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**Promoter variants and transcriptional regulation of the intestinal
fatty acid binding protein gene (*FABP2*)**

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Dipl. oec. troph. Maja Klapper

aus Jena

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Dekan: Prof. Dr. Krieter
1. Berichterstatter: Prof. Dr. S. Wolffram
2. Berichterstatter: Prof. Dr. G. Rimbach
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SUMMARY

The human intestinal fatty acid binding protein (FABP2) is a cytosolic 15 kDa protein and is involved in resorption of long-chain fatty acids. Phenotyping of *FABP2* knock-out mice and association analysis of promoter haplotypes revealed *FABP2* as a susceptibility gene for insulin resistance and related traits. Relevant *FABP2* promoter polymorphisms -80Del>T, -136AGTAG>Del, -168AAG>T, -260G>A, -471G>A, and -778G>T result in two haplotypes A and B. Haplotype B possesses 2-3 fold lower transcriptional activity than A. The aim of this work was to investigate the general and haplotype-specific transcriptional regulation of the human *FABP2* promoter. For this purpose, reporter gene assays in human colonic carcinoma Caco-2, cervix carcinoma Hela and hepatoma Huh7 cells, site-directed mutagenesis, electrophoretic mobility shift assays and supershift-assays were used. The specific binding of hepatic nuclear factor 1 alpha (HNF-1 α), HNF-4 α and GATA factors to *FABP2* promoter haplotypes was investigated. The trans-activation of the promoter by these major intestinally expressed transcription factors was also studied. The factors are involved in differentiation and nutrient dependent gene regulation.

In electromobility shift and supershift assays the presence of two overlapping HNF-1 binding sites within the *FABP2* promoter regions -185/-165 and -169/-149 was shown. Reporter gene analysis in Caco-2 and Hela cells demonstrated that HNF-1 α activates an 836 bp *FABP2* promoter construct by 3.5 and 20-fold, respectively. This induction is identical between *FABP2* promoter haplotypes. Mutational analysis of HNF-1 element -185/-165 resulted in about 50 % reduction of basal activity of the *FABP2* promoter construct in Caco-2 cells. Disruption of the -169/-149 element had marginal effect. Co-transfection experiments in Hela cells with HNF-1 α revealed similar reduction in activity of *FABP2* promoter mutants. Further electromobility shift assays demonstrated that HNF-4 α binds at position -336/-324 of the *FABP2* promoter. Mutation of this HNF-4 binding site abolished the luciferase reporter activity of *FABP2* in postconfluent Caco-2 cells. In Hela cells, this mutation reduced the activation of the *FABP2* promoter by HNF-4 α by about 50 %. Thus, the binding element at position -336/-324 essentially determines the transcriptional activity of the promoter. This series of experiments demonstrates that *FABP2* is strongly regulated

by the transcriptional factor HNF-1 α and HNF-4 α via identified functional recognition elements.

In order to identify the causative polymorphism(s) for different activities of *FABP2* promoter haplotypes, reporter gene assays with a series of chimeric *FABP2* promoter constructs were performed in Caco-2 cells. Data show that polymorphism -80Del>T, together with -136AGTAG>Del and -168AAG>T, essentially determines different activities of *FABP2* promoter haplotypes. In accordance, in electrophoretic mobility shift assays transcriptional factors GATA-5 and -6 bind with higher binding affinities to the *FABP2* promoter region containing the -80A allele compared to B. As functional consequence, haplotype A is twice as much activated by GATA factors than haplotype B in Huh7 cells. Additionally, a construct bearing the -80B allele in the background of haplotype A reversed the activity from A to B. Thus, the GATA mediated differential activation of *FABP2* haplotypes depends on polymorphism -80Del>T.

In conclusion, the data suggest a major role of transcriptional factors HNF-1 α and HNF-4 α in the control of *FABP2* expression in intestine via a functional HNF-1 recognition element within *FABP2* promoter region -185/-165 and a HNF-4 element at position -336/-324. This seems to be of importance in the control of *FABP2* expression by differentiation and nutrients, e.g. dietary lipids. The different activities of *FABP2* promoter haplotypes are essentially determined by GATA factors and are mediated by differential GATA binding to the polymorphic promoter region -80Del>T. Taken together, the work provides insights into the molecular basis of the general and variant specific transcriptional regulation of the diabetes type 2 associated human *FABP2* gene.

ZUSAMMENFASSUNG

Das humane intestinale Fettsäure-bindende Protein (*FABP2*) ist ein cytosolisches 15 kDa Protein. Es ist an der Resorption von langkettigen Fettsäuren beteiligt. Die Phänotypisierung von *FABP2* knock-out Mäusen und Assoziationsstudien mit beschriebenen Promotorhaplotypen zeigten, dass *FABP2* ein Kandidatengen für Insulinresistenz und verwandte Krankheitsmerkmale ist. Die relevanten *FABP2* Promotorpolymorphismen -80Del>T, -136AGTAG>Del, -168AAG>T, -260G>A, -471G>A und -778G>T resultieren in zwei Haplotypen A und B. Haplotyp B weist eine zwei- bis dreifach niedrigere transkriptionelle Aktivität als A auf. In dieser Arbeit wurde die generelle und Haplotyp-spezifische transkriptionelle Regulation des humanen *FABP2* Promotors untersucht. Hierzu wurden Reporteranalysen in humanen Kolonkarzinomzellen Caco-2, Cervixkarzinomzellen Hela und Hepatomzellen Huh7, zielgerichtete Mutagenese und Gel-Retardations-Analysen angewendet. Desweiteren wurde die spezifische Bindung von Hepatozyten Nukleärem Faktor 1 alpha (HNF-1 α), HNF-4 α und GATA Faktoren an die *FABP2* Promotor Haplotypen analysiert. Außerdem wurde die Aktivierung des Promotors durch diese Transkriptionsfaktoren untersucht. Die Faktoren werden im Dünndarm exprimiert und sind an der differenzierungs- und nahrungsabhängigen Genregulation beteiligt.

In Gel-Retardations-Analysen wurden zwei überlappende HNF-1 Bindungsstellen innerhalb der *FABP2* Promotor Regionen -185/-165 und -169/-149 nachgewiesen. Reporteranalysen in Caco-2 und Hela Zellen zeigten, dass HNF-1 α ein 836 bp *FABP2* Promotor-Reporter-Konstrukt 3.5- bzw. 20-fach aktiviert. Diese Aktivierung ist unabhängig von dem *FABP2* Promotor Haplotyp. Eine Mutationsanalyse des HNF-1 Elements -185/-165 führte zu einer Erniedrigung der Basalaktivität des *FABP2* Promotorkonstruktes in Caco-2 Zellen um etwa 50 %. Die Mutation des -169/-149 Elements hatte keine relevante Auswirkung auf die Promotoraktivität. Co-Transfektions-Experimente in Hela Zellen mit HNF-1 α zeigten ähnliche Effekte auf die Aktivität der *FABP2* Promotor Mutanten. Weitere Gel-Retardations-Analysen belegen, dass HNF-4 α an Position -336/-324 des *FABP2* Promotors bindet. Die Mutation dieser HNF-4 α Bindungsstelle führte zu einer vollständigen Inaktivierung der *FABP2* Promotor-Reporter Aktivität in postkonfluenten Caco-2 Zellen. In Hela

Zellen führte diese Mutation zur Erniedrigung der HNF-4 α -induzierten Aktivierung des *FABP2* Promotors um etwa 50 %. Somit wird die transkriptionelle Aktivität des *FABP2* Promotors durch das identifizierte HNF-4 Bindungselement -336/-324 erheblich beeinflusst. Die Daten zeigen insgesamt, dass der *FABP2* Promotor maßgeblich durch die identifizierten funktionellen HNF1 und HNF4 Erkennungssequenzen reguliert wird.

Um die ursächlichen Polymorphismen für die unterschiedlichen Aktivitäten der *FABP2* Promotor Haplotypen zu identifizieren, wurden Analysen mit einer Vielzahl von chimären *FABP2* Promoter-Reporter-Konstrukten in Caco-2 Zellen durchgeführt. Die Daten zeigen, dass hauptsächlich der Polymorphismus -80Del>T, in Kombination mit -136AGTAG>Del und -168AAG>T, die unterschiedlichen Aktivitäten der *FABP2* Promotor-Haplotypen verursacht. In Übereinstimmung mit diesen Befunden zeigten Gel-Retardationsanalysen, dass die Transkriptionsfaktoren GATA-5 und -6 mit höheren Affinitäten an diejenige *FABP2* Promotorregion binden, die das -80A Allel enthält. In Übereinstimmung mit diesem Ergebnis wurde in Huh7 Zellen aufgezeigt, dass die GATA Faktoren den *FABP2* Promoter Haplotyp A doppelt so stark aktivieren wie Haplotyp B. Weiterhin wurde eine *FABP2* Promotormutante vom Haplotyp A hergestellt, die das -80B Allel enthält. Die GATA-5 induzierte Aktivität dieser Mutante ist nahezu identisch mit der Aktivität des Haplotyps B. Somit wird die GATA vermittelte differentielle Aktivierung der *FABP2* Haplotypen vom Polymorphismus -80Del>T verursacht.

Insgesamt zeigt die Untersuchung, dass die Transkriptionsfaktoren HNF-1 α und HNF-4 α maßgeblich die Aktivität des *FABP2* Promotors bestimmen. Dieser Effekt wird durch zwei funktionelle Bindungsstellen vermittelt, die sich im *FABP2* Promoter in Position -185/-165 und -336/-324 befinden. Dieser Befund ist vermutlich für die Kontrolle der *FABP2* Expression durch Differenzierung und Nährstoffe, wie zum Beispiel Nahrungsfette, bedeutsam. Die unterschiedlichen Aktivitäten der *FABP2* Promotor Haplotypen werden im Wesentlichen durch GATA Faktoren und den *FABP2* Promotor Polymorphismus 80Del>T bestimmt. Somit liefert diese Arbeit insgesamt Erkenntnisse zur molekularen Basis der generellen und Variantenspezifischen transkriptionellen Regulation des Diabetes Typ 2 assoziierten humanen *FABP2* Gens.

GENERAL INTRODUCTION

Fatty acid binding proteins (FABPs) are small (14-15 kDa) cytosolic proteins and were first described by Ockner et al. (1). The FABP family belongs together with the lipocalin family to the calycin superfamily, which functions in binding of small hydrophobic molecules. FABPs are conserved throughout the animal kingdom in vertebrates and invertebrates (2), although invertebrate FABPs do not have pronounced sequence motif analogies with vertebrate FABPs (3). In vertebrates, the family comprises 9 members: liver (FABP1), intestinal (FABP2), heart/muscle (FABP3), adipocyte (FABP4), epidermal (FABP5), ileal (FABP6), brain (FABP7), myelin P2 (MP2, FABP8) and testis FABP (FABP9) (4). The names were given according to the tissue, where a FABP was first discovered or is predominantly expressed.

FABPs have a common tertiary structure that comprises 10 antiparallel β -strands forming a β -barrel, which are structured into two nearly orthogonal β -sheets, and two short anti-parallel α -helices that are located over one end of the barrel. This structural feature allows the formation of a binding pocket, which is partially filled with water. In the binding cavity polar amino acid residues interact with carboxylate groups of fatty acids by hydrogen bonding (5). Ligand binding properties differ between single FABPs. Besides saturated and unsaturated long-chain free fatty acids, specific FABPs are able to bind also other ligands, such as acyl-CoAs, bile salts, cholesterol, heme, eicosanoids or other hydrophobic molecules (4).

The cellular functions of FABPs seem to be very versatile and may be specific for single FABPs in dependence on ligand binding and tissue distribution. FABPs influence metabolic pathways by binding and intracellular transport of fatty acids and other ligands, which are delivered to specific cellular compartments and enzymes. They mediate fatty acid signaling by transferring fatty acids to the nucleus as ligands for transcription factors, such as peroxisome proliferator-activated receptors (PPARs), and seem to be involved in regulation of growth and differentiation [reviewed in (6)]. More recently, an antioxidant function was shown for FABP1 (7, 8).

In intestine, the genes encoding FABP1, FABP2 and FABP6 are expressed. The expression patterns of these FABP members are overlapping, even if they show staggered expression maxima (9). Whereas FABP1 and FABP6 are also expressed in other tissues, the expression of FABP2 seems to be restricted to the intestine (6, 10). At a cellular level, FABP2 is absent in crypt cells but is present at high levels in enterocytes of upper villus region (11, 12), where fat absorption takes place. 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 (13-15).

