Effect of dietary protein restriction on liver transcription factors

Norman W. MARTEN*, Frances M. SLADEK† and Daniel S. STRAUS*‡

*Biomedical Sciences Division, Biology Department, and †Environmental Toxicology Graduate Program, University of California, Riverside, CA 92521-0121, U.S.A.

The transcription of several genes that are preferentially expressed in the liver, including the serum albumin, transthyretin and carbamyl phosphate synthetase-I genes, is specifically decreased in animals consuming inadequate amounts of dietary protein. The high level of transcription of these genes in the liver is directed in part by a number of liver-enriched transcription factors, including hepatocyte nuclear factors (HNF)-1, -3, and -4, and proteins of the CCAAT/enhancer-binding protein (C/EBP) family. In the present study, we investigated the possibility that the co-ordinate decrease in transcription of the nutritionally sensitive genes in protein-deprived rats results from altered activity of one or more of the liver-enriched transcription factors. For HNF-4, Western blots indicated no change in the level of nuclear HNF-4 protein in liver of protein-deprived animals, whereas we observed a 40% reduction in the DNA binding activity of HNF-4 as measured by electrophoretic mobility shift assay (EMSA). Furthermore, the binding affinity of HNF-4 for DNA was unaltered by dietary protein deprivation, while the number of HNF-4 molecules able to bind to DNA (B_{max}) was reduced, as determined by Scatchard analysis. This indicates that in the protein-restricted rats a portion of the pool of HNF-4 protein is inactivated or otherwise prevented from binding to DNA. The overall DNA binding activity of C/EBP α and β was increased in protein-restricted animals. This change

occurred in the absence of a change in the amount of the full-length forms of these two proteins, quantified by Western blotting. Interestingly, dietary protein restriction specifically increased the level of a truncated form of C/EBP β (liver-enriched transcriptional inhibitory protein, LIP), which is a potent dominant negative inhibitor of C/EBP function. Analysis of HNF-3 DNA-binding activity by EMSA revealed that HNF-3 α and β DNA binding was increased and that HNF-3 γ DNA-binding activity was unchanged in protein-restricted animals. We also detected two apparently novel shift complexes with the HNF-3 probe by EMSA, both of which were decreased in protein-restricted animals. HNF-1 DNA-binding activity was increased by dietary protein restriction. We also examined the effect of protein restriction on the DNA-binding activity of two ubiquitous transcription factors, NF1 and Sp1. The DNA binding activity of the major NF1 isoforms was unchanged whereas the binding activity of Sp1 was increased in the protein-restricted animals. In summary, restriction of dietary protein resulted in a number of specific changes in the DNA-binding activity of various transcription factors. Because transcriptional activation typically involves the synergistic action of more than one transcription factor, small changes in the amount/activity of several factors could have a strong net effect on the transcription of many genes.

INTRODUCTION

A hallmark characteristic of dietary protein deficiency in humans and other animals is a low concentration of serum albumin [1,2]. Circulating levels of other plasma proteins, including transthyretin (TTR), are also decreased under conditions of dietary protein deficiency [2]. Serum albumin and TTR belong to a group of genes that are transcribed highly in the liver and a limited number of other tissues [3–5]. We showed recently that the mRNA abundance for four such genes that are preferentially expressed in the liver, i.e. albumin, TTR, carbamyl phosphate synthetase I (CPS-I) and class-I alcohol dehydrogenase, was co-ordinately decreased in the liver of protein-restricted animals [6,7]. This decrease was specific, as the mRNA abundance for six other genes was either unchanged or increased. Furthermore, we have demonstrated a decrease in the transcription of three of these liver-specific genes, albumin, TTR and CPS-I, under conditions of dietary protein restriction [6,7].

As the response to low dietary protein appears to occur at the transcriptional level, we wished to determine whether this decrease in transcription was caused by a decrease in the activity of

one or more transcription factors. Transcription of the serum albumin and TTR genes, and other genes that are preferentially expressed in the liver, involves the binding of liver-enriched as well as ubiquitous transcription factors to promoter and enhancer elements. The liver-enriched transcription factors include hepatocyte nuclear factors (HNFs) -1, -3 and -4 as well as CCAAT/enhancer-binding protein (C/EBP) and related factors (for reviews on these factors, see ref. [3]). Whereas all of these transcription factors share a common property, in that they are abundant in the liver and a limited number of other tissue types [3], they differ from each other in their structure. HNF-1 α and HNF-1 β (vHNF1) belong to the POU homeodomain family of transcription factors [8,9]. The HNF-3 family has three members, α , β and γ , all of which bind as monomers and belong to the wing-helix motif group of transcription factors, which are homologous to the *Drosophila* forkhead protein [10]. HNF-4, which binds to DNA as a homodimer, is an orphan receptor that belongs to the nuclear receptor superfamily [11]. C/EBP α and C/EBP β [liver-enriched transcriptional activator proteins (LAPs)] are members of the basic leucine zipper (bZIP) family of transcription factors [12,13]. Both C/EBP proteins have trun-

Abbreviations used: EMSA, electrophoretic mobility shift assay; HNF, hepatocyte nuclear factor; C/EBP, CCAAT/enhancer-binding protein; LAP, liver-enriched transcriptional activator protein; LIP, liver-enriched transcriptional inhibitory protein; NF1, nuclear factor 1; TTR, transthyretin; apoCIII, apolipoprotein CIII; DTT, dithiothreitol; CPS-I, carbamyl phosphate synthetase I.

