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The human intestinal fatty acid binding protein (hFABP2) gene is regulated by HNF-4α

Maja Klapper *, Mike Böhme, Inke Nitz, Frank Döring

Molecular Nutrition, Institute of Human Nutrition and Food Science, Christian-Albrechts-University of Kiel, Heinrich-Hecht-Platz 10, D-24118 Kiel, Germany

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Abstract

The cytosolic human intestinal fatty acid binding protein (hFABP2) is proposed to be involved in intestinal absorption of long-chain fatty acids. The aim of this study was to investigate the regulation of hFABP2 by the endodermal hepatocyte nuclear factor 4α (HNF-4α), involved in regulation of genes of fatty acid metabolism and differentiation. Electromobility shift assays demonstrated that HNF-4α binds at position −324 to −336 within the hFABP2 promoter. Mutation of this HNF-4 binding site abolished the luciferase reporter activity of hFABP2 in postconfluent Caco-2 cells. In HeLa cells, this mutation reduced the activation of the hFABP2 promoter by HNF-4α by about 50%. Thus, binding element at position −336/−324 essentially determines the transcriptional activity of promoter and may be important in control of hFABP2 expression by dietary lipids and differentiation. Studying genotype interactions of hFABP2 and HNF-4α, that are both candidate genes for diabetes type 2, may be a powerful approach.

Keywords: hFABP2; HNF-4α; Fatty acid metabolism; EMSA; Reporter assay

The human intestinal fatty acid binding protein (hFABP2) gene is localized on chromosome 4q28-q31 and contains four exons [1]. The FABP2 gene encodes a 15 kDa cytosolic protein, which is exclusively expressed in enterocytes with highest cellular concentrations in jejunum with falling levels to the proximal duodenum and colon [2,3]. FABP2 is up-regulated during development [4] and crypt-to-villus transition. Whereas FABP2 is absent in crypt cells, highest protein abundance is found in upper villus region [2,5,6], where fat absorption takes place. The function of FABP2 is still not fully elucidated. It is proposed to be involved in intestinal fat absorption by cellular binding and trafficking of newly absorbed non-esterified long-chain fatty acids, which are finally assembled and secreted in triglyceride-rich chylomicrons [7–9].

The homodimeric transcription factor HNF-4α is an abundant nuclear protein in the endoderm derived tissues liver, intestine, pancreas and kidney and controls an unusual high proportion of hepatic and pancreatic expressed genes as shown by ChIP [10]. In enterocytes, HNF-4α seems to be a critical regulator of differentiation processes [11–13]. Cellular differentiation in small intestine leads to a fully functional cell type in villus region that can entirely meets the requirements in absorption of macro- and micronutrients. Accordingly, this differentiated cell type is characterized by a specific expression pattern of several genes, including genes involved in lipid metabolism. Genes that are up-regulated during development and crypt-to-villus differentiation and that are furthermore annotated with the gene ontology (GO) term "lipid metabolism" have an overrepresentation of potential HNF-4 binding sites in their promoters and are correlated with highest levels of lipids in villus tips of intestine [11]. HNF-4α, originally considered as orphan nuclear receptor, was previously shown to bind fatty acid acyl-CoA esters as ligands. These were able to modulate HNF-4α DNA binding activity in dependence on their chain length and saturation grade

* Corresponding author. Fax: +49 (0) 431 8805658.
E-mail address: klapper@molnut.uni-kiel.de (M. Klapper).

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[14]. HNF-4α is involved in regulation of genes that participate in chylomicron synthesis in enterocytes such as MTP [15], Apo B [16], Apo C III [16] Apo A I [17], or Apo A IV [18]. For the Apo A IV gene, it was shown that HNF-4 mediates its transcriptional regulation by dietary lipids [19]. Thus, the cellular fatty acid concentration seems to be linked through HNF-4α with transcriptional control of genes involved in fat metabolism.

In the present study, we wanted to address the question if and how human FABP2 is regulated by HNF-4α. We show the presence of a HNF-4 binding site by electromobility shift assays. Reporter analysis indicates that this element essentially determines the transcriptional activity of a 836 bp hFABP2 promoter luciferase construct in postconfluent Caco-2 cells. Mutations of this HNF-4-binding site nearly abolished the promoter activity of hFABP2 suggesting a major role for HNF-4α in the control of hFABP2 expression in the small intestine.

