/Red1 in spite of the species difference. In particular, the first amino acid of NF1/Red1 is lysine but that of the other family member proteins is threonine. Thus our results indicate that the purified heat-stable L-IIBP is composed of rat NF1-L and the rat counterpart of hamster NF1/Red1.

NF1 family members bind to the L-II element of the PKL gene *in vitro*

We carried out EMSAs using various competitor DNAs and an anti-NF1 serum to verify that NF1 family members bind to the L-II oligonucleotide *in vitro*. When the labelled L-II probe was incubated with rat liver nuclear extract, we detected multiple bands as described above. Although all these bands were competed for by an unlabelled L-II oligonucleotide, only the two fastest migrating bands disappeared in the presence of an excess of an unlabelled NF1-Ade oligonucleotide containing the NF1-binding site of the adenovirus major late promoter gene (Figure 2A). However, these bands were not competed for by an unlabelled AR2 oligonucleotide. When heated nuclear extract was used, two protein–DNA complexes were formed, which were also competed for by the addition of unlabelled L-II and NF1-Ade oligonucleotides, but not by an unlabelled AR2 oligonucleotide (Figure 2B). Incubation of unheated extract with an anti-NF1 serum, but not preimmune serum, produced a supershifted band with concomitant decreases in the abundance of the two fastest migrating bands (Figure 1A). Similar results were obtained with heated nuclear extract. The two protein–DNA complexes were also observed when the labelled NF1-Ade probe was incubated with heated as well as unheated nuclear extracts (Figure 4). The formation of these complexes was competed for by the addition of an excess of an unlabelled NF1-Ade oligonucleotide but not other oligonucleotides, including L-II. A supershifted band was detected by incubation with anti-NF1 serum but not preimmune serum or anti-HNF4 serum. These results indicate that the two heat-stable protein–DNA complexes that interact with the L-II oligonucleotide contain NF1 family proteins and that the binding affinity between NF1 family proteins and the L-II oligonucleotide is much weaker than that between NF1 family proteins and the NF1-Ade oligonucleotide.

Table 2 Peptide sequences from rat heat-stable L-IIBP and comparison with NF1 family members

Undetermined and critical amino acid residues are indicated by x and asterisk respectively. Amino acid sequences for hamster NF1/Red1 and NF1/X, rat NF1-L and human CTF/NF1 are cited in references [34,39,40].

Peptide 1	KYFK	Peptide 2	KRMSK
Hamster NF1/Red1	KYFK	NF1/Red1	KRMSK
Hamster NF1/X	KYFK	NF1/X	KRMSK
Rat NF1-L	KYFK	NF1-L	KRMSK
Human CTF/NF1	KYFK	CTF/NF1	KRMSK
Peptide 3	KxASRLLAK	Peptide 4	KGIPLExxxGERLV*
NF1/Red1	KWASRLLAK	NF1/Red1	KGIPLESTDGERLM
NF1/X	KWASRLLAK	NF1/X	KGIPLESTDGERLY
NF1-L	KWASRLLAK	NF1-L	KGIPLESTDGERLV
CTF/NF1	KWASRLLAK	CTF/NF1	KGIPLESTDGERLV
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Peptide 5	KSPQxxxPxLxVQPHxI	Peptide 6	KxGVFNVxELVRVxR
NF1/Red1	KSPHCTNPALCVQPHHI	NF1/Red1	KSGVFNVSSELVRVSR
NF1/X	KSPQCSNPGLCVQPHHI	NF1/X	TSGVNVVTELVRVVSQ
NF1-L	KSPQCSNPGLCVQPHHI	NF1-L	TSGVFSVTELVRVVSQ
CTF/NF1	KAAQCGHPVLCVQPHHI	CTF/NF1	TSGVFSVTELIQVSR
Peptide 7	(K)DIPMExxK

Figure 4 Characterization of NF1 binding by EMSA

An end-labelled NF1-Ade oligonucleotide was incubated with 10 μ g of liver nuclear extract in the presence or absence of a 200-fold molar excess of the competitor DNAs shown at the top. The heat-treated nuclear extract was incubated at 60 °C for 5 min. Preimmune (Preim) or an antiserum directed against HNF4 or NF1 was added to the binding reaction mixture as indicated. The arrows on the left and right indicate the positions of the NF1 complexes and the supershifted complexes formed with anti-NF1 serum respectively.