‡ To whom correspondence should be addressed.

cated forms which lack the activating portion of the protein but which can still compete with the functional full-length translational products for DNA-binding sites [14,15]. C/EBP α and β can form homodimers, heterodimers with each other, heterodimers with their truncated isoforms, and heterodimers with other factors including members of the AP-1 (Fos, Jun) family [12–16].

An attractive hypothesis to explain the co-ordinate decrease in expression of several liver-specific genes in protein-restricted animals is that this decrease results from reduced activity of one or more of the liver-enriched transcription factors. To test this hypothesis, we examined the effect of dietary protein restriction on the abundance and DNA-binding activity of several liver-enriched transcription factors. We also examined two ubiquitously expressed transcription factors, Sp1 and nuclear factor 1 (NF1). Sp1 was chosen as a control because it is involved in the expression of housekeeping genes that are not regulated by dietary protein restriction [6,17]. NF1 appears to activate the expression of some genes and repress the expression of others. Although expressed ubiquitously, NF1 has been shown to bind to the albumin promoter and enhancer [4,18] and the TTR promoter [19]. Thus, NF1 may play a role in regulating the expression of these two genes, as well as other genes that are preferentially expressed in the liver [20,21].

MATERIALS AND METHODS

Animals and diets

Male Sprague–Dawley rats (4 weeks old) were obtained from Simonsen (Gilroy, CA, U.S.A.). Animals were housed individually, maintained on a 12 h light:12 h dark cycle, and fed *ad libitum* over the course of the experiments. The experimental diets consisted of isocaloric diets containing 20% casein protein (control) or 4% casein protein (protein-restricted) [6,7]. In these diets, starch and fat are held constant, and the energy content is held constant in the low protein diet by increasing the amount of sucrose. All rats were acclimated to the 20% protein diet for 4 days before the start of the experiment. On day 0 of the experiment, animals were randomly assigned to two groups ($n =$ six animals/group), which were then fed the 20% protein diet or 4% protein diet *ad libitum* for a period of 10 days. Details concerning the effect of the diets on growth and food consumption have been presented earlier [6,7]. On day 10, animals were decapitated, and livers were quickly removed, placed on ice, minced, quick-frozen in liquid nitrogen, and stored at -70°C . All protocols for animal research were approved in advance by the University of California, Riverside, Committee on Animal Care.

RNA isolation and Northern blot analysis

Total RNA was prepared from rat livers using the acid–guanidine–thiocyanate method [6]. Northern blot analysis was performed as described previously [6]. Uniform loading of gels was verified by ethidium bromide staining of ribosomal RNA. Blots were probed with a rat HNF-1 α cDNA clone (obtained from Dr. Gerald Crabtree (Stanford University School of Medicine, Stanford, CA, U.S.A.) [8]) or a rat HNF-4 cDNA clone [11].

Preparation of nuclear protein extracts and electrophoretic mobility shift assays (EMSAs)

Nuclear protein extracts were prepared from rat liver as described previously [11,22], with minor modifications as follows. Samples (3 g) of liver were pulverized in liquid nitrogen using a mortar

and pestle. The powdered liver was mixed with 2 ml of Buffer A [10 mM Hepes, pH 7.9, 25 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1.0 mM EGTA, 1.0 mM EDTA, 1 mM dithiothreitol (DTT), 0.5 mM PMSF, 1 mM benzamidine, 0.5 mM leupeptin, 1 $\mu\text{g}/\text{ml}$ pepstatin, 5 $\mu\text{g}/\text{ml}$ aprotinin, 0.32 M sucrose] and homogenized in a 15 ml Dounce homogenizer with five strokes using pestle A followed by five strokes with pestle B. The homogenate was then diluted with 4 ml of Buffer B (same as Buffer A, except 2 M sucrose) and layered over a 4 ml cushion of Buffer B. Nuclei were pelleted by spinning at 28000 rev./min for 1 h at 4°C using a Beckman SW41 Ti rotor. The nuclei were washed in Buffer C [same as Buffer A but 20% glycerol (v/v) in place of sucrose], transferred to a Microfuge tube and brought to 420 mM KCl with Buffer D (same as Buffer C except 1 M KCl). Extracts were dialysed for 1.5 h at 4°C against Buffer E (same as Buffer C except 100 mM KCl) to precipitate chromatin. Chromatin was spun out in a Microfuge tube and supernatants were stored at -80°C . Protein concentration was determined by the Lowry method [23].

EMSAs were performed as follows, unless otherwise noted. Nuclear protein (5 μg) was mixed with 1 μg of poly(dI–dC), 200 ng of sheared genomic *Escherichia coli* DNA and 50000 c.p.m. of labelled probe (approx. 1 ng) in reaction buffer [20 mM Hepes, pH 7.5, 1 mM MgCl_2 , 1 mM DTT, 0.018% (v/v) Nonidet P-40 and 75 mM KCl] in a total volume of 12 μl . In competition experiments a 100-fold molar excess of unlabelled competitor oligonucleotide was added to the mix prior to the addition of nuclear protein extracts. This mixture was allowed to incubate for 30 min at 23°C . Supershifts were performed by adding antiserum to the reaction midway through the incubation. Samples were mixed with 3 μl of running buffer [50% (w/v) sucrose, $0.5 \times$ TBE and 0.1% Bromophenol Blue] and then run on a non-denaturing 10% polyacrylamide gel in $0.25 \times$ TBE buffer, allowing the Bromophenol Blue marker to reach the bottom of the gel. ($1 \times$ TBE buffer is 0.09 M Tris/borate, 0.002 M EDTA, pH 8.0.) The gels were dried and exposed to Kodak XAR-5 X-ray film at 23°C .