Results gained from *in-vitro* and *in-vivo* studies of FABP2 functions are not consistent. Overexpression of FABP2 in cell lines, that endogenously express FABP1 and FABP2 (16-18), led to controversial results. Increased FABP2 expression in colonic carcinoma cell line Caco-2 led to reduction in fatty acid incorporation (19), increased cellular lipid content and reduced secretion (20). A similar approach in another intestinal epithelial cell line (HIEC) resulted in no alteration of fatty acid metabolism (17). Experimental data obtained in non-expressing FABP2 cells seem to be more homogenous. In undifferentiated stably FABP2 expressing ES cells, initial and maximal fatty acid uptake and cytoplasmatic diffusion was increased (21). Diffusion rate was also enhanced in FABP2 transfected L-cell fibroblasts (22). Furthermore, these L-cell fibroblasts showed increased initial fatty acid (oleic acid) esterification into triacylglycerols and elevated triacylglycerol and cholesteryl ester mass (23, 24). Analysis of a *FABP2* knockout mouse did not strengthen the hypothesis that FABP2 is essential for intestinal fat absorption. Mal-absorption of fatty acids was not observed (25). Functional redundancies of FABPs may explain the difficulties to study the functions of a single FABP in Caco-2 cells or knock-out mice.

Functional studies dealing with the *FABP2* exon 2 polymorphism G>A (rs1799883) further support the hypothesis that FABP2 is involved in fat absorption. This polymorphism results in an amino acid exchange of Ala to Thr at codon 54 and causes higher *in-vitro* binding affinity of long-chain fatty acids (26). Transfection of both wild type and variant FABP2 in Caco-2 cells increased cellular fatty acid transport and triglyceride secretion (15). And intriguingly, in intestinal explants of

homozygous carriers of Ala or Thr-encoding alleles the secretion of newly esterified triglycerides and chylomicrons was higher in carriers of Thr-encoding allele (14).

Although the function of FABP2 is not perfectly understood so far, studies with FABP2 knock-out mice as well as association studies provide evidence that *FABP2* is a susceptibility gene for diabetes type 2 and related traits. Male *FABP2* knock-out mice showed key symptoms of the metabolic syndrome such as increased plasma levels of insulin and triglyceride, increased body weight, and changes in liver histology, possibly resulting from impaired lipid metabolism (25, 27). Studies with the exon FABP2 polymorphism revealed that the FABP2 Thr54 variant is associated with parameters of insulin resistance. This was shown in numerous studies with different human populations [reviewed in (28)]. More recently, also the *FABP2* promoter haplotypes were associated with postprandial triglyceride levels (29) and BMI (30). Previous studies including the work presented in [1] revealed that the 5' upstream promoter region of *FABP2* contains insertion/deletion sites -80Del>T, -136AGTAG>Del, -168AAG>T and SNPs -260G>A, -471G>A and -778G>T, which are in complete linkage disequilibrium resulting in only two haplotypes A>B (29-31). The rare *FABP2* promoter haplotype B showed almost 2-3 fold lower transcriptional activity than haplotype A in Caco-2 cells (30, 31).

Since FABP2 is an important gene in regulation of lipid, insulin and glucose homeostasis and its expression level seems to be critical for the development of diabetes type 2, the understanding of transcriptional control of FABP2 is of specific importance. As demonstrated in cell culture as well as in animal models, FABP2 expression is basically dependent on differentiation and developmental processes, whereby it is up-regulated along with differentiation of cells and tissues (11, 12, 16, 18, 32, 33). *FABP2* expression is modulated also by hormones, fatty acids and cytokines. It was increased, when Caco-2 cells were stimulated with hydrocortisone, insulin, leptin, butyric acid, oleic acid, phosphatidylcholine and the cytokine tumor necrosis factor-alpha (TNF- α) (19), and decreased when cells were incubated with epidermal growth factor (EGF) (34). The peptide tyrosine tyrosine (PYY), a gut hormone that is secreted in presence of luminal long-chain fatty acids, increased the FABP2 expression in small intestinal somatic cell hybrids (35, 36).

In rats and mice PPAR-alpha agonists increased FABP2 mRNA level (37). Also knock-out mice gave evidence for regulation of FABP2 by specific signalling molecules. In transforming growth factor β 1 (TGF β 1) knock-out mice FABP2 was down-regulated (38). Maybe, GATA factors (39) are involved in this regulation. Interestingly, FABP2 was also down-regulated in Estrogen related receptor α (ERR α) knock-out mice (40), that may be a clue for gender specific differences in association studies.

The regulation of rat and mouse *FABP2* promoters has been studied to some extent. Cohn et al. shows binding of CCAAT enhancer binding protein (C/EBP) to a proximal promoter element in rat promoter (41). In another study, a direct activation of rat *FABP2* promoter by the hepatocyte nuclear factor 4 (HNF-4) and the apolipoprotein A1 regulatory protein 1 (ARP-1) was demonstrated (38). Furthermore the TGF β -SMAD/GATA-4 signaling was shown to be involved in the up-regulation of mouse *FABP2* promoter activity (29-31). Early *FABP2* promoter studies in transgenic mice with *FABP2* promoter-growth hormone reporter gene have identified promoter elements in rat *FABP2* promoter that regulate *FABP2* expression along duodenal-colonic and crypt-villus axis within the gut epithelium (40).

Previous investigations of human *FABP2* promoter regulation concerned only basal activities of the two FABP2 promoter haplotypes (30, 31), that differ 2-3 fold in Caco-2 cells. To gain more insights in this haplotype specific but also the general regulation of human *FABP2* promoter, this work (4 publications or manuscripts)¹ dealt with the following questions.

1. Is the difference in the basal *FABP2* promoter activities between the two haplotypes also present in postconfluent, semi-differentiated Caco-2 cells, that resemble rather the phenotype of enterocytes of small intestine.
→ 1. Y. Li, E. Fisher, M. Klapper, H. Boeing, A. Pfeiffer, J. Hampe, S. Schreiber, B. Burwinkel, J. Schrezenmeir, F. Döring. **Association between functional FABP2 promoter haplotype and type 2 diabetes.** *Hormone and Metabolic Research.* 2006 May;38(5):300-7

¹ The work was carried out mainly in the Institute of Human Nutrition and Food Science, Department of Molecular Nutrition.

2. Which polymorphism(s) and transcription factor(s) are causative for these different activities.
- 1. Y. Li, E. Fisher, M. Klapper, H. Boeing, A. Pfeiffer, J. Hampe, S. Schreiber, B. Burwinkel, J. Schrezenmeir, F. Döring. **Association between functional FABP2 promoter haplotype and type 2 diabetes.** *Hormone and Metabolic Research*. 2006 May;38(5):300-7
 - 2. M. Klapper, M. Böhme, I. Nitz and F. Döring. **Type 2 diabetes associated Fatty Acid Binding Protein 2 promoter haplotypes are differentially regulated by GATA factors.** *Human Mutation*. in press
3. Which transcription factors mediate the general regulation of human *FABP2* expression.
- 2. M. Klapper, M. Böhme, I. Nitz and F. Döring. **Type 2 diabetes associated Fatty Acid Binding Protein 2 promoter haplotypes are differentially regulated by GATA factors.** *Human Mutation*. in press
 - 3. M. Klapper, M. Böhme, I. Nitz and F. Döring. 2007. **The human intestinal fatty acid binding protein (hFABP2) gene is regulated by HNF-4 alpha.** *Biochemical and Biophysical Research Communication*. 2007 Apr 27;356(1):147-52.
 - 4. M. Klapper, M. Böhme, I. Nitz and F. Döring. **Transcriptional regulation of the Fatty Acid Binding Protein 2 (FABP2) gene by the hepatic nuclear factor 1 alpha (HNF-1 α).** submitted in *Journal of Cellular Physiology*

GENERAL DISCUSSION

Previous studies regarding transcriptional regulation of human *FABP2* were mainly done to characterize the basal activities of the two *FABP2* promoter haplotypes (29-31). The genetic variation in the *FABP2* gene promoter is associated with postprandial triglyceride levels (29), body composition, lipid plasma levels (30) and diabetes (in [1], but not part of this dissertation). As shown previously (30, 31), the basal transcription of the rare *FABP2* promoter haplotype B is about 2-3 fold lower than the common haplotype A. However, little effort was done to reveal regulation of the *FABP2* promoter. The aim of the current work was to investigate the mechanisms of general and differential regulation of *FABP2* promoter haplotypes.

The differences in activities of 836 bp (upstream of translation start) *FABP2* promoter reporter gene constructs were confirmed in this work in post-confluent Caco-2 cells [1]. In these partially differentiated cells, *FABP2* mRNA and protein are expressed. Thus, the differentiated state of Caco-2 cells reflects the enterocytes phenotype in villus region of small intestine, where *FABP2* is expressed. Therefore these data are more conferrable to *in-vivo* situation in small intestine. It should be noted, that there is no evidence for genotype specific expression of *FABP2 in-vivo*, since it is very difficult to obtain healthy tissue samples from the intestine. A first approach to reveal the causal polymorphisms for different transcriptional activities of *FABP2* promoter haplotypes, promoter assays with shortened (388 bp upstream of translation start) promoter constructs, containing only the 4 proximal polymorphisms, were performed [1]. Promoter activities of these shortened constructs exhibit nearly the same differences in their activities as the long (836 bp) promoter version [1]. Therefore the differences in promoter activity seem to be determined by one or more of these 4 proximal promoter polymorphisms. To study these polymorphisms in more detail reporter assays using chimeric *FABP2* promoter constructs were performed [2]. These data show that difference in haplotype activities is essentially determined by the first three proximal *FABP2* promoter polymorphisms, whereby -80Del>T has the greatest impact. Accordingly, clear differences in allele specific binding of nuclear proteins from post-confluent Caco-2 cells were observed for -80Del>T [2]. Furthermore, a higher binding capacity of GATA-5 and GATA-6 to the *FABP2* promoter region containing the -80A allele in comparison to the -80B allele was

demonstrated, that was reflected by higher induction of *FABP2* promoter haplotype A compared to B. Therefore, different transcriptional activities of *FABP2* promoter haplotypes are mainly caused by the -80Del>T polymorphism mediated by GATA factors.

GATA-4, -5 and -6 are zinc finger-containing transcription factors and are present in various meso- and endodermal derived tissues such as intestine, heart, liver, lung and gonad. GATA-4, -5 and -6 are important transcriptional regulators of differentiation and embryonic development and tissue-specific gene expression (42-45). These factors are also present in Caco-2 cells with GATA-6 as the predominant form (43, 46-48). A specific expression pattern of GATA-4, -5 and -6 was described in intestinal cells. GATA-6 is present at high levels in proliferating and less differentiated cells, whereas GATA-4 and -5 are strongly up-regulated along the crypt-villus-axis during cell differentiation (43). GATA factors are essential for general and tissue specific expression (38, 49). Interestingly, GATA factors bind to highly conserved elements in *FABP2* promoters of mouse (*mFABP2*) and zebrafish (*zFABP2*) at regions corresponding to the human *FABP2* promoter segment encompassing the -80Del>T polymorphism. These studies emphasize the crucial role of GATA in regulation of *FABP2* in vertebrates. Interestingly, GATA-4 cooperates with Smad transcription factors, the downstream effectors of the TGF β signalling pathway, to activate the *mFABP2* promoter (38). TGF β is a critical regulator of development and maintenance of intestinal epithelium (50). Taken together, GATA factors differentially induce the *FABP2* promoter haplotypes mediated by the -80Del>T polymorphism encompassing promoter region.

HNF-1 α was identified in this work as an important transcriptional regulator of *FABP2*. It mediates 50 % of *FABP2* promoter activity in Caco-2 cells. The HNF-1 α induced *FABP2* promoter activity in Hela cells is mainly dependent on the *FABP2* promoter region -185 to -165. Interestingly this region contains two HNF-1 α binding sites, whereby only one site seems to be functional. The human *FABP2* promoter segment -185 to -165 has no corresponding element in *FABP2* mouse, rat or bovine promoter sequence at similar position but is conserved within a nearly conserved region in primates. This suggests a unique intestinal regulation of *FABP2* by HNF-1 α in primates. Although the identified HNF-1 α sites are located within the -168AAG>T

polymorphic region, no haplotype specific activation of *FABP2* was observed [4]. Further investigations demonstrated that HNF-4 α is also a key regulator of *FABP2* transcription. It was shown, that HNF-4 α binds at position -336 to -324 of the *FABP2* promoter. This binding essentially determines the transcriptional activity of the promoter. Together, these data emphasize the essential role of HNF-1 α and HNF-4 α as general activators of *FABP2* promoter activity [3].