HNF4 and NF1 family proteins bind to overlapping sequences of the L-II element

It is known that HNF4 interacts with the consensus sequence 5'-TGACCTNTGACCT-3', whereas NF1 family members bind to sequences containing 5'-TGGN₇CCA-3' [34,37,39–41]. The latter also binds to the half-site sequence 5'-TGGCA-3' which contains only one copy of TGG [34]. The L-II region contains a putative

degenerate binding site for both proteins at the same position, 5'-TGGACTCTGGCCC-3'. To examine the possibility that both HNF4 and NF1 family members bind to the same or overlapping sites, we used oligonucleotides mutated at each half-site as competitors in an EMSA. Addition of an excess of an unlabelled L-II oligonucleotide mutated at the 5' site (mL-IIA) did not affect the formation of NF1–L-II complexes (Figure 2). However, an L-II oligonucleotide that was mutated at the 3' site (mL-IIC) competed for the formation of these complexes, although this competition was not as efficient as the wild-type L-II oligonucleotide. Similar results were obtained using the S14-ac oligonucleotide as the probe (Figure 5A), which interacts with NF1 family members (see below).

In contrast, both mutated L-II oligonucleotides competed for HNF4 binding to the probe but to a lesser extent than the wild-type L-II (Figure 2A). The mL-IIA oligonucleotide was a stronger competitor than the mL-IIC oligonucleotide for HNF4 binding. Similar results were obtained using the AR2 oligonucleotide as the probe (Figure 1B). Thus both half-sites of the L-II region are required for high-affinity binding of either NF1 family members or HNF4. The 5' site is more critical than the 3' site for NF1 binding, whereas the 3' site is more important for HNF4 binding.

A factor binding to the accessory site of the rat S₁₄ gene is also a member of the NF1 family

Recently, Shih et al. [29] reported that the L-II-like element neighbouring the ChoRE of the rat S₁₄ gene functioned as an accessory element necessary for glucose-stimulated transcription [29]. They also showed that a novel protein binds to this element as well as to the L-II element of the PKL gene [29]. The sequence of the S₁₄ accessory element is similar to L-II. So, to examine whether NF1 family proteins also bind to the accessory site of the S₁₄ gene, we performed an EMSA using an S14-ac oligonucleotide containing this site as a probe. When the labelled S14-ac oligonucleotide was incubated with rat liver nuclear

Figure 5 Identification of a protein that binds to an accessory factor element of the rat S₁₄ gene

EMSA was carried out with heated or unheated liver nuclear extract and an end-labelled S14-ac oligonucleotide probe. **(A)** The probe was incubated with 5 μ g of the extract in the presence or absence of the indicated molar excess of the competitor DNAs shown at the top. **(B)** Preimmune (Preim) serum or an antiserum directed against HNF4 or NF1 was added to the binding reaction mixture as shown. The arrows on the left and right indicate the positions of the NF1 complexes and the supershifted complexes formed with anti-NF1 serum respectively.

Table 3 NF1/Red1 antagonism of the effect of HNF4 on the PKL gene

The indicated amount of the expression plasmids were transfected into HepG2 cells with pcatL and the reporter plasmid p(L-III-L-II-L-I)Cat62'. The indicated relative CAT activity was normalized with respect to the activity of the luciferase reporter plasmid pcatL. Data are expressed as the percentage of the acetylated form of [¹⁴C]chloramphenicol relative to the value from cells transfected with no expression plasmids (A) or 1 µg of pRSV-HNF4 (B) and are the means ± S.E.M. for three to five experiments.

Plasmid (µg)		Relative CAT activity
A	pRSV-NF1/Red1	
	0	100
	0.01	85 ± 7
	0.1	64 ± 10
	0.5	38 ± 6
	1.0	39 ± 5
B	pRSV-HNF4	pRSV-NF1/Red1
	0	0
	1.0	0
	1.0	0.1
	1.0	0.5
	1.0	1.0
	1.0	2.0

extract, two protein–DNA complexes were detected (Figure 5A). The formation of these complexes was not affected by prior exposure of the nuclear extracts to 60 °C. Formation of these complexes was competed for by unlabelled S14-ac, L-II and NF1-Ade but not by AR2 or mL-IIA oligonucleotides. The mL-IIC oligonucleotide also competed for the formation of these complexes but to a lesser extent. Conversely, the S14-ac oligonucleotide competed for the binding of NF1 family proteins to a labelled L-II oligonucleotide but could not compete for the binding of HNF4 to the AR2 and L-II oligonucleotides (Figures 1B and 2). Incubation with anti-NF1 serum, but not preimmune serum or anti-HNF4 serum, produced a supershifted band concomitant with decreases in the abundance of the original two bands (Figure 5B). These results indicate that the S14 accessory factor of the rat *S₁₄* gene is a member of the NF1 family.