Oligonucleotide probes for EMSA

Double-stranded oligonucleotides ranging in length from 20 to 28 bp were end-labelled with T4 polynucleotide kinase (Gibco/BRL), using [γ - ^{32}P]ATP (3000 Ci/mmol). Oligonucleotides were as follows: for HNF-4, the APF1 and LF-A1 oligonucleotides were derived from the human apolipoprotein CIII (apoCIII) gene (–66 to –87) and human α 1-antitrypsin gene (–101 to –128) respectively [11]; for HNF-1, a probe was derived from the rat serum albumin gene proximal element (–59 to –40) [24]; for HNF-3, a probe was derived from the rat TTR gene HNF-3 strong binding site (–111 to –85) [25]. For C/EBP, a palindromic oligonucleotide representing a C/EBP high-affinity binding site [26] was purchased from Santa Cruz Biotechnology. For NF1 and Sp1, double-stranded oligonucleotides containing the general consensus sites for these factors [27,28] were purchased from Promega.

Western blot analysis

Nuclear protein samples (20 μg) were denatured by boiling in loading buffer [50 mM Tris, pH 6.8, 2% (v/v) SDS, 10% (v/v) glycerol, 100 mM DTT, 3% (v/v) 2-mercaptoethanol, 0.5% Bromophenol Blue] for 3 min and then loaded on to a 12% SDS/polyacrylamide gel. Samples were run at 15 V/cm until the Bromophenol Blue marker reached the bottom of the gel. The protein was then transferred to nitrocellulose (Schleicher & Schuell) by electroblotting overnight with a Bio-Rad 200/2.0

Transblot at 15 V and 0.2 A. Blots were probed with primary antibody as indicated and developed with goat anti-(rabbit IgG) antibody conjugated to horseradish peroxidase. Peroxidase activity was detected using the ECL light-based detection system (Amersham) following the manufacturer's instructions

Quantification of results

Results of some EMSAs were quantified by phosphorimage analysis using a Molecular Dynamics PhosphorImager. Results of other EMSAs, as well as the Northern and Western blot results, were quantified by densitometric scanning of X-ray films using an LKB UltroScan laser densitometer.

Antibodies

Antisera to the various transcription factors were the gifts from the following individuals: antibody to ARP-1, Sotirios Karathanasis (American Cyanamid Co., Pearl River, NY, U.S.A.) [29]; antibody to HNF-3 β , Robert Costa (University of Illinois College of Medicine, Chicago, IL, U.S.A.); antibody to HNF-3 γ , Eseng Lai (Memorial Sloan Kettering Cancer Center, New York, NY, U.S.A.) [30]; HNF-1 α and β antibodies, Gerald Crabtree [8,9]; antibody to NF1, Peter Van der Vliet (University of Utrecht, Utrecht, The Netherlands) [31]. Antibodies to C/EBP α , C/EBP β and Sp1 transcription factors were from Santa Cruz Biotechnology Inc. Antibody to HNF-4 (α 445) was as described previously [11].

Statistics

The significance of the difference between two means was determined using the unpaired *t*-test. Phosphorimage results are expressed in pixel intensity units. $P < 0.05$ was considered significant. Data for all six animals in each experimental group were used in all cases for calculating means. The scanning densitometry results are expressed in integrated image density units, which represent peak area (in absorbance units $\times$ mm).

RESULTS

HNF-4

To determine whether dietary protein restriction affected the level of HNF-4 mRNA, a Northern blot was prepared using RNA extracted from livers of control and protein-restricted rats and probed with a rat HNF-4 cDNA. The results indicated that there was no significant change in HNF-4 mRNA (Figure 1A, 10.0 ± 0.72 for control animals versus 9.88 ± 0.25 for restricted animals, $P > 0.05$). HNF-4 protein, measured by Western blot analysis, was also not significantly changed in the protein-restricted animals as compared with the control animals (Figure 1B, 3.66 ± 0.12 for control versus 3.10 ± 0.38 for restricted animals, $P > 0.05$). The level of ARP-1, another member of the nuclear receptor family which binds to some but not all HNF-4 binding sites [29,32; see below], also was not changed by dietary protein restriction (Figure 1C, controls 0.89 ± 0.09 versus restricted 1.07 ± 0.08 , $P > 0.05$).

To examine the effect of dietary protein restriction on HNF-4 DNA-binding activity, EMSAs were performed using the APF1 oligonucleotide, which corresponds to the well-characterized HNF-4-binding site in the apoCIII gene promoter. Both HNF-4 and ARP-1 have been shown previously to bind this oligonucleotide [11,32]. Two bands were detected in the EMSAs with rat liver nuclear protein extracts (Figure 2, upper left-hand panel). The upper band corresponds to HNF-4, and the lower band corresponds to ARP-1, as determined by supershift analysis

Figure 1 Northern and Western blot analysis of HNF-4 mRNA and HNF-4 and ARP-1 protein levels

(A) Northern blot analysis of total RNA (20 μ g/lane) isolated from the livers of six rats fed a 20% protein diet (lanes 1–6) and six rats fed a 4% protein diet (lanes 7–12). The blot was probed with an HNF-4 cDNA. The intensity of the bands was quantified by scanning densitometry. (B and C) Western blot analysis of crude nuclear protein extracts from the livers of the same animals as in (A). Blots shown in (B) and (C) were probed with antisera to HNF-4 and ARP-1, respectively. The intensity of the bands on both blots was quantified by scanning densitometry.

with antibodies specific for HNF-4 and ARP-1 (Figure 2, upper left-hand panel).