Synergistic actions of transcription factors

Taking into account that transcriptional regulation is not the result of binding of single transcription factors to isolated motifs, synergistic actions between the investigated and other intestinal expressed factors should be considered. Synergistic actions between transcription factors are mediated for example by physical interactions of factors and/or binding to neighbouring sites. GATA factors may differentially regulate *FABP2* promoter haplotypes in synergistic action with HNF-1 α , HNF-4 α and CDX-2. Synergistic interactions between these factors are responsible for tissue-specific high expression of intestinal genes (51-56). This possible cooperative activation of *FABP2* promoters by GATA factors with HNF-1 α and other factors very likely results in high expression of *FABP2*, whereby the difference in transcriptional activities of *FABP2* haplotypes may be essentially determined by GATA factors. Since GATA factors play a major role in differentiation processes of intestine and are supposed to be important for intestine-specific high expression (42-45), an *in-vivo* expression that is similar robustly regulated with respect to the differential regulation of *FABP2* promoter haplotypes may be proposed. HNF-4 α was previously described to synergistically interact with HNF-1 α (57, 58) and other factors, such as Sp1 (59) or HNF-3 (60) to induce reporter activity of apoCIII, FABP1 and apoA1 promoters, respectively. HNF-4 α binding sites were proven to be essential for functional synergism with these factors. Since the here investigated GATA factors, HNF-1 α and HNF-4 α may also function on *FABP2* promoter in synergistic action, they may be in concerted action responsible for tissue-specific high expression of *FABP2*.

Genotype interactions between genes

FABP2 is a candidate gene for diabetes type 2 (non-insulin dependent diabetes mellitus, NIDDM) and related traits. The *FABP2* T54 allele of exon 2 A54T polymorphism could be linked to insulin resistance in different populations (28). Also

FABP2 promoter haplotypes were associated with NIDDM [1], body weight (30) and triglyceride levels (29). Interestingly, GATA-4 cooperates with Smad transcription factors, the downstream effectors of the TGF β signalling pathway, to activate the mouse *FABP2* promoter (38). Notably, polymorphic sites in the TGF β gene are associated with diabetes and obesity phenotypes, e.g. body composition, increased insulin and glucose levels (61, 62). Furthermore, also HNF-1 α and HNF-4 α are candidate genes for diabetes type 2. HNF-1 α is discussed to be associated with late onset form of diabetes type 2 (63-65). The identified coding SNPs of HNF-1 α I27L and A98V alter its transcriptional activity (64). Although there is no differential regulation of *FABP2* promoter haplotypes by HNF-1 α , the combination of particular *FABP2* and *HNF-1 α* genotypes in individuals may be of importance for differences in expression level of *FABP2 in-vivo*. In HNF-4 α , 3 SNPs near the P1 promoter and exons 1-3 could be associated with diabetes type 2 in a Finnish population (66). In conclusion, on basis of the here demonstrated functional interaction of *FABP2* and GATA and very likely TGF β , HNF-1 α and HNF-4 α it seems to be a powerful approach for genetic epidemiologists to study effects of genotype combinations of these genes on the development of NIDDM and other traits of the metabolic syndrome.

Possible dietary regulation of FABP2

An influence of dietary factors on HNF-1 α and HNF-4 α by modulating signalling pathways was provided by several observations and mechanisms. Firstly, HNF-1 α is involved in the glucose-dependent transcriptional regulation of sucrose-isomaltase (67-69) and sodium-dependent glucose transporter (70). Secondly, expression and DNA binding of HNF-1 α in livers of protein restricted rats were increased (71). Thirdly, glucose as well as fatty acids stimulated insulin secretion resulting in decreased activity of cAMP-dependent protein kinase A (PKA). Phosphorylation of HNF-4 α by PKA results in decreased binding affinity to DNA (72). Fourthly, an increased ATP level, that reflects the energy status of a cell, inhibit 5'-AMP dependent kinase (AMPK) (73). AMPK was shown to suppress the expression of several transcription factors including HNF-1 α and HNF-4 α (74) and phosphorylates HNF-4 α which leads to reduced protein stability, dimer formation and DNA binding (75). Expression and stability of HNF-1 α and binding of HNF-1 β to DNA, as previously shown with sucrose-isomaltase promoter, is also dependent on protein

phosphatase 1a/2a activity (76). Together, diet induced changes in ratio of cellular protein kinase and phosphatase activity seem to regulate transcription factor activities, including HNFs. Intriguingly, HNF-4 α , originally considered as orphan nuclear receptor, was previously shown to bind fatty acid acyl-CoA esters as ligands. These were able to modulate HNF-4 α DNA binding activity in dependence on their chain length and saturation grade (77, 78). Interestingly, HNF-4 α is involved in transcriptional regulation of ApoA-IV by lipid micelles, when micelles supplied to apical pole of Caco-2 cells.

As shown recently, the peroxisome proliferator activated receptors (PPARs) activate *FABP2* promoter (79), whereas sterol-regulatory binding protein (SREBP) decreases activity (data not shown). These factors are regulated by fatty acids. Interestingly, HNF-4 and PPARs are inversely regulated. Whereas HNF-4 is inhibited by polyunsaturated fatty acids (PUFAs) (77, 78), PPARs are activated by these ligands (80). SREBP 1a and 1c are negatively regulated at transcriptional as well as post-translational, proteolytic processing level by PUFAs (81-83). Therefore, HNF-1 α and HNF-4 α in complex interplay with other transcription factors may be general mediators of nutritional signals and cellular energy status for transcriptional adaptations of *FABP2* expression in enterocytes. Based on this hypothesis it also seems to be interesting to study the influence of *FABP2* promoter haplotypes with respect to nutrients, such as fat intake in an epidemiological approach.

Although the investigation of the general and promoter haplotype specific regulation is of interest and importance for knowledge about general regulatory mechanisms, the presented data are difficult to use for specific modulation of (genotype dependent) expression of *FABP2*. As discussed above, a possible dietary prevention could be mediated by several, partly opposing mechanisms. Because of potential imbalances of transcription factors and general artificial conditions in cell culture, investigation regarding nutrient dependent regulation of *FABP2* promoter in Caco-2 cells is of limited practical relevance. Furthermore, specific modulation of an isolated gene in complex transcriptional regulation processes is not feasible. It should also be considered, that transcriptional regulation is only one possible regulatory mechanism among many to modulate cellular protein level and activity. These posttranscriptional mechanisms include for example mRNA degradation, translational inhibition by non-

coding and micro RNAs, protein phosphorylation or protein degradation. However, the implemented experimental system seems to be very powerful for future studies to investigate principles of nutrient dependent and variant specific transcriptional regulation of the diabetes type 2 associated human *FABP2* gene.

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PUBLICATIONS AND SUBMITTED MANUSCRIPTS

Y. Li¹
E. Fisher²
M. Klapper¹
H. Boeing²
A. Pfeiffer²
J. Hampe³
S. Schreiber³
B. Burwinkel⁴
J. Schrezenmeir⁵
F. Döring¹

Association Between Functional FABP2 Promoter Haplotype and Type 2 Diabetes

Abstract

Fatty acid-binding protein 2 (FABP2) is a cytosolic protein expressed exclusively in epithelial cells of the small intestine. Some, albeit not conclusive, evidence indicates that the Thr-allele of FABP2 Ala54Thr polymorphism is associated with type 2 diabetes. More recently, common FABP2 promoter polymorphisms have shown association with postprandial increase of triglycerides, body composition and plasma lipid levels. Therefore, we reasoned that variants in the FABP2 promoter may also predispose to type 2 diabetes mellitus. In our Caucasian study population, we found three SNPs and three insertion-deletion polymorphisms that are in complete linkage disequilibrium defining promoter haplotype A and B within 1 kb 5' of the FABP2 initiation codon. Haplotype calculations indicated that the FABP2 promoter and Ala54Thr variants were strongly linked. Functional analysis of promoter fragments demonstrated that haplotype difference is caused by polymorphisms within 260 bp downstream of the FABP2 initiation codon. Using a prospective case-control study nested within the EPIC-Potsdam cohort of 192 incident

type 2 diabetes cases and 384 sex-/age-matched controls, male subjects carrying the FABP2 haplotype B allele showed significantly decreased risk of type 2 diabetes when adjusted for BMI (OR = 0.50, 95% CI = 0.28–0.87, $p < 0.05$) and additional covariates (OR = 0.42, 95% CI 0.22–0.81, $p < 0.01$). Further adjustment for the Ala54Thr polymorphism revealed an OR of 0.18 (95% CI 0.06–0.49, $p < 0.001$). Similarly, Ala/Ala homozygote males carrying the promoter haplotype B had decreased risk (0.33, 0.11–0.94, $p < 0.05$) of type 2 diabetes after stratification for the Ala54Thr polymorphism. FABP2 promoter haplotypes or genotype combinations defined by the promoter and Ala54Thr polymorphism were not associated with BMI, body fat, leptin, HbA_{1c}, total cholesterol or HDL. In conclusion, our findings suggest that the functional FABP2 promoter haplotype may contribute to type 2 diabetes in a sex-specific manner.

Key words

Fatty acid-binding protein · polymorphism · case-control study · SEAP assay

Introduction

Fatty acid-binding proteins (FABP) represent a family of small (14–15 kDa) cytosolic non-enzymatic proteins that play an essential role in a) intracellular transport and metabolism of free

fatty acids, b) fatty acid-signaling pathways, and c) cell growth and differentiation [1]. More than 20 different FABP genes are expressed in a tissue-specific manner. The intestinal isoform FABP-2 (I-FABP) has a high affinity for saturated and unsaturated long-chain fatty acids, and participates in absorption of dietary

Affiliation

¹ Molecular Nutrition, Christian-Albrechts University, Kiel, Germany

² Departments of Epidemiology (E.F. and H.B.) and Clinical Nutrition (AF), German Institute of Human Nutrition, Potsdam-Rehbruecke, Nuthetal, Germany

³ Institute for Clinical Molecular Biology, Christian-Albrechts University, Kiel, Germany

⁴ Molecular Genetic Epidemiology, German Cancer Research Center, Heidelberg, Germany

⁵ Institute for Physiology and Biochemistry of Nutrition, Federal Research Centre for Nutrition and Food, Kiel, Germany

Correspondence

Frank Döring · Molecular Nutrition · University of Kiel · Hermann-Weigmann-Strasse 1 · 24103 Kiel · Germany · Fax: +49 (0) 431-6092472 · E-Mail: doering@molmat.uni-kiel.de

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fatty acids [2]. Target inactivation of the *FABP2* gene in mice leads to hyperinsulinemia, suggesting that *FABP2* has a central function in fat and hormone metabolism [3].

A significant linkage between the *FABP2* locus and insulin resistance has been identified in Pima Indians using a positional candidate gene approach [4]. Since *FABP2* is a potent candidate gene for hyperinsulinemia at this locus, a subsequent search for polymorphisms was initiated and an Ala54Thr (G→A) substitution in exon 2 was identified [5]. The Thr54 allele could be linked to dyslipidemia and insulin resistance in different populations [6–11] such as Mexican-Americans, Japanese and Caucasians. In contrast to that, an association between the Ala54Thr polymorphism and a diabetes-related phenotype has not been found in other populations [12,13]. In addition to the original study on Pima Indians, the associations between Ala54Thr polymorphism and various quantitative traits related to insulin sensitivity have not been found in other studies [14,15]. More recently, polymorphisms in the promoter of *FABP2* were investigated in three studies [16–18]. *FABP2* promoter polymorphisms are associated with postprandial increase of triglycerides, body composition and plasma lipid levels in non-diabetic subjects. Therefore, we investigated whether *FABP2* promoter polymorphisms are associated with the risk of type 2 diabetes. A case-control study on 192 incident cases of type 2 diabetes and 384 control subjects embedded in the EPIC-Potsdam study were employed for genotyping. Ala54Thr genotypes were taken into account while analyzing the effect of the promoter polymorphism. In addition, the functionality of promoter polymorphisms was investigated by reporter assays in *FABP2*-expressing Caco-2 cells.