Materials and methods

Cell culture. HeLa cells and Caco-2 were purchased by German National Resource Centre for Biological Material (DSMZ, Braunschweig, Germany) and American Type Culture Collection (ATCC, Rockville, MD), respectively. Cells were maintained in MEM (Invitrogen, Carlsbad, CA), supplemented with 10% (HeLa) and 20% fetal calf serum (Caco-2) (Invitrogen) and 1 mM non-essential amino acids (PAA, Cölbe, Germany) in a humidified incubator at 37 °C under an atmosphere of 5% CO2. Cells were passaged at preconfluent densities by use of 0.05% trypsin/0.02% EDTA solution (Biochrom AG, Berlin, Germany) every 2–3 days.

hFABP2 promoter luciferase constructs. The dual luciferase system was used (Promega, Madison, WI). Cloning procedures were performed using Gateway Technology (Invitrogen), described previously [20]. attR-sites were inserted into a *Eco*RV blund-end site of the reporter plasmid (pGL4.10[luc2]) encoding *Firefly* luciferase. A 836 bp fragment upstream from initiation codon of the hFABP2 promoter (haplotype A) was subcloned by recombination with LR Clonase into attR-sites of pGL4.10[luc2] from vector pENTR-hFABP2-Prom-attL contained attL-sites resulting in final pGL4.10[luc2]-hFABP2 promoter reporter construct. The empty pGL4.10[luc2] vector served as a negative control. Introduction of mutation into HNF-4 consensus site of pGL4.10[luc2]-hFABP2 resulting in pGL4.10[luc2]-hFABP2-MutHNF-4 was performed using Quick Change™ in vitro mutagenesis Kit (Stratagene, La Jolla, CA). All primers were purchased from MWG Biotech AG (Ebersberg, Germany). Sequences of primers are available on request. Verification of constructs was performed by sequencing.

Expression plasmids. Expression vectors pRc/CMV-HNF-1α and pRc/CMV-HNF-4α2 were a kind gift from Dr. G. Ryffel (University of Essen, Germany). Open reading frames of HNF-1α and HNF-4α2 were amplified by PCR and subcloned in expression vector pDest40 using Gateway technology. Preparation of human PPARγ2 expression vector is described by Nitz et al. [21]. Human expression plasmid RXRa was kindly provided by Dr. J. Weitzel (University Medical Center Hamburg-Eppendorf, Germany).

Transient transfections and reporter assays. Transient transfections were performed with FuGene6 (Roche, Basel, Switzerland) according to the manufacturer's instructions. 1×10^4 Caco cells were plated in 96-well plates. Cells were co-transfected with 47.5 ng pGL4.10[luc2]-hFABP2, pGL4.10[luc2]-hFABP2-MutHNF-4 or pGL4.10[luc2] as negative control and 2.5 ng pGL4.74[hRluc/TK] vector encoding *Renilla* luciferase as internal control. Luciferase activities were measured 120 h after transfection by Dual-Luciferase® Reporter Assay System (Promega). 4×10^3 HeLa cells/96 well were used for co-transfections with HNF-4α2, HNF-1α, and PPARγ/RXRα. Cells were co-transfected with 30 ng pGL4.10[luc2]-hFABP2-promoter construct, pGL4.10[luc2]-hFABP2-Prom-MutHNF-4, or pGL4.10[luc2], 66 ng of the expression vectors pDest40-HNF-4α2 and pDest40-HNF-1α, with 33 ng of PPARγ2 and RXRα each or 66 ng empty pDest40 vector and 3 ng pGL4.74[hRluc/TK] vector. Each experiment was repeated at least twice, and each sample was studied in triplicate.