HNF4 and NF1 proteins contribute to the regulation of the transcription of the PKL gene in an opposing manner

To examine the role of NF1 family members in the transcription of the PKL gene, we carried out co-transfection experiments using p(L-III-L-II-L-I)Cat62' as a reporter. This plasmid contains the three *cis*-acting elements of the PKL gene ligated to the CAT reporter gene and has been shown to regulate reporter genes in a manner similar to the intact promoter [6]. We also used the following constructs as effector plasmids: pGM4, which is the parent expression vector; pRSV-NF1/Red1, which expresses the hamster NF1/Red1 protein; pRSV-NFL21, which expresses the rat NF1-L protein; pRSV-HNF4, which expresses the rat HNF4 protein. When the reporter plasmid was co-transfected into HepG2 cells with increasing amounts of pRSV-NF1/Red1, CAT expression was repressed in a concentration-dependent manner (Table 3). Maximal inhibition (about 62%) was obtained with 0.5 µg of pRSV-NF1/Red1. Co-transfection of the HNF4 expression plasmid (pRSV-HNF4) into HepG2 cells resulted in 5.2-fold stimulation of the reporter gene expression (Table 3). This stimulation was inhibited in a concentration-dependent manner by co-transfection with increasing amounts of pRSV-NF1/Red1. A similar inhibition was observed when pRSV-NF1/Red1 was replaced with pRSV-NFL21 in the same

experiment (results not shown). In contrast, when pCat 62', which lacks the three *cis* elements, was used as a reporter, no CAT expression was detected after co-transfection with pRSV-HNF4, pRSV-NF1/Red1 or pRSV-NFL21 (results not shown). These results, together with those described above, indicate that HNF4 and the NF1 family are involved in the transcriptional regulation of the PKL gene through the L-II element in an opposing manner.

DISCUSSION

In this study we analysed proteins bound to the L-II element of the rat PKL gene and found that at least two factors with distinct heat stabilities interact with this element. The heat-stable L-IIBP was found to be comprised of the NF1 family proteins, NF1-L and NF1/Red1, by purification and determination of its partial amino acid sequence. The heat-labile factor was determined to be HNF4 protein, which has previously been shown to bind to this element [12,25,26]. We also demonstrated that HNF4 and NF1 family proteins bind to overlapping sequences of the L-II element and regulate PKL gene transcription in an opposing manner.

The NF1 family has four genetic loci designated NF1/Red1, NF1/X, NF1-L and CTF/NF1 [34,39,40]. These NF1 proteins show nearly complete sequence identity in the N-terminal region from residues 1 to 190 and diversity in their C-terminal portions. The N-terminal portions exhibit DNA-binding, dimerization and DNA-replication activities [41,42]. NF1 family members can function as transactivators or transrepressors. These functions are dependent on the context of the regulatory elements in the promoter [43]. On the other hand, the C-terminal portions are responsible for transactivation activity and interaction with other proteins [44–46]. NF1-L purified from rat liver has a molecular mass of 30 kDa, and NF1-L and NF1/Red1 from hamster liver are purified as 35 and 33 kDa forms respectively [34,39]. Since the molecular masses of NF1-L and NF1/Red1 calculated from their cDNAs are much larger than these values, and since all of the peptides isolated from purified proteins correspond to the N-terminal portions, it has been suggested that purified proteins are degradation products corresponding to the N-terminal halves of NF1 family proteins [34,39]. In agreement with these observations, six of seven peptides derived from a purified L-IIBP preparation, which showed two protein bands migrating at about 33 kDa on an SDS/polyacrylamide gel, were identified as portions of the N-terminal halves of NF1-L and NF1/Red1. Since peptide 7 did not correspond to any transcription factor, including NF1 family members or HNF4, this fragment might be the carboxyl region of a unique NF1 protein. Another possibility that we cannot exclude is that peptide 7 is from a contaminating protein. We suggest that the two bands from the purified L-IIBP correspond to NF1-L and NF1/Red1, although we did not sequence the two purified proteins separately.