Research Design and Methods

Subjects

Subjects were taken from the European Prospective Investigation into Cancer and Nutrition (EPIC) Potsdam study, a population-based, longitudinal study comprising a total of 27,548 participants from the area of Potsdam, Germany [19]. Baseline examinations, including anthropometric measurements, blood sampling, a self-administered food frequency questionnaire, and a personal interview on lifestyle habits and medical history were conducted between 1994 and 1998. During the first follow-up, on average 2.3 years after recruitment, 192 incident cases of type 2 diabetes mellitus were identified and confirmed by the primary care physician [20]. Cases were matched with 2 control subjects each by age and sex ($n = 384$). Gender distribution of study participants was 59% male and 41% female subjects with a mean age of 55.5 years (35 to 65 years). All study participants had given informed consent and the genotype assessment was agreed to by the local ethic committee. Body weight, height and fat percent were determined at recruitment by means of standardized procedures as described previously [19]. BMI was estimated as weight (kg) divided by height (m) squared. Detailed information on drug use was obtained at baseline and consisted of all medications being taken during the previous four weeks on the level of medication name. Sports activities were calculated from hours/week in summer and winter.

Genotyping and sequencing of the *FABP2* promoter polymorphisms

At baseline, 30 ml venous blood was taken from each study participant and DNA was extracted by the spin-column method (E.Z.N.A. Blood DNA Kit, PeqLab, Germany). Genotyping of insertion/deletion promoter polymorphisms rs5861423 (-136 bp upstream from the *FABP2* initiation codon) and rs5861422 (-80) was performed by pyrosequencing using the PSQ 96 instrument (Biotage AB, Uppsala) and PSQ 96 SNP reagents. Genotyping of SNPs rs6857641 (-260) and rs2282688 (-471) was performed with the TaqMan system (ABI, Foster City, CA, USA). Fluorescence was measured using the ABI Prism 7900 HT sequence detection system. Direct sequencing of genomic DNA samples from six unrelated subjects confirmed published SNPs [16–18] in the 5' region of *FABP2*. A 838 bp fragment upstream from the *FABP2* (M18079) start codon was PCR-amplified, inserted into pENTR/D-TOPO vector (Invitrogen, Karlsruhe, Germany) and sequenced by terminator cycle sequencing using Big Dye chemistry and ABI 3700 capillary DNA sequencer (Applied Biosystems, Foster City, CA, USA). All PCR and sequencing primers were designed using the OLIGO software and primers were purchased from MWG Biotech AG (Ebersberg, Germany). Sequences of pyrosequencing primers, PCR primers as well as TaqMan assay primers and probes are available on request.

FABP2 promoter SEAP reporter constructs

The secreted alkaline phosphatase (SEAP) reporter gene system was used (BD Biosciences Clontech, Palo Alto, CA, USA). Cloning procedures were performed using Gateway Technology (Invitrogen). 836 bp and 388 bp fragments upstream from initiation codon of *FABP2* encompassing all six or the first four (-80, -136, -168, -260) promoter polymorphisms were amplified using Pfu-Polymerase (Invitrogen) from genomic DNA of subjects homozygous for promoter haplotype A or B. Amplified products were inserted into pENTR/D-TOPO vector and sequenced. The resulting plasmids pENTR-*FABP2*-Prom-*attL* contained *attL*-sites allowing recombination reaction with *attR*-sites. *attR*-sites (were inserted into the *SrfI* blunt-end site of the reporter plasmid pSEAP-Basic. Subsequent recombination, catalyzed by LR Clonase, of the *attL*-containing vector pENTR-*FABP2*-Prom-*attL* with *attR*-containing vector pSEAP-Basic-*attR* resulted in final *FABP2* promoter SEAP reporter constructs. The pSEAP2-Basic plasmid lacking *FABP2* promoter fragments served as a negative control vector.

Cell culture

CaCo-2 and Hela cells were provided by American Type Culture Collection (ATCC, Rockville, MD) and were used between passage 70 and 110. Cells were cultured and passaged in MEM supplemented with 10% (HeLa) or 20% (Caco-2) fetal calf serum and 1 mM non-essential amino acids in a humidified incubator at 37°C under an atmosphere of 5% CO₂. Cells were passaged at pre-confluent densities using a solution containing 0.05% trypsin and 5 mM EDTA.

Reverse transcriptase (RT)-PCR and Western-blot analysis

For RT-PCR, total RNA was isolated from pre-confluent and post-confluent (day 1–6) Caco-2 cells with the RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Oligonucleotide primer pairs for *FABP2* were designed based on GenBank sequence accession number M18079. Sequences of pri-

mers are available on request. For western-blotting, total protein extracts from pre-confluent and post-confluent (day 1–6) cells were separated by SDS-PAGE (12–20%) using the XCell-Sure-Lock Mini Gelsystem (Invitrogen, Germany), transferred to nitrocellulose, and probed with anti-FABP2 antibody (Santa Cruz Biotechnology, USA).

Transfection and SEAP reporter assay

Cells were plated in 24-well plates and grown overnight to 40–50% confluence. Transient transfection was performed using FuGene6 (Roche) according to the manufacturer's protocol. Cells were transfected with 0.192 μ g (Caco-2) or 0.384 μ g (HeLa) of the reporter constructs or control plasmid pSEAP2-Basic. 0.008 μ g (Caco-2) or 0.016 μ g (HeLa) luciferase-containing plasmid pGL3-control (Promega) was co-transfected with the reporter constructs to standardize for transfection efficiency. Caco-2 culture media were exchange 48 h after transfection. Cells were grown another 72 h before media were taken for the SEAP reporter assay. Media from HeLa cells were taken for the SEAP assays 48 h after transfection. The SEAP reporter assay was carried out with the Great EscAPE SEAP Chemiluminescence kit (Clontech) on a white opaque 96-well flat-bottom microplate according to the manufacturer's protocol. SEAP activities were normalized by luciferase activities measured from lysed cell pellets using commercial Bright-Glo luciferase assay system (Promega). Activities of the empty control vector pSEAP-Basis were subtracted from SEAP reporter activities.

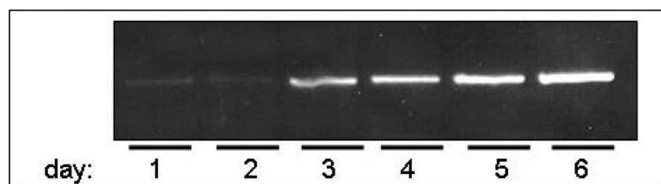
Statistical analysis

The study population was tested as a whole and cases/controls individually for the distribution of genotypes according to the Hardy-Weinberg-equilibrium with a χ^2 test (1 degree of freedom). Statistics were computed using SAS software 9.1 (SAS Institute, Cary, NC). Data on biochemical and genetic measurements and measurements of body composition were missing in ten type 2 diabetes mellitus and twelve control subjects; therefore, analysis of covariance (ANCOVA) was performed on a final study sample of 182 cases and 372 controls. Mean values were adjusted for sex, age, body mass index and waist-hip-ratio or in the case of anthropometric variables for sex and age; p-values were corrected for multiple comparisons. Associations between disease and each SNP were assessed by conditional logistic regression analysis. Significance level was set at $p < 0.05$ (1 degree of freedom). Multivariate logistic regression analysis was conducted adjusting for body mass index (kg/m^2), waist-hip ratio, sports activities (h/week), total energy (kJ/d) and alcohol intake (g/d) and other non-continuous covariates – presence of co-morbidities (hypertension or/and hyperlipidemia), smoking (current smoker, non-smoker and former smoker), use of lipid-lowering drugs (statins, fibrates, CSE-inhibitors, others) and antihypertensives (ACE-inhibitors, beta-blockers, Ca^{2+} -channel blockers, others).

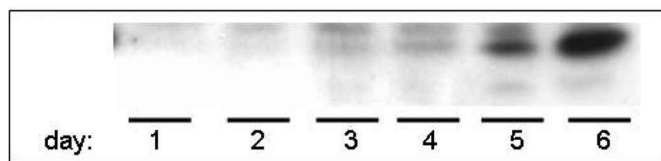
Results

Functional FABP2 promoter haplotypes

The 5' upstream region of the FABP2 (M18079) initiation codon contains three single nucleotide polymorphisms and three insertion/deletion polymorphisms [16–18]. In our study, direct sequencing of the FABP2 5' region as well as genotyping of poly-

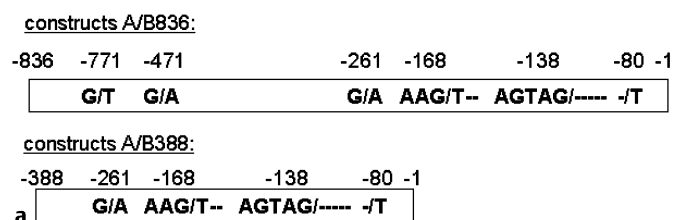


a

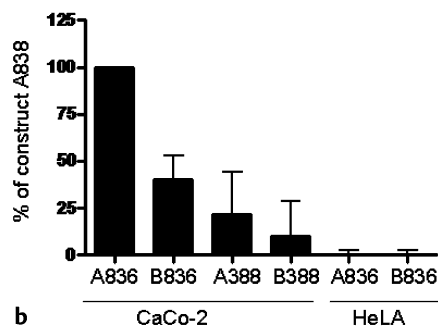


b

Fig. 1 FABP2 mRNA and protein expression in post-confluent Caco-2 cells. RNA and protein were isolated from pre-confluent and post-confluent (day 1–6) Caco-2 cells. FABP2 mRNA (a) was analyzed by RT-PCR. Western-blot (b) was used for protein detection.



a



b

Figure 2 Transcriptional activity in the reporter gene assay for various FABP2 constructs. a Schematic presentation of reporter gene constructs used in transient transfection. The 5' downstream region of the FABP2 initiation codon contains six polymorphic sites at position –80 bp, –136, –168, –260, –471, and –778. These sites are in complete linkage disequilibrium and define FABP2 haplotype A and B. Positions of the polymorphisms were determined from the genomic FABP2 reference sequence M18079 and based on numbering 5' from the start codon. b Caco-2 cells or HeLa were transiently transfected with FABP2-SEAP reporter gene constructs haplotype A (A) and haplotype B (B). 836 bp and 388 bp FABP2 promoter fragments upstream from initiation codon of FABP2, encompassing all six (838) or the first four (388) promoter polymorphisms, was used. The resulting SEAP activity was measured and normalized for transfection efficiency (Luciferase). Activities of the empty control vector pSEAP-Basic were subtracted from SEAP reporter activities. Results are reported in relation to the activity of the 836 bp haplotype A. Data are given as mean \pm SEM from five experiments; $p = 0.0012$ as determined by a paired t-test.

morphisms confirmed previous findings in Caucasians [16–18] that the six promoter polymorphisms are in complete linkage disequilibrium (Fig. 2a). To verify and extend reported effects [16–18] of FABP2 promoter polymorphisms, 836 bp and a 388 bp 5' fragments of the FABP2 gene carrying either the A or B allele were inserted into the SEAP reporter plasmid. The Caco-2 cell

Table 1 Distribution of FABP2 promoter genotypes in incident type 2 diabetes subjects and controls

Subjects	AA	AB	BB	B-allele
Cases (n = 188)	66 (35.1%)	87 (46.3%)	35 (18.6%)	41.8%
men (n = 112)	41 (36.6%)	48 (42.9%)	23 (20.5%)	41.9%
women (n = 76)	25 (32.9%)	39 (51.3%)	12 (15.8%)	41.4%
Controls (n = 376)	114 (30.3%)	182 (48.4%)	80 (21.3%)	45.5%
men (n = 224)	59 (26.3%)	118 (52.7%)	47 (21.0%)	47.3%
women (n = 152)	55 (36.2%)	64 (42.1%)	33 (21.7%)	42.8%

line was selected for testing FABP2 promoter activities. As shown in Fig. 1, RT-PCR and Western-blot analysis revealed robust FABP2 mRNA and protein expression in post-confluent CaCo-2 cells. Under these conditions, the 836 FABP2 haplotype B showed $40.2 \pm 7.3\%$ ($p = 0.0012$) lower SEAP activity than the 836 FABP2 haplotype A (Fig. 2b). The short 388 FABP2 constructs produced generally lower activities in comparison to the long constructs. However, the short alleles containing polymorphisms -80, -136, -168 and -260 showed similar proportions in activity as observed in the long promoter versions (Fig. 2b).