When the extracts from the protein deprivation experiment were analysed by EMSA using the APF1 probe, the amount of protein–DNA complex formed for both HNF-4 and ARP-1 was decreased in extracts from the protein-restricted animals (Figure 2, upper right-hand panel). When the two shift complexes were quantified together by phosphorimage analysis, the integrated image for the 20% protein group was $(4.31 \pm 0.08) \times 10^4$ versus $(2.99 \pm 0.14) \times 10^4$ for the 4% protein group ($P < 0.05$).

In order to more accurately quantify the binding activity of HNF-4, we next performed EMSAs using an oligonucleotide (LF-A1) corresponding to the HNF-4-binding site of the α_1 -antitrypsin promoter. Consistent with previously published results [11], two shift complexes were obtained with this oligonucleotide, both of which were completely supershifted with antiserum to HNF-4, indicating that both shift complexes corresponded to HNF-4 (Figure 2, lower left-hand panel). When the extracts from the protein deprivation experiment were assayed with the LF-A1 probe, the result was similar to that with the APF1 probe: the HNF-4-binding activity was decreased by nearly 40% in the 4% dietary protein group $(6.74 \pm 0.89) \times 10^5$ as compared with the 20% dietary protein group $(10.33 \pm 0.64) \times 10^5$ ($P < 0.05$) (Figure 2, lower right-hand panel).

The above results indicated that in the protein-restricted animals, HNF-4 DNA-binding activity was decreased in the

APF1 probe

LF-A1 probe

Figure 2 EMSA of protein restriction experiment using the APF1 and LF-A1 (HNF-4) probes

Upper left-hand panel: EMSAs were performed using the APF1 probe and nuclear extract from a control rat liver. A 100-fold molar excess of unlabelled APF1 oligonucleotide was used as specific competitor (S) (lane 2) and the HNF-1 oligonucleotide was used as non-specific competitor (NS) (lane 3). Supershifts were performed using antisera to HNF-4 (lane 4) or ARP-1 (lane 5). Upper right-hand panel: EMSAs were performed using the APF1 probe and 5 μ g of nuclear protein extract from six animals fed the 20% protein diet (lanes 1–6) or the 4% protein diet (lanes 7–12). DNA–protein complexes were quantified by phosphorimaging. Lower left-hand panel: supershifts and competitions using the LF-A1 probe and liver nuclear protein extract (1 μ g per reaction). A 50-fold molar excess of unlabelled LF-A1 oligonucleotide was used as specific competitor (S) (lane 2), and an oligonucleotide corresponding to positions –175 to –148 of the mouse TTR promoter [11] was used as non-specific competitor (NS) (lane 3). Supershifts using antiserum to HNF-4 (lane 4) verified that both complexes contained HNF-4. Lower right-hand panel: EMSAs were performed with the LF-A1 probe and nuclear extracts from animals fed either the 20% protein diet (lanes 1–6) or 4% protein diet (lanes 7–12). The shift complexes representing HNF-4 binding were quantified by phosphorimaging.

absence of a significant change in HNF-4 protein. To determine whether the decrease in DNA binding resulted from a decrease in binding affinity or a change in the number of HNF-4 molecules that could bind DNA, saturation binding experiments were performed with nuclear extracts from control and protein-

restricted animals, and the data were subjected to Scatchard–Rosenthal analysis (Figure 3). The results indicated that there was a 61% decrease in the $B_{\max}$ in the protein-restricted animals, with no significant change in the K_d (2.2×10^{-8} M for controls versus 1.8×10^{-8} M for the restricted animals). Since the amount

Figure 3 Scatchard plot of HNF-4 saturation binding experiments

Nuclear extract (5 μ g) from either a 20% protein diet animal ($\blacktriangle$) or a 4% protein diet animal ($\triangle$) was mixed with a fixed amount of labelled LF-A1 probe (40 000 c.p.m.) and increasing amounts of unlabelled LF-A1 oligonucleotide. Levels of bound and free probe were determined by phosphorimage analysis and are expressed in pixel intensity units (multiplied by 10^{-4} for bound or by 10^3 for bound/free).

of HNF-4 protein measured by Western blot analysis was not decreased in the protein-restricted animals, the data shown in Figure 3 suggest that protein restriction results in the loss of DNA-binding activity of 61% of the HNF-4 molecules, without altering the DNA-binding affinity of the remaining 39% of the HNF-4 molecules.

HNF-3

An oligonucleotide corresponding to the strong HNF-3-binding site in the TTR promoter (HNF-3 oligonucleotide) was used for EMSA analysis of liver nuclear protein extracts. Analysis of the rat protein deprivation experiment using this probe revealed five distinct shift complexes that represented specific binding (Figure 4). The slowest migrating shift complex is unidentified. A similar shift complex has been reported previously to correspond to the binding of AP-1 to a site adjacent to the HNF-3-binding site on this oligonucleotide [33]. However, we were unable to eliminate this complex using excess unlabelled AP-1 oligonucleotide (results not shown) or a non-specific oligonucleotide (HNF-1) (Figure 4, lanes 6 and 7). Only an excess of the unlabelled HNF-3 oligonucleotide itself completely eliminated this complex (Figure 4, lanes 4 and 5).