We confirmed by EMSA that NF1 family members as well as HNF4 bind to the L-II oligonucleotide. Purified heat-stable nuclear extract produced two bands on incubation with a labelled L-II probe. These two bands containing NF1 proteins were also detected after incubation with crude liver nuclear extract with labelled L-II, NF1-Ade or S14-ac oligonucleotides as shown by supershift and competition experiments. Therefore the two bands were probably formed with NF1-L and NF1/Red1, which had been cleaved proteolytically during preparation of the extract. The L-II region contains putative binding sequences (TGGACTCTGGCCC) for both NF1 family members and HNF4 at the same position. This was examined using mutated oligonucleotides as competitors for EMSA. Mutations within either half-site resulted in decreased competition for NF1 as well

as HNF4 binding to probes, indicating that HNF4 and NF1 family members bind to overlapping sequences of the L-II element. However, a mutation in the 5' half-site influenced NF1 binding more strongly than one in the 3' site, whereas HNF4 binding was preferentially affected by the 3' mutation. This raises the question of whether NF1 family members and HNF4 can form a heterodimer on binding to the L-II element. It has been reported that NF1 forms a heterodimer as well as a homodimer, whereas HNF4 forms only a homodimer [47,48]. Although the NF1 proteins appear to be proteolytically cleaved during the preparation of liver extract, the remaining N-terminal portion is reported to contain a dimerization domain [41,42]. However, preincubation of liver nuclear extract with anti-HNF4 serum or anti-NF1 serum decreased only the abundance of the complexes formed with the respective proteins (Figure 1A). We conclude that HNF4 and NF1 family members do not form a heterodimer with each other on binding to the L-II element.

What is the physiological role of NF1 family members in the regulation of the PKL gene? While HNF4 stimulates PKL gene transcription, co-transfection experiments revealed that NF1 family members antagonized HNF4. Therefore the ratio of HNF4 to NF1 may be important for the transcriptional activity of the PKL gene. Tyrosine phosphorylation of HNF4 is important for its DNA-binding and transactivation activities [49], but phosphorylation of the NF1 family has not been described. Transcription of the PKL gene is repressed by glucagon via cAMP and polyunsaturated fatty acids. The L-III element is the regulatory element responsible for the cAMP effect [24], and the L-II element is responsive to polyunsaturated fatty acids [30]. Thus NF1 family members and/or HNF4 may be involved in the polyunsaturated fatty acid regulation of PKL gene transcription.

We found that a factor that binds to the accessory site of the S_{14} gene is a member of the NF1 family (Figure 5). The L-II element is necessary for the PKL gene to respond to carbohydrates through the L-III element. Similarly, the S_{14} gene requires an accessory site in order to respond to carbohydrates through the S_{14} ChoRE. It has been reported that the same member of the basic helix-loop-helix family protein binds to both L-III and ChoRE, and that HNF4 is involved in carbohydrate regulation by binding to the L-II element of the PKL gene [24,26]. However, the present study raises the possibility that NF1 proteins, rather than HNF4, are involved in the transcriptional regulation of the PKL and S_{14} genes by carbohydrates. As mentioned above, NF1 family members repress HNF4 activity, presumably by competing for binding to the L-II element. However, HepG2 cells may not contain the transcription factor that interacts with the L-III element involved in carbohydrate regulation, since PKL gene transcription in this cell line is not regulated by carbohydrates (K. Yamada, T. Tanaka and T. Noguchi, unpublished work). Therefore interactions between an unidentified L-III-binding protein and NF1 family proteins may cause stimulation of PKL gene transcription. In fact, interaction between CTF/NF1 and USF contributes to the transcriptional activation of the type-I plasminogen activator inhibitor gene by transforming growth factor- β [44]. It has also been shown that CTF/NF1 physically interacts with USF and binds to the transforming growth factor- β responsive unit of the collagen $\alpha 2(I)$ gene [45], and that CTF/NF1 family proteins bind to the vitamin D response element of the *c-fos* gene in conjunction with the nuclear hormone receptors, vitamin D receptor and retinoid X receptor α [46].

Kahn's group insists that USF is involved in the carbohydrate regulation of the PKL gene by interacting with the L-III element [50]. However, substitution of the L-III element with an upstream stimulatory element (USF-binding site) from the adenovirus

major late promoter results in a loss of carbohydrate responsiveness [25,50]. In addition, we found that an L-III-binding protein differed from USF in terms of heat stability and immunoreactivity (K. Yamada, T. Tanaka and T. Noguchi, unpublished work). Moreover, USF did not stimulate the basal level of transcription from a reporter plasmid p(L-III-L-II-L-I)cat62' in HeLa cells or HepG2 cells when co-transfected with or without pRSV-HNF4, pRSV-NF1/Red1 or pRSV-NFL21 (results not shown). Thus isolation of a non-USF L-III-binding protein is required to gain further understanding of the physiological role of NF1 family proteins in the regulation of the PKL gene transcription.