Association of the FABP2 polymorphisms with type 2 diabetes

To examine whether the functional FABP2 promoter variant contributes to type 2 diabetes, we genotyped the polymorphisms in a prospective nested case-control study taken from the EPIC-Potsdam cohort. The baseline characteristics of 192 incident type 2 diabetes subjects and 384 age and sex-matched controls have already been described [20]. The resulting allele frequencies (Table 1) were 55.8% for the FABP2 haplotype A and 44.2% for the haplotype B in the overall study population. These frequencies are similar to those previously reported in Caucasians [16,17]. The genotype distribution was in compliance with the Hardy-Weinberg equilibrium ($p = 0.56$). In women, the frequency of the FABP2 haplotype B was similar in cases (41.4%) and controls (42.8%), whereas haplotype B was more frequent in controls

(47.3%) than in type 2 diabetes subjects (41.9%) in the male subgroup, but this difference did not reach significance ($p = 0.2$).

To evaluate the association between the FABP2 promoter polymorphisms and risk of type 2 diabetes, crude and adjusted odds ratios (ORs) and their 95% CIs were calculated using conditional logistic regression models (Table 2). Assuming co-dominant inheritance with the A/A-genotype as a reference group, the FABP2 promoter polymorphism showed no significant association with the risk of type 2 diabetes in the overall study population in an unadjusted model (Table 2, model A). Since gender may play a critical role in the effect of the FABP2 polymorphisms [16,21], analysis was performed separately on males and females. No significant associations were found in females independently of the model used (Table 2). In males, the heterozygote FABP2 promoter genotype A/B was significantly associated with decreased risk (OR = 0.48, 95% CI = 0.27–0.87, $p = 0.04$) of type 2 diabetes when adjusted for BMI (model B). Comparable and significant ORs were obtained for FABP2 genotypes A/B (0.45, 0.23–0.9, $p = 0.02$) and B/B (0.41, 0.17–0.99, $p = 0.05$) when adjusted for BMI, total energy intake, sport activities, lipid-lowering and antihypertensive drug intake and concomitant diseases (model C). Male subjects carrying the FABP2 haplotype B allele (genotype AB+BB) showed significantly decreased risk of type 2 diabetes when adjusted to BMI (OR = 0.50, 95% CI = 0.28–0.87, $p < 0.05$) and additional covariates (0.42, 0.22–0.81, $p < 0.01$).

Haplotype analysis indicated that the FABP2 promoter and exon 2 (Ala54Thr) polymorphisms were not linked completely in our Caucasian study populations (Table 3). Similar results were obtained by Formanack et al. (2004). However, comparison of calculated haplotypes revealed no significant differences between cases and controls (Table 3). Therefore, we extended our association study with respect to the Ala54Thr polymorphisms. When analyses further adjusted for the exon 2 polymorphism, the promoter AB (OR = 0.18, 0.06–0.49, $p < 0.01$) and BB (0.09, 0.02–0.41, $p < 0.01$) genotypes were strongly associated with reduced risk of diabetes in males. Similar results were obtained after stratification for the Ala54Thr polymorphism (Table 4). As shown in

Table 2 Association between FABP2 promoter genotypes and type 2 diabetes

Model OR	Gender OR (95% CI)	A/A OR (95% CI)	A/B OR (95% CI)	B/B	A/B+B/B
A	all	1.00	0.84 (0.57–1.23)	0.81 (0.49–1.31)	0.83 (0.58–1.19)
	men	1.00	0.59 (0.35–0.99)*	0.72 (0.38–1.37)	0.62 (0.38–1.01)
	women	1.00	1.37 (0.76–2.48)	0.84 (0.39–1.84)	1.18 (0.68–2.04)
B	all	1.00	0.72 (0.46–1.11)	0.51 (0.28–0.91)*	0.65 (0.43–0.99)*
	men	1.00	0.48 (0.27–0.87)*	0.54 (0.26–1.15)	0.50 (0.28–0.87)*
	women	1.00	1.33 (0.67–2.66)	0.38 (0.14–1.01)	0.93 (0.50–1.73)
C	all	1.00	0.79 (0.50–1.26)	0.49 (0.26–0.94)*	0.69 (0.44–1.06)
	men	1.00	0.45 (0.23–0.90)*	0.41 (0.17–0.99)*	0.42 (0.22–0.81)**
	women	1.00	1.52 (0.72–3.21)	0.32 (0.10–1.03)	1.03 (0.52–2.03)
D	all	1.00	0.56 (0.29–1.08)	0.32 (0.12–0.86)*	0.55 (0.29–1.06)
	men	1.00	0.18 (0.06–0.49)**	0.09 (0.02–0.41)**	0.18 (0.06–0.49)+
	women	1.00	2.51 (0.8–7.9)	0.81 (0.16–4.09)	2.11 (0.70–6.34)

* $p < 0.05$; ** $p < 0.01$; + $p < 0.001$; Model A, crude; Model B, adjusted for BMI; Model C, adjusted for BMI, total energy (kJ/d) and alcohol intake (g/d), physical activity level, smoking, presence of hypertension or/and hyperlipidemia, use of lipid-lowering drugs and antihypertensives; Model D, adjusted for covariates as used in model C and the FABP2 Ala54Thr SNP.

the male subgroup, Ala/Ala homozygote males carrying promoter haplotype B showed decreased risk (0.33, 0.11–0.94, $p < 0.05$) of developing type 2 diabetes.

We also tested for associations between FABP2 promoter polymorphisms and common traits of the insulin resistance syndrome. As shown in Table 5, non-parametric covariance analysis

adjusted for age, physical activity level and smoking revealed no significant differences among the three FABP2 promoter genotypes and BMI, body fat percent, plasma levels of leptin, HbA_{1c}, total cholesterol or HDL-cholesterol. Genotype combinations defined by the FABP2 promoter and exon 2 polymorphisms were also not associated with these quantitative traits (Table 6)

Discussion

The present study provides the first evidence that the FABP2 promoter polymorphism is associated with type 2 diabetes. Interestingly, the association was found in men, but not women. In non-diabetic control subjects, the FABP2 promoter polymorphism was marginally associated with fat mass and triglycerides in men only [16]. Sex-specific differences in triglyceride levels were also found in an association study accounting for the FABP2 Ala54Thr polymorphism [21], which is strongly linked to the promoter polymorphisms. In agreement, FABP2 knockout

Table 3 Calculated FABP2-promoter/Ala54Thr haplotype frequencies

Haplotype	All subjects	Cases	Controls	<i>p</i> *
A Ala	0.549	0.574	0.538	0.3
A Thr	0.008	0.003	0.009	0.2
B Ala	0.145	0.131	0.152	0.3
B Thr	0.298	0.292	0.301	0.8

* χ^2 -test for haplotype-trait association

Table 4 Association of FABP2 promoter genotypes with type 2 diabetes in subjects homozygous for the Ala54 variant

FABP2		A/A Ala/Ala	A/B Ala/Ala	B/B Ala/Ala	A/B+B/B Ala/Ala
all	Ca/Co (No.)	65/111	25/60	1/8	26/68
	OR(95%CI) ¹	1.00	0.66 (0.30–1.46)	0.14 (0.01–1.82)	0.57 (0.27–1.21)
men	Ca/Co (No.)	40/58	14/44	1/4	15/48
	OR(95%CI) ¹	1.00	0.32 (0.11–0.94)*	0.47 (0.01–28.39)	0.33 (0.11–0.94)*
women	Ca/Co (No.)	25/53	11/16	0/4	11/20
	OR(95%CI) ¹	1.00	3.97 (0.63–24.95)	–	1.29 (0.38–4.41)

* $p < 0.05$; ¹ adjusted for BMI; Ca, cases; Co, controls.

Table 5 ANCOVA on anthropometric and metabolic variables according to FABP2 promoter genotypes stratified by gender

	A/A	A/B	B/B	<i>p</i> **	$\geq B$ allele	<i>p</i> **
Men/Women (no.)	98/82	162/102	69/44		231/146	
BMI¹						
Men	27.85 (27.13, 28.57)	28.32 (27.75, 28.89)	27.71 (26.84, 28.57)	0.8	28.14 (27.67, 28.61)	0.5
Women	27.47 (26.38, 28.55)	27.89 (26.91, 28.86)	28.63 (27.15, 30.12)	0.2	28.11 (27.3, 28.92)	0.4
Body fat (%)¹						
Men	25.4 (24.3, 26.4)	26.1 (25.3, 27.0)	25.4 (24.2, 26.6)	1.0	25.91 (25.24, 26.59)	0.4
Women	37.0 (35.7, 38.2)	37.1 (35.9, 38.2)	37.3 (35.6, 39.0)	0.7	37.13 (36.2, 38.06)	0.8
Leptin (ng/ml)¹						
Men	5.1 (4.2, 6.2)	5.0 (4.3, 5.9)	3.9 (3.1, 4.9)	0.08	4.65 (4.08, 5.29)	0.5
Women	14.1 (12.0, 16.5)	16.2 (14.0, 18.7)	16.9 (13.5, 21.1)	0.2	16.38 (14.52, 18.48)	0.1
HbA_{1c} (%)²						
Men	5.41 (5.15, 5.68)	5.07 (4.88, 5.27)	5.23 (4.93, 5.54)	0.4	5.12 (4.96, 5.28)	0.06
Women	5.05 (4.85, 5.25)	4.94 (4.76, 5.11)	4.76 (4.51, 5.02)	0.09	4.88 (4.74, 5.03)	0.2
TC (mmol/l)³						
Men	4.63 (4.45, 4.81)	4.74 (4.60, 4.89)	4.63 (4.41, 4.85)	0.9	4.71 (4.6, 4.84)	0.4
Women	5.04 (4.85, 5.24)	4.87 (4.70, 5.04)	4.90 (4.64, 5.17)	0.4	4.88 (4.74, 5.02)	0.2
HDL-C (mmol/l)³						
Men	0.92 (0.89, 0.96)	0.89 (0.87, 0.92)	0.90 (0.85, 0.94)	0.3	0.90 (0.87, 0.92)	0.2
Women	1.10 (1.04, 1.15)	1.09 (1.05, 1.15)	1.11 (1.04, 1.20)	0.7	1.10 (1.06, 1.14)	0.9

Data expressed as means (95% confidence levels). * Log_e transformed for statistical analysis, geometric means presented for transformed data; ¹ adjusted for age, physical activity level, and smoking; ² adjusted for age, physical activity level, smoking, and BMI; ³ adjusted for age, physical activity level, smoking, BMI, lipid-lowering drug intake and hyperlipidemia; ** *p*-value refers to the difference of means between AA and BB (or AB+BB) diplotypes.

Table 6 ANCOVA on anthropometric and metabolic variables according to FABP2 promoter genotypes stratified by gender in subjects homozygous for the 54Ala variant

	A/A+Ala/Ala	A/B+Ala/Ala	B/B+Ala/Ala	p**	≥ B allele+Ala/Ala	p**
Men/Women (no.)	96/80	57/26	5/4		62/30	
BMI¹						
Men	27.8 (27.1, 28.5)	28.6 (27.6, 29.5)	27.0 (23.7, 30.3)	0.6	28.4 (27.5, 29.4)	0.3
Women	27.5 (26.6, 28.5)	26.6 (24.9, 28.2)	27.1 (22.7, 31.5)	0.8	26.6 (25.04, 28.2)	0.3
Body fat (%)¹						
Men	25.4 (24.3, 26.4)	26.6 (25.2, 28.0)	24.4 (19.7, 29.0)	0.7	26.4 (25.1, 27.8)	0.2
Women	37.0 (35.8, 38.3)	36.0 (33.7, 38.2)	36.7 (31.0, 42.5)	0.9	36.1 (34.0, 38.1)	0.4
Leptin (ng/ml)^{* 1}						
Men	5.14 (4.26, 6.21)	5.15 (4.04, 6.56)	2.17 (0.96, 4.87)	0.04	4.8 (3.8, 6.1)	0.7
Women	14.05 (11.97, 16.49)	13.76 (10.38, 18.23)	15.18 (7.30, 31.55)	0.8	13.93 (10.72, 18.11)	1.0
HbA_{1c} (%)^{* 2}						
Men	5.40 (5.13, 5.68)	5.16 (4.83, 5.51)	5.10 (4.07, 6.33)	0.6	5.15 (4.84, 5.49)	0.3
Women	5.03 (4.81, 5.26)	5.10 (4.71, 5.52)	4.59 (3.74, 5.65)	0.4	5.03 (4.67, 5.42)	1.0
TC (mmol/l)^{* 3}						
Men	4.63 (4.43, 4.84)	4.79 (4.53, 5.08)	4.48 (3.70, 5.43)	0.7	4.77 (4.51, 5.04)	0.4
Women	5.10 (4.89, 5.28)	5.04 (4.71, 5.39)	5.40 (4.54, 6.43)	0.5	5.09 (4.78, 5.41)	1.0
HDL-C (mmol/l)^{* 3}						
Men	0.93 (0.89, 0.97)	0.91 (0.87, 0.97)	0.99 (0.82, 1.18)	0.5	0.92 (0.87, 0.97)	0.8
Women	1.11 (1.06, 1.17)	1.05 (0.96, 1.15)	1.17 (0.92, 1.49)	0.7	1.07 (0.98, 1.16)	0.4

Data expressed as means (95% confidence levels). * Log_e transformed for statistical analysis, geometric means presented for transformed data; ¹ adjusted for age, physical activity level, and smoking; ² adjusted for age, physical activity level, smoking, and BMI; ³ adjusted for age, physical activity level, smoking, BMI, lipid-lowering drug intake and hyperlipidemia; ** p-value refers to the difference of means between AA and BB (or AB+BB) diplotypes.

mouse showed sex-specific alterations in body weight with dietary fat content taken into account [3]. In a family study, a maternal effect was observed at FABP2 [22]. In consideration of this evidence, sex-specific effects of FABP2 polymorphisms on type 2 diabetes and related phenotypes seem apparent, although a plausible mechanism has not been found so far. Maybe the FABP2 expression is regulated by sex hormones that may modify the different activities of FABP2 promoter haplotypes. This hypothesis should be tested by using our FABP2 promoter constructs.