Electromobility shift assay (EMSA). The biotinylated probe was prepared by end labeling oligonucleotides with biotin by the Biotin 3′ End DNA Labeling Kit (Pierce, Rockford, IL). Annealing of the unlabeled and labeled oligomers was done following the technical resource "Annealing complementary pairs of oligonucleotides" (Pierce). The following oligonucleotides were used as DNA probes: −700/−687: 5′-CAT GGCAGATGTTTAAAGCTCATTCTTTCTTT-3′, −468/−480: 5′-GAATCTTATTAACTTTAGCTTTTCAAC-3′, −336/−324: 5′- CAAC ATAGTCTGCACTTTGAACTTAGAAAAAC-3′, mutated −336/−324: 5′-CAACATAGTCTGCAAGGGTAACTTAGAAAAAC-3′, −120/−102: 5′-GTGATTTCCTGAACTTTAAGCTTCCACATCAC-3′ and unrelated oligo: 5′-CAGAACGAGAATTAAGAATTAATAAGAATA AGAATTAATTAATTGCTTG-3′. Nuclear extracts were prepared from postconfluent Caco2 cells with the NE-PER Nuclear and Cytoplasmic Extraction Reagents Kit (Pierce). The EMSAs were performed with the LightShift Chemiluminescent EMSA Kit (Pierce) according to manufactures instructions. The reaction mixture (20 μl) contained 1 mM Tris, 50 mM KCl, 1 mM DTT, 1 μg Poly(dI–dC), 2.5% Glycerol, and 0.1 mM EDTA. The DNA-transcription factor complexes were resolved on a native 4% Tris-borate-EDTA polyacrylamide gel in 0.5× TBE buffer and transferred to a nylon membrane (Amersham Bioscience, Piscataway, NJ). Biotinylated DNA was detected with streptavidin-horseradish peroxidase conjugate and chemiluminescent substrate Luminol using Chemiluminescence Nucleic Acid Detection Module. In supershift reactions the following antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were used: HNF-4α(C-19): sc-6556 and HNF-1α(C-19): sc-6547.

Statistical analyses. Statistical analyses were performed in GraphPad Prism 4 (Graphpad Software, Inc., San Diego, CA) using ANOVA and *t*-test. All values were expressed as means ± SD. Significant differences were considered for p values less than 0.05.

Results and discussion

In silico analysis of the hFABP2 gene promoter region

As a first approach to identify HNF-4 binding sites within the human FABP2 gene promoter region, a database search was performed with an 836 bp segment upstream of the translation start by using the TRANSFAC Professional database [22,23]. As cut-offs minimisation of sum of false positives and negatives and scores of core and matrix match for HNF-4 recognition sites of at least 0.9 were chosen. Three putative HNF-4 binding sites were found at positions −700/−687, −336/−324, and −120/−102 of the hFABP2 promoter. In addition, we proved an element, which was already postulated as conserved HNF-4 binding sites in rodent and human FABP2 promoters, at position −480/−468 [24]. Taken together, as shown in Fig. 1, four putative HNF-4 binding sites were found in the hFABP2 promoter.

EMSA confirms binding of HNF-4 to position −324/–336 of the hFABP2 gene promoter

To prove binding of HNF-4 to the four putative sites within the hFABP2 gene promoter, we performed EMSAs

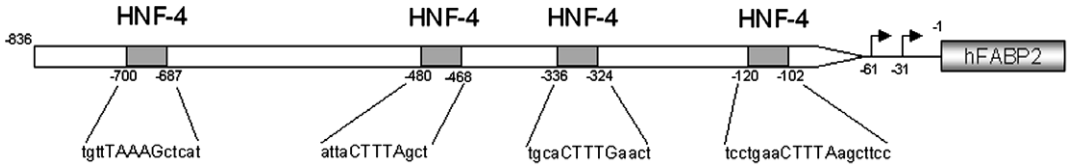

Fig. 1. Putative HNF-4 binding sites in the human intestinal fatty acid binding protein 2 (hFABP2) gene promoter. The nucleotide sequence –836 to –1 relative to translation start site is shown. The sequence was analysed with TRANSFAC database and compared with the HNF-4 factor matrix. An element, which was already postulated as conserved HNF-4 binding sites in rodent and human FABP2 promoters [24], at position −480/−468 was also proved. The sequence on (+)-strand of the suggested consensus site is given. Capitals indicate the core sequence, the most conserved region. As cut-offs were chosen: to minimize the sum of false positives and negatives. The scores of core and matrix match for HNF-4 were at least 0.9. Arrows indicate the transcriptional start site.