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REFERENCES

- 1 Tanaka, T., Harano, Y., Sue, F. and Morimura, H. (1967) *J. Biochem. (Tokyo)* **62**, 71–91
- 2 Imamura, K. and Tanaka, T. (1972) *J. Biochem. (Tokyo)* **71**, 1043–1051
- 3 Nakashima, K., Miwa, S., Oda, S., Tanaka, T., Imamura, K. and Nishina, T. (1974) *Blood* **43**, 537–548
- 4 Noguchi, T., Yamada, K., Yamagata, K., Takenaka, M., Nakajima, H., Imai, E., Wang, Z. and Tanaka, T. (1991) *Biochem. Biophys. Res. Commun.* **181**, 259–264
- 5 Noguchi, T., Yamada, K., Inoue, H., Matsuda, T. and Tanaka, T. (1987) *J. Biol. Chem.* **262**, 14366–14371
- 6 Yamada, K., Noguchi, T., Matsuda, T., Takenaka, M., Monaci, P., Nicosia, A. and Tanaka, T. (1990) *J. Biol. Chem.* **265**, 19885–19891
- 7 Tremp, G., Boquet, D., Ripoche, M.-A., Cognet, M., Lone, Y.-C., Jami, J., Kahn, A. and Daegelen, D. (1989) *J. Biol. Chem.* **264**, 19904–19910
- 8 Yamada, K., Noguchi, T., Miyazaki, J., Matsuda, T., Takenaka, M., Yamamura, K. and Tanaka, T. (1990) *Biochem. Biophys. Res. Commun.* **171**, 243–249
- 9 Noguchi, T., Okabe, M., Wang, Z., Yamada, K., Imai, E. and Tanaka, T. (1993) *FEBS Lett.* **318**, 269–272
- 10 Cuif, M.-H., Cognet, M., Boquet, D., Tremp, G. L., Kahn, A. and Vaulont, S. (1992) *Mol. Cell. Biol.* **12**, 4852–4861
- 11 Cuif, M.-H., Porteu, A., Kahn, A. and Vaulont, S. (1993) *J. Biol. Chem.* **268**, 13769–13772
- 12 Vaulont, S., Puzeat, N., Levrat, F., Cognet, M., Kahn, A. and Raymondjean, M. (1989) *J. Mol. Biol.* **209**, 205–219
- 13 Noguchi, T., Inoue, H., Chen, H.-L., Matsubara, K. and Tanaka, T. (1983) *J. Biol. Chem.* **258**, 15220–15223
- 14 Inoue, H., Noguchi, T. and Tanaka, T. (1984) *J. Biochem. (Tokyo)* **96**, 1457–1462
- 15 Noguchi, T., Inoue, H. and Tanaka, T. (1985) *J. Biol. Chem.* **260**, 14393–14397
- 16 Vaulont, S., Munnich, A., Decaux, J.-F. and Kahn, A. (1986) *J. Biol. Chem.* **261**, 7621–7625
- 17 Munnich, A., Lyonnet, S., Chauvet, D., Van Schaftingen, E. and Kahn, A. (1987) *J. Biol. Chem.* **262**, 17065–17071
- 18 Ogier, H., Munnich, A., Lyonnet, S., Vaulont, S., Reach, G. and Kahn, A. (1987) *Eur. J. Biochem.* **166**, 365–370
- 19 Matsuda, T., Noguchi, T., Takenaka, M., Yamada, K. and Tanaka, T. (1990) *J. Biochem. (Tokyo)* **107**, 655–660
- 20 Matsuda, T., Noguchi, T., Yamada, K., Takenaka, M. and Tanaka, T. (1990) *J. Biochem. (Tokyo)* **108**, 778–784
- 21 Decaux, J.-F., Antoine, B. and Kahn, A. (1989) *J. Biol. Chem.* **264**, 11584–11590
- 22 Jump, D. B., Clarke, S. D., Thelen, A. and Liimatta, M. (1994) *J. Lipid Res.* **35**, 1076–1084
- 23 Thompson, K. S. and Towle, H. C. (1991) *J. Biol. Chem.* **266**, 8679–8682
- 24 Bergot, M.-O., Diaz-Guerra, M.-J., Puzeat, N., Raymondjean, M. and Kahn, A. (1992) *Nucleic Acids Res.* **20**, 1871–1878
- 25 Liu, Z., Thompson, K. S. and Towle, H. C. (1993) *J. Biol. Chem.* **268**, 12787–12795
- 26 Diaz-Guerra, M.-J., Bergot, M.-O., Martinez, A., Cuif, M.-H., Kahn, A. and Raymondjean, M. (1993) *J. Biol. Chem.* **268**, 7725–7733