The next complex (which is actually a doublet) consists of HNF-3 α and HNF-3 β . These two factors are roughly the same size and run together on shift gels [30]. Their identity was verified by supershift analysis with anti-(HNF-3 β) serum, which shifts a portion of the band, leaving behind HNF-3 α (Figure 4, lanes 8 and 9). The shift complexes corresponding to HNF-3 α and β were increased by 50% in the protein-deprived group (Figure 4 and Table 1).

The third shift complex from the top could not be identified and will be referred to as HNF-3-like protein #1 (HNF-3L1, Figure 4). This complex represented specific binding, as it was competed for by the unlabelled HNF-3 oligonucleotide but not by the HNF-1 oligonucleotide. The HNF-3L1 complex was decreased by 25% in the protein-deprived animals (Figure 4 and Table 1).

The fourth shift complex was identified as HNF-3 γ using anti-

HNF-3 probe

Figure 4 EMSA of protein restriction experiment using an HNF-3 probe

EMSA were performed using the HNF-3 oligonucleotide as probe and nuclear extract from an animal fed the 20% protein diet (lanes 2, 4, 6, 8 and 10) or the 4% protein diet (lanes 3, 5, 7, 9 and 11). A 100-fold molar excess of unlabelled oligonucleotides was used for competition analysis: the HNF-3 oligonucleotide was used as specific competitor (S) (lanes 4 and 5) and the HNF-1 oligonucleotide as non-specific competitor (NS) (lanes 6 and 7). Supershifts were performed using antiserum to either HNF-3 β (lanes 8 and 9) or HNF-3 γ (lanes 10 and 11). In addition to HNF-3 α , β , and γ , two other shift complexes which bound specifically to the probe were identified and are indicated as HNF-3L1 and HNF-3L2.

Table 1 Effect of dietary protein restriction on the DNA-binding activity of HNF-3 isoforms and related proteins

EMSA analysis was performed using the HNF-3 oligonucleotide, and the intensity of the shift complexes was quantified by phosphorimage analysis. Each number represents the mean $\pm$ S.E.M. for nuclear protein extracts from six different rats.

Shift complex	20% protein group	4% protein group
HNF-3 α + HNF-3 β	1291 $\pm$ 152	1956 $\pm$ 189*
HNF-3L1	1855 $\pm$ 103	1374 $\pm$ 110*
HNF-3 γ	1351 $\pm$ 124	1263 $\pm$ 90
HNF-3L2	1575 $\pm$ 145	649 $\pm$ 89*

* Significantly different from mean for 20% protein group ($P < 0.05$).

(HNF-3 γ) serum (which does not supershift the band but instead prevents HNF-3 γ from binding, thereby eliminating the complex [30]) (Figure 4, lanes 10 and 11). HNF-3 γ displayed no significant change in binding activity between the control and protein-restricted groups (Figure 4 and Table 1).

The fastest migrating shift complex (fifth band from the top) has not been identified previously, but, like HNF-3L1, binds the HNF-3 oligonucleotide specifically (Figure 4). It is referred to as HNF-3-like protein #2 (HNF-3L2). The amount of HNF-3L2 complex appeared to be highly regulated by protein deprivation,

Figure 5 EMSA and Western blot analysis of C/EBP levels in the protein restriction experiment

Western blots of nuclear protein extracts from six rats fed the 20% protein diet (lanes 1–6) and six rats fed the 4% protein diet (lanes 7–12) were probed with antiserum (left-hand panels) to either C/EBP α (A) or C/EBP β (B). The intensity of the bands on both blots was quantified by scanning densitometry. Middle panel: EMSAs were performed using the C/EBP oligonucleotide as probe and nuclear extract from a control rat liver. Competitions were performed using a 100-fold molar excess of either the C/EBP oligonucleotide as specific competitor (S) (lane 3) or the HNF-1 oligonucleotide as non-specific competitor (NS) (lanes 4). Supershifts are shown using antiserum to C/EBP α (lane 5) and C/EBP β (lane 6). Right-hand panel: EMSAs were performed with the C/EBP oligonucleotide as probe and nuclear extracts from six animals fed the 20% protein diet (lanes 1–6) or the 4% protein diet (lanes 7–12). Supershift analysis was performed with anti-C/EBP α serum (lane 13) or anti-C/EBP β serum (lane 14). The intensity of the shift complexes was quantified by phosphorimaging.

as indicated by a greater than 2-fold decrease in bound protein in the 4% diet group (Figure 4 and Table 1).

C/EBP

C/EBP α mRNA has two alternative translation start sites, corresponding to AUG codons located at positions +133 and +484 relative to the cap site, which produce proteins of 42 kDa and 30 kDa, respectively [14]. The 42 kDa form is fully functional, while the 30 kDa protein lacks the major transcriptional activation portion of the protein [14]. Western blot analysis of nuclear protein extracts probed with anti-C/EBP α serum showed no significant change in full-length C/EBP α (42 kDa) in the protein-deprived animals (0.61 ± 0.10 for the 4% group versus 0.55 ± 0.08 for the 20% group, $P > 0.05$) [Figure 5, left-hand panels (A)]. The truncated version of C/EBP α (30 kDa) was somewhat decreased by protein restriction (0.41 ± 0.07 for control group versus 0.20 ± 0.06 for restricted group, $P < 0.05$). The 30 kDa form comprises about 10% of the total C/EBP α protein.