with nuclear protein extracts from post-confluent and differentiated Caco-2 cells. In this cell line HNF-4α is up-regulated by differentiation [25]. We observed no binding for hFABP2 promoter elements at positions −700/−687, −468/−480, and −120/−102 (data not shown). In contrast to Rottman and Gordon [24] who detected a HNF-4 binding site in a conserved rat FABP2 element position (−82/−69) we could not detect binding of HNF-4α to this promoter element at corresponding position −120/102 in human FABP2. However, promoter sequence is not fully conserved and binding affinity of HNF-4α may be drastically lower.

As shown in Fig. 2A, we could detect a strong and specific shift in case of HNF-4 site at position −336/−324 of the hFABP2 gene promoter. Lane 2 shows a specific shift that could be competed by 100 molar excess of unlabeled probe (lane 3). An excess of unlabeled probe containing a 5-bp mutation (lane 4) and of unrelated oligonucleotide (lane 5) could not interfere with binding of proteins. Addition of HNF-4α antibody abolished the strong specific shift completely. A supershift of the signal was not detected (Fig. 2B, lane 2). This may be either due to impairment of HNF-4-binding to consensus site by HNF-4α antibody or supershift signal may be located in unspecific signals. To assure that the effect was caused specifically by HNF-4α antibody, HNF-1 was added to binding reaction (lane 3). In this case formation of the specific complex was not disturbed. Therefore, we conclude that HNF-4α binds

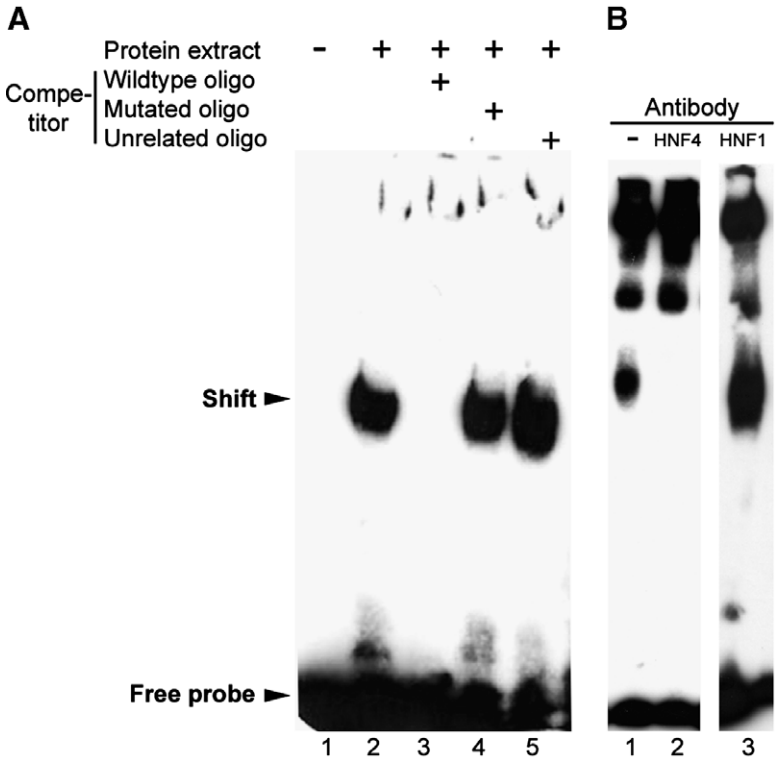

Fig. 2. Specific interaction of HNF-4α to the human hFABP2 gene promoter at position −336/−324. Two microliters of nuclear extracts isolated from postconfluent Caco2 cells were used for each binding reaction with 20 fmol of biotinylated oligos (no extract was added in B, lane 1) in electromobility shift assays. (A) Competition experiments were carried out without unlabeled oligo (lane 2), with 100-fold excess of unlabeled oligo (lane 3), mutated oligo (lane 4) and unrelated oligo (lane 5). Specific shift is indicated with an arrowhead. (B) Supershift was performed with 2 μg of HNF-4α antibody (lane 2). HNF-1 antibody was used as control (lane 3).

specifically to oligonucleotide corresponding to positions −336/−324. This element has no corresponding element in rat or mouse promoter sequence at similar position [1].