Functional analysis revealed a 2.5-fold decreased promoter activity of FABP2 haplotype B in comparison to haplotype A in post-confluent CaCo-2 cells. Previously, we obtained opposite results [18] in human embryonic intestinal cell line INT 407 (ATCC CCL-006) with similar, but not identical promoter-reporter constructs. This line was originally thought to be derived from normal embryonic intestinal tissue [23], but subsequent analysis [24,25] of isoenzymes, chromosomal markers and DNA fingerprinting revealed HeLa cell contamination. Therefore, the INT 407 cell line does not seem to be appropriate for promoter assays and expression analysis, although we cannot exclude cell-specific differences in FABP2 promoter activity. Post-confluent CaCo-2 cells showed endogenous expression of FABP2 (Fig. 1), which makes this cell line particularly suitable for characterization of the FABP2 promoter. The low activity of the promoter haplotype B was also found by other groups [16,17]. We could also demonstrate that four of six polymorphisms within 1kb 5' of the FABP2 initiation codon are responsible for different haplotype activities. The four variants comprise the region up to -260 bp 5' upstream of the FABP2 initiation codon. This region contains several bind-

ing sites including PPAR/RXR. Evidence of the involvement of PPAR δ / RXR α in the regulation of FABP2 has been reported in rats [26]. Assuming similar transcriptional regulation in humans, the insertion/deletion variants could lead to altered binding affinity of PPAR δ / RXR α to FABP2 promoter haplotypes. This potentially results in different transcriptional activity, which is consistent with our FABP2 promoter reporter assay. Of course, we do not know whether the *in vitro* effect of the FABP2 promoter polymorphisms also occur *in vivo* and/or do affect heterozygote individuals. Comparing the relative gene expression levels from individuals with different FABP2 promoter genotypes may address this problem.

Assuming altered FABP2 expression levels depending on promoter genotypes *in vivo*, these may considerably affect fat metabolism and downstream effects on insulin resistance. Postprandial concentrations of chylomicrones and triacylglycerol after ingestion of olive oil have been shown to be even higher in subjects carrying the FABP2 Thr54 allele [27]. Because the "risk" Thr54 allele is strongly linked to the "prevention" haplotype B promoter allele, we propose independent effects of these functional variants on the development of type 2 diabetes. Future studies in larger populations will be necessary and more informative in revealing the relative contribution of both polymorphisms in influencing type 2 diabetes and metabolic parameters. In our incident case-control study, we found no evidence for any association between the FABP2 promoter polymorphism and parameters linked to insulin resistance at *baseline*, although the variant was associated with type 2 diabetes in men. Damcott et al. showed a marginal association between promoter variants and body composition as well as plasma cholesterol in non-diabetic control

subjects [16]. Overall, the physiological mechanism by which the functional FABP2 promoter influences the risk of type 2 diabetes remains obscure, most notably when sex-specific effects and exon 2 polymorphism are taken into account. However, there is some evidence, that FABP2 is not only expressed in enterocytes but probably also in intestinal epithelial L-cells [28]. These cells secrete the anti-diabetic hormones GLP1 and GLP 2 after fat ingestion. The expression level of FABP2 in L-cells could therefore influence insulin sensitivity via fatty acid dependent GLP secretion. This hypothesis should be tested by intervention studies [29,30] with different FABP2 genotypes.

In this study, we found an association between the FABP2 promoter haplotype B and reduced risk of type 2 diabetes in men. The lower FABP2 promoter variant B expression level observed could be functionally related to a short fragment of the haplotype B containing a PPAR δ /RXR α -binding site. This may alter PPAR-dependent activation of the FABP2 promoter. There was some evidence of intragenic interaction between the functional FABP2 promoter and exon 2 Ala54Thr variants, but these results need to be interpreted with caution and should be replicated in future studies.

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Abbreviations

FABP2 fatty acid-binding protein 2
SEAP secreted alkaline phosphatase

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**Type 2 diabetes associated Fatty Acid Binding Protein 2 promoter haplotypes are
differentially regulated by GATA factors**

Short title: Regulation of FABP2 haplotypes via GATA factors

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

***Corresponding author**

Maja Klapper, Molecular Nutrition, Institute of Human Nutrition and Food Science,
Christian-Albrechts-University of Kiel, Heinrich-Hecht-Platz 10, 24118 Kiel, Germany. Tel.:
049 (0) 431-8805661; Fax: 049 (0) 431-8805658; E-mail: klapper@molnut.uni-kiel.de

Abstract

The human intestinal fatty acid binding protein 2 (FABP2) mediates fat absorption by binding and intracellular trafficking of long-chain free fatty acids. Studies with knock-out mice and association analysis of polymorphisms revealed that *FABP2* is a susceptibility gene for diabetes type 2 and related traits. Relevant *FABP2* promoter polymorphisms c.-80_-79insT (rs5861422), c.-136_-132delAGTAG (rs5861423), c.-168_-166delAAGinsT (rs1973598), c.-260G>A (rs6857641), c.-471G>A (rs2282688), c.-778G>T (rs10034579) result in two haplotypes A and B, whereby B possesses 2-3 fold lower transcriptional activity than A. We show in luciferase reporter gene assays by a series of chimeric *FABP2* promoter constructs in intestinal Caco-2 cells that polymorphism c.-80_-79insT essentially determines different activities of *FABP2* promoter. In accordance, in electrophoretic mobility shift assays transcriptional factors GATA-5 and -6 bind with higher binding affinities to the *FABP2* promoter region containing the -80A allele compared to B. As functional consequence, haplotype A is twice as much more activated by GATA factors than haplotype B in liver Huh7 cells. Additionally, a construct bearing the -80B allele in the background of haplotype A reversed the activity from A to B. Thus, the GATA mediated differential activation of *FABP2* haplotypes depends on polymorphism c.-80_-79insT. This provides the molecular basis for the variant specific transcriptional regulation of the diabetes type 2 associated *FABP2* gene.

Keywords

fatty acid binding protein 2, *FABP2*, Type 2 diabetes, promoter haplotypes, GATA factors

Introduction

Human fatty acid binding proteins (FABPs) represent a multigene family encoding ~15 kDa cytosolic proteins with nine members (Hertzel and Bernlohr, 2000) that are involved in central cellular processes. FABPs influence metabolic pathways by binding and intracellular transport of long-chain free fatty acids, which are delivered to specific cellular compartments and enzymes. They mediate fatty acid signaling by transferring fatty acids to the nucleus as ligands for transcription factors, such as peroxisome proliferator-activated receptors (PPARs), and seem to be involved in regulation of growth and differentiation (Hauerland and Spener, 2004).

The human intestinal fatty acid binding protein 2 gene (FABP2, OMIM accession number: 134640; GenBank: NM_000134.2) is located on chromosome 4q28-q31, contains four exons and codes for a 15.1 kDa protein comprising 132 amino acid residues (Sweetser, et al., 1987). It is exclusively expressed in intestine with highest cellular concentrations in jejunum with falling levels to the proximal duodenum and colon (Cohn, et al., 1992; Sweetser, et al., 1988). *FABP2* is up-regulated during development (Gordon, et al., 1985) and crypt-to-villus transition. Whereas FABP2 is absent in crypt cells, highest protein abundance is found in enterocytes of upper villus region (Shields, et al., 1986; Sweetser, et al., 1988), where fat absorption takes place. The particular 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 (Baier, et al., 1996; Levy, et al., 2001; Lowe, et al., 1987).

FABP2 is a candidate gene for diabetes type 2 (non-insulin dependent diabetes mellitus, NIDDM). According to this, a huge number of studies in different human populations

revealed associations between the minor allele of the *FABP2* Ala54Thr polymorphism and risk parameters of insulin resistance (Weiss, et al., 2002). Functional studies showed increased binding affinity of the variant protein to long-chain fatty acids (Baier, et al., 1995). More recently, *FABP2* promoter haplotypes were associated with postprandial triglyceride levels (Geschonke, et al., 2002), BMI (Damcott, et al., 2003) and type 2 diabetes (Li, et al., 2006). The *FABP2* coding and promoter polymorphisms are in strong or complete linkage disequilibrium (D' 0.6-1.0) (Damcott, et al., 2003; Formanack and Baier, 2004; Li, et al., 2006). In accordance with the association studies, male *FABP2* knock-out mice showed key symptoms of the metabolic syndrome such as increased plasma levels of insulin and triglyceride, increased body weight, and changes in liver histology, maybe resulting from impaired lipid metabolism (Agellon, et al., 2006; Vassileva, et al., 2000). Therefore, the expression level of *FABP2* seems to be critical for insulin sensitivity and triglyceride metabolism.

Because *FABP2* is a susceptibility gene for diabetes type 2 and related traits, its variant specific transcriptional regulation is of special interest. Previous studies (Damcott, et al., 2003; Formanack and Baier, 2004; Li, et al., 2006) revealed that the 5' upstream promoter region of *FABP2* contains insertion/deletion sites c.-80_-79insT, c.-136_-132delAGTAG, c.-168_-166delAAGinsT and SNPs c.-260G>A, c.-471G>A and c.-778G>T, which are in complete linkage disequilibrium resulting in only two haplotypes A>B. As demonstrated by us (Li, et al., 2006) and others (Damcott, et al., 2003; Formanack and Baier, 2004) the rare *FABP2* promoter haplotype B showed almost 2-3 fold lower transcriptional activity than haplotype A in Caco-2 cells. In the present study we intended to reveal the causative polymorphism(s) and transcription factor(s) for these different activities. We show by a site-directed mutagenesis approach, transfections and reporter assays of a series of *FABP2* promoter constructs in Caco-2 cells that polymorphism c.-80_-79insT had the greatest impact

in determining different activities of *FABP2* promoter haplotypes. Based on electrophoretic mobility shift and transfection assays, the low activity of *FABP2* promoter haplotype B is essentially determined by the -80B allele via GATA-5 and -6. Thus, we provide a molecular basis for the variant specific regulation of the type 2 diabetes associated *FABP2* gene.

Material and Methods

Mutation Nomenclature – All positions of the polymorphisms are numbered relative to the A of the ATG translation initiation codon of the *FABP2* reference sequence (GenBank: NM_000134.2).

Cell culture - HeLa and Caco-2 cells were purchased by German National Resource Centre for Biological Material (DSMZ, Braunschweig, Germany) and American Type Culture Collection (ATCC, Rockville, MD), respectively, and Huh7 cells were kindly provided by D. Hartwig (University Medical Center Schleswig-Holstein, Luebeck, Germany). 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. Huh7 cells were cultured in DMEM containing 4,5 g/L glucose and 10% fetal calf serum. Cells were passaged at confluent densities by use of 0.05% trypsin/5 mM EDTA (Biochrom AG, Berlin, Germany) solution every 2-3 days.

FABP2 promoter luciferase constructs - The dual luciferase system was used (Promega, Madison, WI). Cloning procedures were performed using Gateway Technology (Invitrogen), described previously (Klapper, et al., 2007; Li, et al., 2006). 836 bp fragments upstream from initiation codon of the *FABP2* promoter haplotypes A and B were subcloned by recombination with LR Clonase into attR-sites of pGL4.10[luc2] from vector pENTR-

FABP2-Prom-attL resulting in final promoter reporter constructs pGL4.10[luc2]-FABP2(A) and pGL4.10[luc2]-FABP2(B). The empty pGL4.10[luc2] vector served as a negative control.