Although encoded by a different gene, C/EBP β is similar to C/EBP α in that its mRNA possesses alternative start sites for translation [15]. The larger full-length version of C/EBP β migrates at 30 kDa and is also called LAP. The truncated translational product is approx. 20 kDa in size and has been referred to as LIP (liver-enriched transcriptional inhibitory protein). As with the truncated C/EBP α , the 20 kDa C/EBP β protein is missing its transactivation domain. The full-length version of C/EBP β was unchanged by dietary protein deprivation (controls 1.29 ± 0.05 versus restricted 1.19 ± 0.06 , $P > 0.05$). Interestingly, however, the truncated 20 kDa form of C/EBP β

(LIP) was increased significantly by protein deprivation (0.79 ± 0.11 in the 20% animals as compared with 1.35 ± 0.13 in the 4% animals, $P < 0.05$) [Figure 5, left-hand (B)].

EMSAs performed with the high-affinity C/EBP oligonucleotide demonstrated specific binding of both C/EBP α and C/EBP β , as determined by supershifts and competitions. Supershifts with antisera to C/EBP α and β indicated that the upper region of the shift complex consists primarily of C/EBP α , while the lower region is comprised mostly of C/EBP β (Figure 5, middle panel). More than one shift complex was supershifted by the C/EBP α and β antibodies: these presumably correspond to α/α homodimers, β/β homodimers, and various heterodimers [13–15]. Qualitative examination of EMSAs performed with nuclear extracts from control and protein-restricted rats indicated that all shift complexes were increased in the protein-restricted animals (Figure 5, right-hand panel). This suggests that the binding activities of C/EBP α and C/EBP β were both increased in the protein-restricted animals. Quantifying all shift complexes together, total C/EBP-binding activity was increased from $(3.79 \pm 0.60) \times 10^5$ in the control group to $(6.73 \pm 0.40) \times 10^5$ in the protein-restricted group ($P < 0.05$).

NF1

Binding sites for the ubiquitous transcription factor NF1 are present in the albumin enhancer and promoter and TTR promoter, and evidence suggests that NF1 may play a role in activating the expression of the albumin, TTR and other liver genes. Therefore, we next considered the possibility that the repression of several liver-specific genes that occurs in protein-

Figure 6 EMSA and Western blot analysis of CTF/NF1 levels in the protein-restriction experiment

Upper panel: Western blot analysis of liver nuclear protein extracts from six rats fed the 20% protein diet (lanes 1–6) or the 4% protein diet (lanes 7–12), using antiserum directed against NF1 as probe. The intensity of the bands was quantified by scanning densitometry. Middle panel: EMSAs were performed using the CTF/NF1 oligonucleotide as probe and nuclear extract from a control rat liver. Competitions were performed using a 100-fold molar excess of either unlabelled NF1 oligonucleotide as specific competitor (S) (lane 3) or HNF-1 oligonucleotide as non-specific competitor (NS) (lane 4). A supershift is shown using NF1 antiserum (lane 5). Three other specifically bound complexes were identified in addition to the shift complex identified as NF1-L. These complexes are marked as NF1-L2, NF1-L3 and NF1-L4 respectively. Lower panel: EMSAs were performed using the CTF/NF1 oligonucleotide as probe and nuclear extracts from six animals fed the 20% protein diet (lanes 1–6) or the 4% protein diet (lanes 7–12). The intensity of all complexes was determined by phosphorimaging.

restricted animals is caused by a decrease in NF1. Western blots of the nuclear protein extracts from the rat protein deprivation experiment were probed with antiserum directed to the CTF/NF1 N-terminus, which contains the DNA-binding domain that is highly conserved among CTF/NF1 isoforms (Figure 6, upper panel). This antiserum has been shown previously to bind to the rat NF1-Liver (NF1-L) protein [34]. A single major 42 kDa band was identified that was slightly decreased in the protein-restricted animals (2.95 ± 0.10 for controls versus 2.29 ± 0.25 for the restricted animals, $P < 0.05$). In addition, there were five minor bands between 30 and 40 kDa which were detected with the CTF/NF1 antibody. Of these bands, the 32 and 38 kDa bands were down-regulated in the protein-deprived animals, with the 32 kDa form being decreased by 7-fold (control animals 0.63 ± 0.10 versus restricted animals 0.085 ± 0.012 , $P < 0.05$).

Analysis of competitions and supershifts using liver nuclear extracts revealed four DNA–protein complexes which were specific for the NF1 consensus oligonucleotide (Figure 6, middle panel). The slowest migrating of these included at least two major complexes, both of which were specific for the NF1 oligonucleotide. Only the more slowly migrating complex of the two was supershifted with the anti-NF1 serum and appears to be equivalent to a complex previously designated NF1-L [34]. Migrating ahead of the NF1-L complex, there were three other shift complexes that we have designated NF1-L2, NF1-L3 and NF1-L4. All of these NF1-L complexes were specific, as demonstrated by competitions with unlabelled oligonucleotide, although we could not clearly supershift these complexes with the NF1 antiserum.