The HNF-4 binding site in position −324 to –336 is essential for transcriptional activity of hFABP2 promoter in Caco-2 cells

To investigate the functional importance of HNF-4 binding site at promoter position −336/−324 for hFABP2 expression, we performed promoter-reporter assays with dual luciferase system. The *Firefly* luciferase constructs contained an functional 836 bp segment of the hFABP2-promoter [20]. A 5-bp mutation was introduced into the core sequence, CTTTG → AGGGT, of HNF-4 consensus at position −336/−324 within reporter construct pGL4.10[luc2]-hFABP2 resulting in pGL4.10[luc2]-hFABP2-MutHNF-4. Luciferase activities were measured 6 days after seeding, when Caco-2 cells were 4 days post-confluent. As shown by us previously, both, FABP2 mRNA as well as protein level are clearly up-regulated in Caco-2 cells at this time point [20], very likely due to spontaneous differentiation in this cell line after having reached confluence [26]. Also HNF-4α expression is notably increased at this stage [25]. As shown in Fig. 3A, the activity obtained from the FABP2 promoter construct was 44-fold higher in comparison to the control construct. The mutation in HNF-4 binding site at position −336/−324 nearly abolished the *Firefly* luciferase activity in Caco-2 cells. This result is consistent with the report that FABP2 is absent in visceral endoderm of embryonic bodies of differentiated ES-cells of HNF-4α −/− mice [27]. Together these findings give rise to assumption that HNF-4α is essential for FABP2 expression. This dramatic effect may be explained by synergistic action of HNF-4α with other transcription factors. HNF-4α was previously described to synergistically interact with Sp1 [28], HNF-1 [29,30], or HNF-3 [31] to induce reporter activity of apoCIII, FABP1 and apoAI promoters, respectively, and HNF-4 binding sites were proved to be essential for functional synergism with these factors.

Mutation in positions −336/−324 reduced the activation of the hFABP2 promoter by HNF-4α in HeLa cells

In order to study the influence of position −336/−324 on HNF-4α induced hFABP2 promoter activity directly we used HeLa cells that do not express FABP2 [24] and HNF-4α. As shown in Fig. 3B, hFABP2 promoter exhibits a 7.3-fold increase in luciferase activity when co-transfected with HNF-4α plasmid. A mutation in the HNF-4 binding site at positions −336/−324 reduced the activation of the hFABP2 promoter by HNF-4α of about 50%. If activation of mutant is attributable to more HNF-4 binding sites in the analysed promoter region or if activation of other transcription factors by HNF-4α overexpression leads to induction of reporter activity, we cannot specify. The specificity of the differential regulation of hFABP2 promoter constructs by HNF-4 was shown by activation experiments with HNF-1. We observed no difference in HNF-1 induced activities of hFABP2 wild type and mutant promoter. The HNF-4 consensus element encompasses an direct repeat of 6 bases spaced by one nucleotide (DR-1) on (-) strand (agttca(a)agttca). Because DR-1 repeats can be recognized not only by HNF-4α but also by PPAR/RXR heterodimers [32,33] we tested the activation of FABP2 promoter