Chimera constructs – Mutations in haplotype A (pGL4.10[luc2]-FABP2(A)) and haplotype B (pGL4.10[luc2]-FABP2(B)) containing promoter constructs were introduced to generate chimeras with single polymorphisms of haplotype B in the background of haplotype A (A-xB) and vice versa (B-xA). Single polymorphism exchange chimera constructs are shortly named as follows: for exchange of polymorphism c.-80_-79insT of haplotype A (rs5861422): A-80B; for c.-136_-132delAGTAG (rs5861423): A-136B; for c.-168_-166delAAGinsT (rs1973598): A-168B, c.-260G>A (rs6857641): A-260B c.-471G>A (rs2282688): A-471B; c.-778G>T (rs10034579): A-778B and vice versa. For introduction of exchanges at two polymorphic sites single mutant chimeras were used to generate double mutant chimeras. For instance, A-80B was used to introduce an additional exchange at polymorphism c.-136_-132delAGTAG resulting in chimera A-80/136B. For generation of triple mutant chimeras double mutants were used. Introduction of mutations 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 for mouse GATA factors pcDNA-GATA-5 and pcDNA-GATA-6 were kindly provided by Dr. J. Philippe (University Hospital Geneva, Switzerland).

Transient transfections and reporter assays - Transient transfections were performed with FuGene6 (Roche, Basel, Switzerland) according to the manufacturers instructions. 1×10^4 Caco-2 cells were plated in 96-well plates. Cells were co-transfected with 47,5 ng

pGL4.10[luc2]-FABP2-Promoter constructs 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). 5×10^3 Huh7 cells were used for co-transfections with mouse GATA-5 and -6. Cells were co-transfected with 30 ng pGL4.10[luc2]-FABP2-Promoter constructs or pGL4.10[luc2], 66 ng of the expression vectors pcDNA3-GATA-5, pcDNA3-GATA-6 or 66 ng empty pcDNA3 vector and 3 ng pGL4.74[hRluc/TK] vector. Relative luciferase activities of negative control pGL4.10[luc2] were determined for each condition and were subtracted from corresponding activities for pGL4.10[luc2]-FABP2-Promoter constructs. Each experiment was repeated at least three times, and each sample was studied in triplicate. HeLa cells were used for preparation of nuclear extracts containing GATA-5 and -6. 4×10^5 cells were seeded in T25 flasks, transfected with 4 µg expression plasmid and extracts were prepared 48 h after transfection.

Electrophoretic mobility 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). Nuclear extracts were prepared from HeLa and postconfluent Caco-2 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 manufacturers instructions. The reaction mixture (20 µl) contained 1 mM Tris, 50 mM KCl, 1 mM DTT, 1 µg Poly (dI-dC), 2,5 % Glycerol, 0,1 mM EDTA. The DNA-transcription factor complexes were resolved on a native 4 % Tris-borate-EDTA polyacrylamide gel in 0.5 x 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.

Statistical analyses - Statistical analyses were performed with GraphPad Prism 4 (Graphpad Software, Inc., San Diego, CA) using ANOVA and unpaired t-tests. The differences between haplotype A and B activities were tested with two-tailed t-tests. For comparison of chimera activities with haplotype A and B we used one-tailed t-tests because we hypothesized that activities of the chimera promoter constructs have to be below or above promoter activities of haplotype A and haplotype B, respectively. All values were expressed as means \pm SEM. Significant differences were considered for P values less than 0.05.

Results

The proximal polymorphisms c.-80_-79insT, c.-136_-132delAGTAG, and c.-168_-166delAAGinsT essentially determine activity of FABP2 promoter haplotypes in post-confluent Caco-2 cells.

As an initial approach to identify the causal polymorphism(s) for the differential activity of *FABP2* promoter haplotypes we performed promoter-reporter analysis. By means of site-directed mutagenesis we sequentially introduced alleles of haplotype B into haplotype A and vice versa (Fig. 1). We aimed to find minimal necessary change to achieve the activity of the other haplotype, respectively. Reporter *Firefly* luciferase activities of the *FABP2* promoter haplotypes and from it derived chimeras A-xB and B-xA were determined 120 h after transient transfection, when Caco-2 cells were 4 days post-confluent. As shown previously by us, both, mRNA as well as protein of *FABP2* are clearly up-regulated in differentiated Caco-2 cells at this time point (Li, et al., 2006).

As previously reported (Darimont, et al., 1998; Formanack and Baier, 2004; Li, et al., 2006), *FABP2* haplotype B possessed 28 % activity of haplotype A (Fig. 1). *FABP2* promoter

chimeras A-80B, A-136B, A-168B, A-260B, A-471B, and A-778B showed activities of 48, 55, 65, 69, 73, and 78 %. Since highest reduction of haplotype A activity was caused by proximal polymorphisms, we generated *FABP2* promoter chimeras A-80/136B, A-80/168B, and A-136/168B. As shown in Fig. 1, these constructs had the same low activity as haplotype B. Next, we performed reporter analysis starting from *FABP2* promoter haplotype B with consideration of the three polymorphisms c.-80_-79insT, c.-136_-132delAGTAG, and c.-168_-166delAAGinsT. As shown in Fig. 1, activities of chimeras B-80A, B-136A reached 59, 42 and 42 % of haplotype A. Chimeras B-80/136A, B-80/168A, and B-136/168A could revert the activity of haplotype B into A up to 84 %. However, only a simultaneous introduction of alleles -80, -136, and -168 of haplotype A polymorphisms into haplotype B (A-80/136/168B) was sufficient to obtain the full activity of A. Thus we reasoned the different activities of *FABP2* promoter haplotypes are essentially determined by the polymorphisms at positions -80, -136 and -168. Because single chimeras A-80B and B-80A have the greatest impact onto their activities, the polymorphism c.-80_-79insT is of particular importance for transcriptional regulation of *FABP2* promoter haplotypes.

EMSAs confirmed functional importance of FABP2 promoter polymorphisms c.-80_-79insT and c.-168_-166delAAGinsT. To see if *FABP2* promoter regions containing the alleles of the two haplotypes showed differences in transcriptional factor binding capacity, we tested nuclear protein extracts from post-confluent Caco-2 cells using EMSA (Fig. 2B). Sequences of used oligonucleotides representing the different alleles of *FABP2* haplotypes are shown in Fig. 2A. EMSA revealed no specific signals with probes containing alleles of polymorphisms c.-260G>A. For oligonucleotides comprising polymorphisms c.-471G>A and c.-778G>T longer film exposure times were necessary to reveal specific shifts (Fig. 2B, over-exposure). A very faint signal was obtained with the -136A but not with the -136B probe. In the case of *FABP2* promoter oligonucleotides containing alleles of SNP c.-168_-166delAAGinsT two specific shifts with high molecular weight were detected for haplotype A and B but no clear

differences could be observed in their binding capacities. The strongest signals were obtained for -80 probes, whereby one main complex was formed (Fig. 2B). Interestingly, the oligonucleotide containing the -80 allele of haplotype B bound less nuclear proteins than A. Taken together, EMSA revealed strong binding of transcription factors to proximal *FABP2* promoter polymorphisms c.-80_-79insT and c.-168_-166delAAGinsT. Clear differences in binding capacity between the regions encompassing the polymorphisms were obtained for the -80 probes with strongest protein interaction with the -80A probe. Thus, EMSAs support the results from the reporter gene analysis with chimeric promoter constructs and reinforce the functional importance of polymorphism c.-80_-79insT in determining different activities of *FABP2* haplotype A and B.

Transcription factors bind with higher affinity to the FABP2 promoter region containing the -80 allele of haplotype A in comparison to B. To further analyze the binding characteristics of the *FABP2* promoter region containing the c.-80_-79insT polymorphism we performed EMSA with nuclear protein extract from Caco-2 cells designed as competition and cross-competition experiments. As shown in Fig. 3A, we observed a complete displacement of transcription factor binding to probes -80A (lane 1-5) and -80B (lane 6-9) with 50 (lane 2, 8) or 100 (lane 4, 5, 9) molar excess of unlabelled probes. However, longer film exposure times (Fig. 3A, lower panel) showed that binding to the -80A probe is more eliminated by a 100 molar excess of competitor -80A (lane 4) in comparison to -80B (lane 5). To compare affinities more clearly, lower concentrations of competitors were used. As shown in Fig. 3B, specific shifts obtained by the -80A probe were competed away by gradually increasing excess of unlabelled probes. Already a 20-fold excess of unlabelled -80A probe completely abolished the shift, whereas a 50-fold excess of competitor B was necessary. Therefore, we conclude that nuclear proteins seem to bind with higher affinity to the *FABP2* promoter region containing the -80A allele of haplotype in comparison to B.

GATA factors show stronger interaction with FABP2 promoter region containing the allele -80A in comparison to -80B. To reveal putative transcription factor binding site(s) in the -80 region of the *FABP2* promoter we performed an *in-silico* analysis using Transfac database (Kel, et al., 2003; Matys, et al., 2006). A GATA consensus site (A/T)GATA(A/G) (Molkentin, 2000) was found that bears within its matrix the c.-80_-79insT polymorphism. Whereas the five most conserved bases of GATA consensus matches with both variants completely, the score for matrix match is slightly higher for haplotype A (0.995) than B (0.981) suggesting differential binding of GATA factors.

Based on these results we investigated binding of GATA-5 and 6 to *FABP2* -80A and -80B probes. EMSA were carried out with nuclear protein extracts from Hela cells transfected with GATA-5 and 6 because these factors are not expressed in this particular cell line. As shown in Fig. 4, no shifts were detected in non-transfected cells (lane 3, 4). Specific shifts for probes -80A (lane 5, 6, 9, 10) and -80B (lane 7, 8, 11, 12) were obtained with extracts from GATA-5 and GATA-6 transfected cells. The Gata-6 DNA complexes showed exactly the same size as shifts obtained with extracts from Caco-2 cells (lane 1, 2) whereas the complex formed with GATA-5 extract was detected slightly below. This difference corresponds with the predicted molar mass of 42 and 45 kDa for GATA-5 and -6, respectively. Even more interesting, *FABP2* oligonucleotide containing the -80A allele shows higher binding capacity for both factors, GATA-5 and GATA-6 in comparison to the -80B allele (compare lanes 5 and 7, 9 and 11, respectively). Thus, these data confirm the competition experiments with nuclear extracts from post-confluent Caco-2 cells.

The GATA-induced two times lower activation of FABP2 promoter haplotype A in comparison to B is determined by polymorphisms c.-80_-79insT. To prove, if different binding capacities of GATA factors to *FABP2* promoter region containing the c.-80_-79insT polymorphism reflect different activation potential of *FABP2* haplotype A and B we performed reporter gene analysis. To circumvent the problem of high and different activities

of *FABP2* promoter haplotypes as detected in Caco-2 cells, GATA-5 and -6 induced activities of haplotypes were analyzed in hepatic Huh7 cells. As shown in Fig. 5, this cell line shows very low and similar activity of *FABP2* promoter haplotypes. GATA-5 as well as GATA-6 increased clearly basal activities of both *FABP2* promoter haplotypes, whereby GATA-5 overall induced higher transcriptional activity than GATA-6. Interestingly, *FABP2* promoter haplotype A is two times more activated by GATA factors than haplotype B. This difference reflects nearly those obtained in Caco-2 cells. To analyse the specific importance for polymorphism c.-80_-79insT for different GATA5-induced activation of *FABP2* haplotype A and B we analyzed relevant chimeras A-80B, B-80A, and B-80/136/168A. As shown in Fig. 5, chimeras B-80A and B-80/136/168A show similar activation by GATA-5 than haplotype B. Consistent with different binding affinities of GATA factor to the -80 region, a construct bearing the -80B allele in the background of haplotype A reversed the activity from A to B.

Discussion

Here we provide a comprehensive functional investigation regarding promoter haplotype depending regulation of human *FABP2*. These haplotypes are associated with type 2 diabetes, postprandial triglyceride levels, and BMI (Damcott, et al., 2003; Geschonke, et al., 2002; Li, et al., 2006). As shown previously by us (Li, et al., 2006) and others (Damcott, et al., 2003; Formanack and Baier, 2004), the basal transcription of the rare *FABP2* promoter haplotype B is about 2-3 fold lower than the common haplotype A. Consequently, we investigated which polymorphism(s) and transcription factor(s) are causal for this different regulation.