When the NF1 oligonucleotide was used for EMSA analysis of nuclear extracts from the protein-deprivation experiment, no change in binding activity was found for NF1-L [$(1.70 \pm 0.10) \times 10^5$ for control diet versus $(1.71 \pm 0.13) \times 10^5$ for the restricted diet, $P > 0.05$] (Figure 6, lower panel). However, all three of the shift complexes designated NF1-L2, 3 and 4 were down-regulated in the protein-deprived animals by at least 40% [NF1-L2, $(4.36 \pm 0.22$ versus $2.45 \pm 0.23) \times 10^4$; NF1-L3, $(2.48 \pm 0.10$ versus $1.28 \pm 0.12) \times 10^4$; NF1-L4, $(2.02 \pm 0.13$ versus $0.90 \pm 0.08) \times 10^4$ for control and protein-restricted animals respectively, $P < 0.05$ in each case].

Sp1

EMSA assays performed with an Sp1 oligonucleotide produced one shift complex that could be specifically eliminated by addition of unlabelled oligonucleotide and supershifted with anti-Sp1 serum (results not shown). Examination of the protein restriction experiment using this probe showed that Sp1 binding increased by 25% in the protein-deprived as compared with control animals [control animals $(1.19 \pm 0.03) \times 10^5$ versus restricted animals $(1.49 \pm 0.10) \times 10^5$, $P < 0.05$].

HNF-1

EMSA analysis of HNF-1 binding was performed initially with nuclear extract prepared from the liver of a control animal. Consistent with results reported previously by others [8,9], these assays yielded a single complex which could be partially supershifted with anti-HNF-1 α serum and partially supershifted with anti-HNF-1 β serum, indicating that the complexes containing these two proteins run together on a shift gel (Figure 7, upper panel).

EMSAs were performed with samples from the protein-deprivation experiment (Figure 7, middle panel). Since complexes containing HNF-1 α and HNF-1 β run together on gels, the shift

Figure 7 EMSA and Northern blot analysis of protein restriction experiment using probes for HNF-1

Upper panel: EMSAs were performed using the HNF-1 oligonucleotide as probe and nuclear extract from a control rat liver. Competitions were performed using a 100-fold molar excess of either the HNF-1 oligonucleotide as specific competitor (S) (lane 3) or the HNF-3 oligonucleotide as non-specific competitor (NS) (lane 4). Supershifts were performed with antiserum directed against HNF-1 α (lane 5) or HNF-1 β (lanes 6 and 7) (1/5 β implies a 1/5 dilution of the antiserum). Middle panel: EMSAs were performed with the HNF-1 oligonucleotide as probe and nuclear extracts from six animals fed the 20% protein diet (lanes 1–6) or the 4% protein diet (lanes 7–12). The DNA–protein complexes were quantified by scanning densitometry. Lower panel: Northern blot analysis was performed with total RNA (20 μ g per lane) isolated from the livers of six rats fed the 20% protein diet (lanes 1–6) and six rats fed the 4% protein diet (lanes 7–12). The blot was probed with the HNF-1 α cDNA. The intensity of the bands representing the two transcripts was measured by scanning densitometry.

complex which was scanned represents the binding of the probe to both factors. Overall binding of both factors was increased in the protein-deprived animals (5.57 ± 0.79) versus the control animals (3.57 ± 0.23) ($P < 0.05$).

Northern blot analysis of RNA prepared from liver of the control and restricted animals showed two major species of HNF-1 α mRNA (Figure 7, lower panel: 4.3 kb and 3.7 kb), as reported previously [8]. Quantifying the two species together by scanning densitometry, the abundance of HNF-1 α mRNA was

3.90 ± 0.57 for controls versus 8.74 ± 0.64 for restricted animals, $P < 0.05$. The increase in HNF-1 α mRNA is consistent with the increase in overall HNF-1 binding activity detected by EMSA.

DISCUSSION

The purpose of the present project was to test the hypothesis that the decreased expression of several liver-specific genes in protein-restricted rats is caused by a reduction in the activity of one or more of the liver-enriched transcription factors. One interesting finding was that restriction of dietary protein led to a significant decrease in the binding of HNF-4 to its DNA target, as measured by EMSA using two different oligonucleotide probes. The experimental diet did not appear to have a significant effect on HNF-4 at either the level of HNF-4 mRNA or total nuclear HNF-4 protein. Furthermore, dietary protein restriction did not decrease the DNA binding affinity of the overall pool of HNF-4 protein. Rather, Scatchard analysis of binding data indicated that protein restriction resulted in a decreased B_{max} . Since the amount of HNF-4 protein was unchanged, these results suggest that in protein-restricted animals a portion of the nuclear pool of HNF-4 protein was incapable of binding to DNA.

HNF-4 is an orphan receptor which contains the ligand-binding domain found in other members of the nuclear receptor superfamily. If the binding of a ligand to HNF-4 modulates its binding to DNA or transcriptional activation function, then regulation of this ligand would be an obvious control point for HNF-4 activity. A possible explanation for the regulation by dietary protein restriction of HNF-4-binding activity is that this reflects regulation of the (as yet unknown) HNF-4 ligand.

A second possibility is that one of the other related members of the nuclear receptor superfamily directly competes for HNF-4 binding. Supershifts with antiserum to HNF-4 verified that only HNF-4 bound to the LF-A1 oligonucleotide, and no additional shift complexes were seen when using this probe. These results argue against the possibility of direct competition by a related factor. The alternative possibility of HNF-4 heterodimerizing with another factor, thereby reducing the binding of the heterodimer to HNF-4 cognate sites, is very unlikely [35].