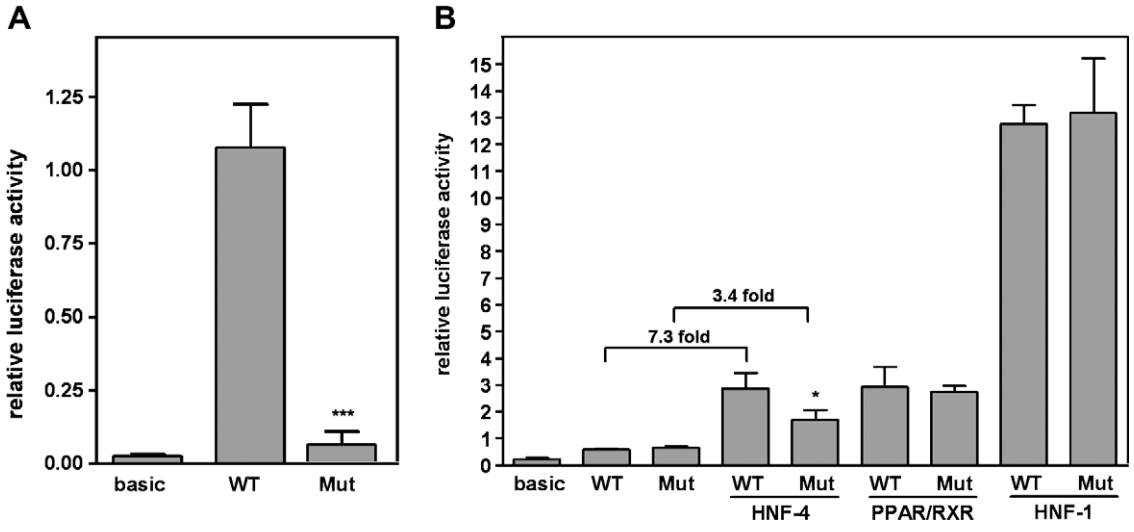

Fig. 3. Reporter assay of hFABP2 promoter wild type (WT) and HNF-4 mutant (Mut) constructs after transient transfection using dual luciferase assay. (A) Basal activities of FABP2 promoter constructs were assessed in postconfluent Caco-2 cells 120 h after transfection. $^{***}p < 0.001$, as compared with wild type FABP2 promoter activity. (B) Induction of promoter activity of hFABP2 promoter constructs by HNF-4 was assessed in HeLa cells. Luciferase activities were measured 48 h after transfections. $^{*}p < 0.05$, as compared with wild type FABP2 HNF-4α induced promoter activity. The *Firefly* luciferase activities were normalized to *Renilla* luciferase activities and are given as relative luciferase activities. Each experiment was performed in triplicate for each sample. The results are expressed as means ± SD for two (A) or three (B) independent experiments.

constructs by PPARγ/RXRα. No differences in activation of wild type and mutant with or without 2 μM of PPARγ ligand Rosiglitazone were found (Fig. 3), indicating that PPAR/RXR heterodimers do not bind to this DR-1 repeat. This result is consistent with EMSA that revealed only one DNA–protein complex.

Previous investigations in transcriptional regulation of human FABP2 concerned basal activities of two FABP2 promoter haplotypes [20,34–36]. This genetic variation in the hFABP2 gene is associated with postprandial triglyceride levels [36], body composition, lipid plasma levels [34], and diabetes [20]. However, little effort was done to reveal the substantial promoter regulation of hFABP2. FABP2 expression was shown to be induced by oleic acid in Caco-2 cells; however, no mechanism mediating this regulation has been proposed [37]. As already mentioned, HNF-4α is involved in transcriptional regulation of Apo A IV by lipid micelles, when micelles supplied to apical pole of Caco-2 cells. If HNF-4α also plays a role in regulation of hFABP2 by dietary lipids needs to be proved.

Interestingly, HNF-4α and hFABP2 are candidate genes for diabetes type 2 (non-insulin dependent diabetes mellitus, NIDDM). The hFABP2 T54 allele of exon 2 A54T polymorphism could be linked to insulin resistance in different populations [38–40]. Also hFABP2 promoter haplotypes were associated with NIDDM [20]. In HNF-4α, 3 SNPs near the P1 promoter and exons 1–3 could be associated with diabetes type 2 in a finish population [41]. On the basis of the here demonstrated functional interaction of hFABP2 and HNF-4α it seems to be a powerful approach for genetic epidemiologists to study effects of genotype combinations of both genes on the development of NIDDM.

In conclusion, in the present study we have systematically investigated the presence of HNF-4α binding sites in the human FABP2 promoter and identified a functional HNF-4α element at the position −336/−324. The modulation of FABP2 expression by different types of fatty acids and especially the physiological importance of the HNF-4α binding site in regulation of FABP2 by dietary lipids should be in focus of future investigations.

Acknowledgments

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