-
- 27 Jump, D. B., Bell, A. and Santiago, V. (1990) *J. Biol. Chem.* **265**, 3474–3478
- 28 Shih, H. and Towle, H. C. (1992) *J. Biol. Chem.* **267**, 13222–13228
- 29 Shih, H., Liu, Z. and Towle, H. C. (1995) *J. Biol. Chem.* **270**, 21991–21997
- 30 Liimatta, M., Towle, H. C., Clarke, S. and Jump, D. B. (1994) *Mol. Endocrinol.* **8**, 1147–1153
- 31 Hagenbüchle, O. and Wellauer, P. K. (1992) *Nucleic Acids Res.* **20**, 3555–3559
- 32 Frain, M., Swart, G., Monaci, P., Nicosia, A., Stampfli, S., Frank, R. and Cortese, R. (1989) *Cell* **59**, 145–157
- 33 Hata, S., Tsukamoto, T. and Osumi, T. (1992) *Biochim. Biophys. Acta* **1131**, 211–213
- 34 Gil, G., Smith, J. R., Goldstein, J. L., Slaughter, C. A., Orth, K., Brown, M. S. and Osborne, T. F. (1988) *Proc. Natl. Acad. Sci. U.S.A.* **85**, 8963–8967
- 35 Wang, Z., Takenaka, M., Imai, E., Yamada, K., Tanaka, T. and Noguchi, T. (1994) *Eur. J. Biochem.* **220**, 301–307
- 36 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- 37 Sladek, F. M., Zhong, W., Lai, E. and Darnell, Jr., J. E. (1990) *Genes Dev.* **4**, 2353–2365
- 38 Hardon, E. M., Frain, M., Paonessa, G. and Cortese, R. (1988) *EMBO J.* **7**, 1711–1719
- 39 Paonessa, G., Gounari, F., Frank, R. and Cortese, R. (1988) *EMBO J.* **7**, 3115–3123
- 40 Santoro, C., Mermod, N., Andrews, P. C. and Tijan, R. (1988) *Nature (London)* **334**, 218–224
- 41 Nagata, K., Guggenheimer, R. A., Enoto, T., Lichy, J. H. and Hurwitz, J. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 6438–6442
- 42 Jones, K. A., Kadonaga, J. T., Rosenfeld, P. J., Kelly, T. J. and Tijan, R. (1987) *Cell* **48**, 79–89
- 43 Eskild, W., Simard, J., Hansson, V. and Guerin, S. L. (1994) *Mol. Endocrinol.* **8**, 732–745
- 44 Riccio, A., Pedone, P. V., Lund, L. R., Olsen, T., Olsen, H.-S. and Andreasen, P. A. (1992) *Mol. Cell. Biol.* **12**, 1846–1855
- 45 Alevizopoulos, A., Dusserre, Y., Tsai-Pflugfelder, M., von der Weid, T., Wahli, W. and Mermod, N. (1995) *Genes Dev.* **9**, 3051–3066
- 46 Candeliere, G. A., Jurutka, P. W., Haussler, M. R. and St-Arnaud, R. (1996) *Mol. Cell. Biol.* **16**, 584–592
- 47 Kruse, U. and Sippl, A. E. (1994) *FEBS Lett.* **348**, 46–50
- 48 Jiang, G., Nepomuceno, L., Hopkins, K. and Sladek, F. M. (1995) *Mol. Cell. Biol.* **15**, 5131–5143
- 49 Ktistaki, E., Ktistakis, N. T., Paradogeorgaki, E. and Talianidis, I. (1995) *Proc. Natl. Acad. Sci. U.S.A.* **92**, 9876–9880
- 50 Lefrançois-Martinez, A.-M., Martinez, A., Antoine, B., Raymondjean, M. and Kahn, A. (1995) *J. Biol. Chem.* **270**, 2640–2643
-