Another possibility is that the decrease in binding of HNF-4 results from a post-translational modification of the HNF-4 protein. Proteolysis of HNF-4 would most likely remove a significant portion of the HNF-4 protein, thereby changing its molecular size as observed on SDS/polyacrylamide gels. Since a change in size was not observed, a more probable modification would be phosphorylation or dephosphorylation, which might alter the conformation of HNF-4 and thus prevent it from binding to DNA. We have shown recently that HNF-4 is phosphorylated (F. M. Sladek, unpublished work).

NF1 has been shown to bind to the albumin promoter, albumin enhancer and TTR promoter [4,18,19]. Diverse roles have been demonstrated for NF1 in the regulation of various genes. NF1 has been shown to be an activator of transcription in certain contexts [36] and an inhibitor of transcription in other contexts [37]. NF1 also promotes DNA replication of the adenovirus genome [38]. The wide range of NF1 actions most likely lies in its variable C-terminus. While it has been shown that the various members of the CTF/NF1 family of transcription factors are extremely homologous at the N-terminus (the DNA-binding region), they show more divergence in their mid region to C-terminus [39]. This divergence appears to occur at several levels. First, the multiple factors that bind to the NF1 consensus sequence may be encoded by different genes. Four genes for proteins in the NF1 family have been identified in chicken and are thought to be homologous to various mammalian NF1

genes, which encode the CTF/NF1 and NF1-L proteins [39]. Furthermore, the mammalian CTF/NF1 mRNAs can be differentially spliced, providing for different protein sequences [39]. Finally, it is possible that several of the smaller isoforms of NF1 arise from proteolytic cleavage of larger NF1 species [40].

Although expressed ubiquitously, NF1 has been shown to bind to promoter and enhancer elements of several liver-expressed genes [4,18–21]. Since NF1 can apparently act as both an inhibitor or an activator of transcriptional activity, it is possible that the different species we have identified serve to play multiple roles in gene regulation, some acting as activators and other species functioning as negative regulators of transcription. We have demonstrated that the DNA-binding activity of NF1-L is unchanged, but that the DNA-binding activity of a number of smaller NF1-like proteins is substantially decreased by protein deprivation.

Both C/EBP α and C/EBP β can be alternatively translated to produce either a functional full-length protein (42 and 30 kDa respectively) or a truncated protein which lacks the activation domain. One interesting finding in the present study is the differential regulation of the truncated as compared with the full-length forms of both C/EBP α and C/EBP β . Protein restriction did not result in any significant change in the full-length form of either protein; however, it resulted in a significant decrease in truncated C/EBP α and increase in truncated C/EBP β . Since the full-length and truncated proteins are translated from the same mRNA, the most likely explanation for this differential regulation would be a change in the relative frequencies for initiation of translation at the upstream as compared with the downstream AUGs.

We also observed an increase in net C/EBP α and β binding in the absence of a significant change in the level of full-length C/EBP α or β protein. Since there was an increase in truncated C/EBP β (LIP), some of the increase in net C/EBP β binding could have been caused by an increase in binding of heterodimers composed of full-length and truncated C/EBP β . Recent studies, in which amounts of expressed LIP were accurately quantified, have indicated that LIP is a potent dominant negative inhibitor of C/EBP function [41]. Therefore, even a small increase in LIP relative to full-length C/EBP β could have a strong repressive effect on transcription.

It is also possible that some of the change in binding of C/EBP β may have been caused by a change in the phosphorylation state of this protein. Phosphorylation of C/EBP β on certain serine residues inhibits DNA binding; thus, dephosphorylation of these residues would enhance binding [42,43]. On the other hand, dephosphorylation of Ser¹⁰⁵ would decrease the transactivation activity of C/EBP β [42,43]. Therefore, an overall decrease in the phosphorylation of C/EBP β on serine residues could actually increase binding and simultaneously decrease transcription activation.

We also examined the effect of protein restriction on the binding of the HNF-3 family of transcription factors. The results indicated that HNF-3 γ binding was not affected, whereas the binding of HNF-3 α and β was increased by 50%. Interestingly, in the EMSAs performed with the HNF-3 oligonucleotide, we also noted the binding of two proteins that have not been described previously (HNF-3L1 and HNF-3L2). The binding of these proteins was specific for the HNF-3 oligonucleotide and was decreased by protein restriction. The identity of these two proteins and their effect on transcription remains to be determined.

In conclusion, restriction of dietary protein results in a number of specific changes in the binding of various transcription factors to their cognate sites, including increased binding of some

factors and decreased binding of others. To our knowledge, this is the first systematic examination of the effect of changes in dietary protein intake on transcription-factor activity. Although there was no single change that clearly explained the co-ordinate repression of transcription of several liver-specific genes that occurs in protein-restricted animals, perhaps the most important consideration in the changes we have observed is that transcription factors are capable of acting synergistically. For example, C/EBP and HNF-1 are both involved in activating the albumin promoter [44]. It has been shown that in HeLa cells, the activation of the albumin promoter by the two factors together is at least 10-fold greater than the activation by either of the factors individually [45]. HNF-4 and C/EBP have also been shown to act synergistically in activating transcription of the ornithine transcarbamylase and apoprotein B (apoB) genes [46,47]. This indicates that the transactivating potential of certain combinations of transcription factors may be far greater than that of the individual factors. Due to this co-operativity, a minor alteration in the activation potential in one or more transcription factors may have a very large effect on an individual promoter. Further, as each promoter or enhancer is made up of binding sites for different combinations of liver-enriched and ubiquitous factors, this allows for fine tuning potential where small changes in a number of transcription factors can create a large number of specific changes in expression in a variety of genes.

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