By means of reporter assays using chimeric *FABP2* promoter constructs we found that difference in haplotype activities is essentially determined by the first three proximal *FABP2* promoter polymorphisms, whereby c.-80_-79insT has the greatest impact. Accordingly, clear

differences in allele specific binding of nuclear proteins from post-confluent Caco-2 cells were observed for c.-80_-79insT, whereas regions containing other *FABP2* promoter polymorphisms showed no substantial (c.-778G>T, c.-471G>A, -260G>A) or allele specific (c.-168_-166delAAGinsT) binding. Further, we demonstrated higher binding capacity of GATA-5 and GATA-6 to the *FABP2* promoter region containing the -80A allele in comparison to the -80B allele. These GATA factors induce the activity of *FABP2* promoter haplotype A two times more than haplotype B. Therefore, different transcriptional activities of *FABP2* promoter haplotypes are mainly caused by the c.-80_-79insT polymorphism mediated by GATAs. GATA-4, -5 and -6 are zinc finger-containing transcription factors and are present in various meso- and endoderm derived tissues such as intestine, heart, liver, lung and gonad. GATA-4, -5 and -6 are important transcriptional regulators of differentiation and embryonic development and tissue-specific gene expression (Bossard and Zaret, 1998; Bosse, et al., 2006; Gao, et al., 1998; Molkenin, 2000). These factors are also present in Caco-2 cells with GATA-6 as the predominant form (Fang, et al., 2001; Fitzgerald, et al., 1998; Gao, et al., 1998; Kamitani, et al., 2000). A specific expression pattern of GATA-4, -5 and -6 were described in intestinal cells. GATA-6 is present at high levels in proliferating and less differentiated cells, whereas GATA-4 and -5 are strongly up-regulated along the crypt-villus-axis during cell differentiation (Gao, et al., 1998). *FABP2* expression is also highest expressed in villus region (Shields, et al., 1986; Sweetser, et al., 1988), reinforcing GATA induced activation of the *FABP2* promoter. Furthermore, GATA factors bind to highly conserved elements in *FABP2* promoters of mouse (*mFABP2*) and zebrafish (*zFABP2*) at regions corresponding to the human *FABP2* promoter segment encompassing the c.-80_-79insT polymorphism (Belaguli, et al., 2007; Her, et al., 2004). Mutations of this site abolished the *mFABP2* promoter reporter activity after transient transfection completely (Belaguli, et al., 2007). Intriguingly, mutation in the highly conserved GATA site of the *zFABP2* promoter resulted in a non-specific expression of a *zFABP2* reporter in other tissues

(Her, et al., 2004). These studies emphasize the crucial role of GATA in regulation of *FABP2* in vertebrates. Interestingly, GATA-4 cooperates with Smad transcription factors, the downstream effectors of the TGF β signalling pathway, to activate the *mFABP2* promoter (Belaguli, et al., 2007). TGF β is a critical regulator of development and maintenance of intestinal epithelium (Hauck, et al., 2005). Notably, polymorphic sites in the TGF β gene are associated with diabetes and obesity phenotypes, e.g. body composition, increased insulin and glucose levels (Long, et al., 2003; Rosmond, et al., 2003). Therefore on the basis of possible functional interaction between TGF β , GATA factors and *FABP2* it seems to be a promising approach to study effects of polymorphism combinations in these genes on traits of the metabolic syndrome.

Although our experiments strongly indicate a predominant role of c.-80_-79insT polymorphism in determining different activities of *FABP2* promoter haplotypes, we have not ignored that polymorphisms c.-136_-132delAGTAG and c.-168_-166delAAGinsT are also of functional relevance. This assumption is based on two observations. Firstly, reporter assays using chimeras of *FABP2* promoter haplotypes revealed, that double or triple substitutions at positions -80, -136, and -168 are necessary for complete reconstitution of haplotype activity. This is particular true for chimeras which bear haplotype B alleles in the background of haplotype A. Secondly, although chimera A-80B reversed the GATA induced activation from haplotype A into that of B, the reverse chimera could not re-establish the activation potential of A. Also, the GATA-5 induced activity of chimera B-80/136/168A is similar to *FABP2* promoter haplotype B and not to A. We cannot fully explain this data. However, taking into account that transcriptional regulation is not the result of binding of single transcription factors to isolated motifs, GATA factors may differentially regulate *FABP2* promoter haplotypes in synergistic action with other factors. Interactions of GATA with abundantly expressed CDX-2 and HNF-1 have been already described and are responsible for tissue-

specific high expression of intestinal genes (Boudreau, et al., 2002; Divine, et al., 2004; Krasinski, et al., 2001; van Wering, et al., 2004; van Wering, et al., 2002). *In-silico* analysis as well as experimental verifications (Klapper et al, unpublished) revealed functional but not allele specific HNF-1 binding sites in the *FABP2* promoter region bearing the polymorphism c.-168_-166delAAGinsT. We propose a synergistic activation of *FABP2* promoters by GATA factors, HNF1, and CDX-2 resulting in high expression of *FABP2*, whereby difference in transcriptional activity of *FABP2* haplotypes may be essential determined by GATA factors. It should be noted, that there is no evidence for genotype specific expression of *FABP2 in-vivo*, since it is very difficult to obtain healthy tissue samples from the intestine. However, since GATA factors play an essential role in differentiation processes of intestine and supposed to be important for intestine-specific high expression (Bossard and Zaret, 1998; Bosse, et al., 2006; Gao, et al., 1998; Molkentin, 2000), we propose that *in vivo* expression is similar robustly regulated with respect onto the differential regulation of *FABP2* promoter haplotypes A and B.

Thus, the GATA mediated differential activation of *FABP2* haplotypes depends on polymorphism c.-80_-79insT. This provides the molecular basis for the variant specific transcriptional regulation of the diabetes type 2 associated *FABP2* gene.

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Figure legends

Figure 1. Reporter activities of *FABP2* promoter haplotypes and from it derived chimeras in post-confluent CaCo-2 cells. Alleles of each polymorphism in the *FABP2* promoter haplotypes A>B are given. The positions of polymorphisms c.-80_-79insT (rs5861422), c.-136_-132delAGTAG (rs5861423), c.-168_-166delAAGinsT (rs1973598), c.-260G>A (rs6857641), c.-471G>A (rs2282688), c.-778G>T (rs10034579) are named as -80, -136, -168, -260, -471, -778, respectively (positions relative to translation start codon). Luciferase activities of *FABP2* promoter haplotypes (A, B) and from it derived chimeras (A-xB, B-xA) were assessed 120 h after transfection using dual luciferase assay. The *Firefly* luciferase activities were normalized to *Renilla* luciferase activities and are given as relative luciferase activities in % of *FABP2* promoter haplotype A. Each experiment was performed in triplicate for each sample. The results are expressed as mean \pm SEM for at least three independent experiments. ***, $p < 0.001$, **, $p < 0.01$, *, $p < 0.05$, as compared with haplotype A activity. +++, $p < 0.001$, ++, $p < 0.01$, +, $p < 0.05$, as compared with haplotype B activity.

Figure 2. Differential interactions of transcription factors with *FABP2* promoter regions containing the alleles A and B of polymorphisms c.-80_-79insT (rs5861422), c.-136_-132delAGTAG (rs5861423), c.-168_-166delAAGinsT (rs1973598), c.-260G>A (rs6857641), c.-471G>A (rs2282688), c.-778G>T (rs10034579) were analyzed with EMSAs. The oligonucleotides used in EMSAs were shortly named with -80A/B, -136 A/B, -168 A/B, -260 A/B, -471 A/B and -778A/B. **A**, The sequences for oligonucleotides are given. **B**, Interaction of nuclear proteins from post-confluent Caco-2 cells with *FABP2* probes bearing the 6 promoter polymorphisms. 2 μ l nuclear extract isolated from post-confluent Caco-2 cells were used for each binding reaction with 20 fmol of biotinylated oligonucleotides. Specific shifts were diminished by 100-fold excess of unlabelled oligo and are indicated with an arrowhead.

EMSA with nearly equal film exposure times (1-2 min) are shown. The lower images represent results of EMSAs for -471A/B and -778A/B probes after longer exposure times (40 min) to reveal weak protein-DNA complexes.

Figure 3. Transcription factors bind with higher affinity to -80A (A) than to -80B (B) oligo (see Fig. 1A). 2 μ l nuclear extract isolated from postconfluent Caco2 cells were used for each binding reaction (no extracts were added in lanes 1 and 6) with 20 fmol of biotinylated oligos in EMSA. Competition and cross-competition experiments were carried out. **A** Biotinylated A probe was competed away with 50-fold (lane 3) and 100-fold excess of unlabelled A (lane 4) and B oligo (lane 5). Biotinylated B probe was competed away with 50-fold (lane 8) and 100-fold excess of unlabelled B (lane 9). The lower image represents film overexposure marked italic numbered lanes. **B** Biotinylated A probe were competed away with increasing amounts of A or B oligo as indicated.

Figure 4. GATA factors show stronger interaction with -80A (A) than with -80B (B) oligo (see Fig.1A). 2 μ l nuclear extracts isolated from post-confluent Caco2 cells (lanes 1 and 2), HeLa cells (lanes 3 and 4) and HeLa cells transfected with GATA-5 (lanes 5-8) and GATA-6 (lanes 9-14) were used for binding reactions with 20 fmol of biotinylated oligos A or B probe. Specific shifts were diminished by 100-fold excess of unlabelled A or B oligo and are indicated with an arrowhead.

Figure 5. *FABP2* promoter variant A is more activated by GATA factors than variant B in Huh7 cells. Luciferase activities of *FABP2* promoter haplotypes A and B and chimeric constructs of both variants were assessed 48 h after transfection in Huh7 cells using dual luciferase assay. The *Firefly* luciferase activities were normalized to *Renilla* luciferase activities and are given as relative luciferase activities in % of GATA-5 activated variant A.

Each experiment was performed in triplicate for each sample. The results are expressed as mean \pm SEM for at least three independent experiments. ***, $p < 0.001$, **, $p < 0.01$, *, $p < 0.05$, as compared with haplotype A activity. +, $p < 0.05$, as compared with haplotype B activity.

Figure 1

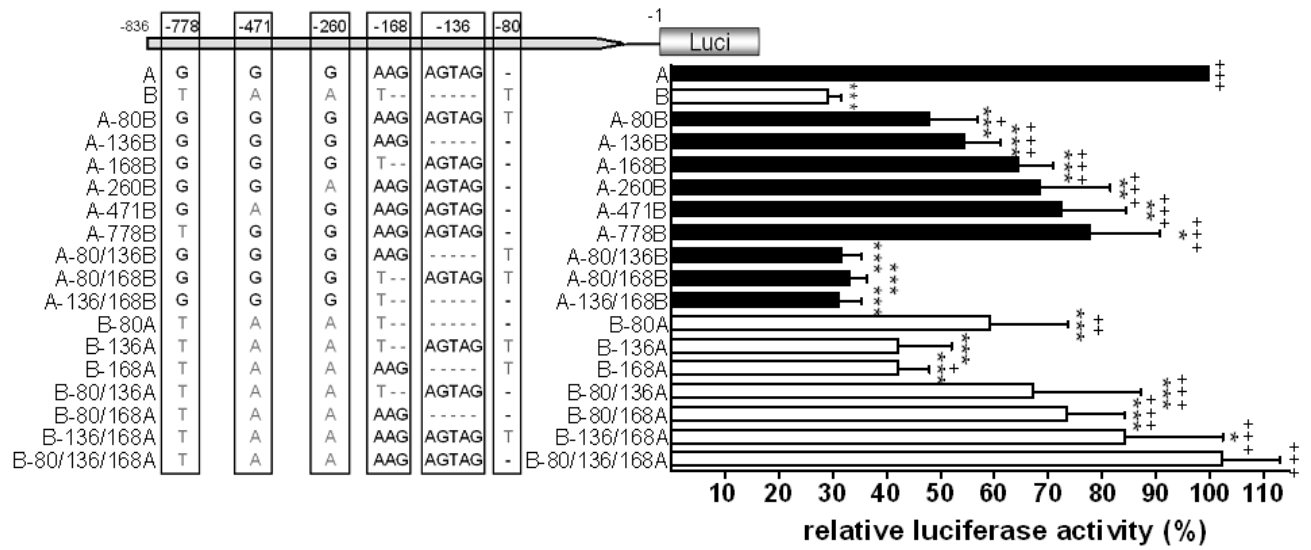


Figure 3

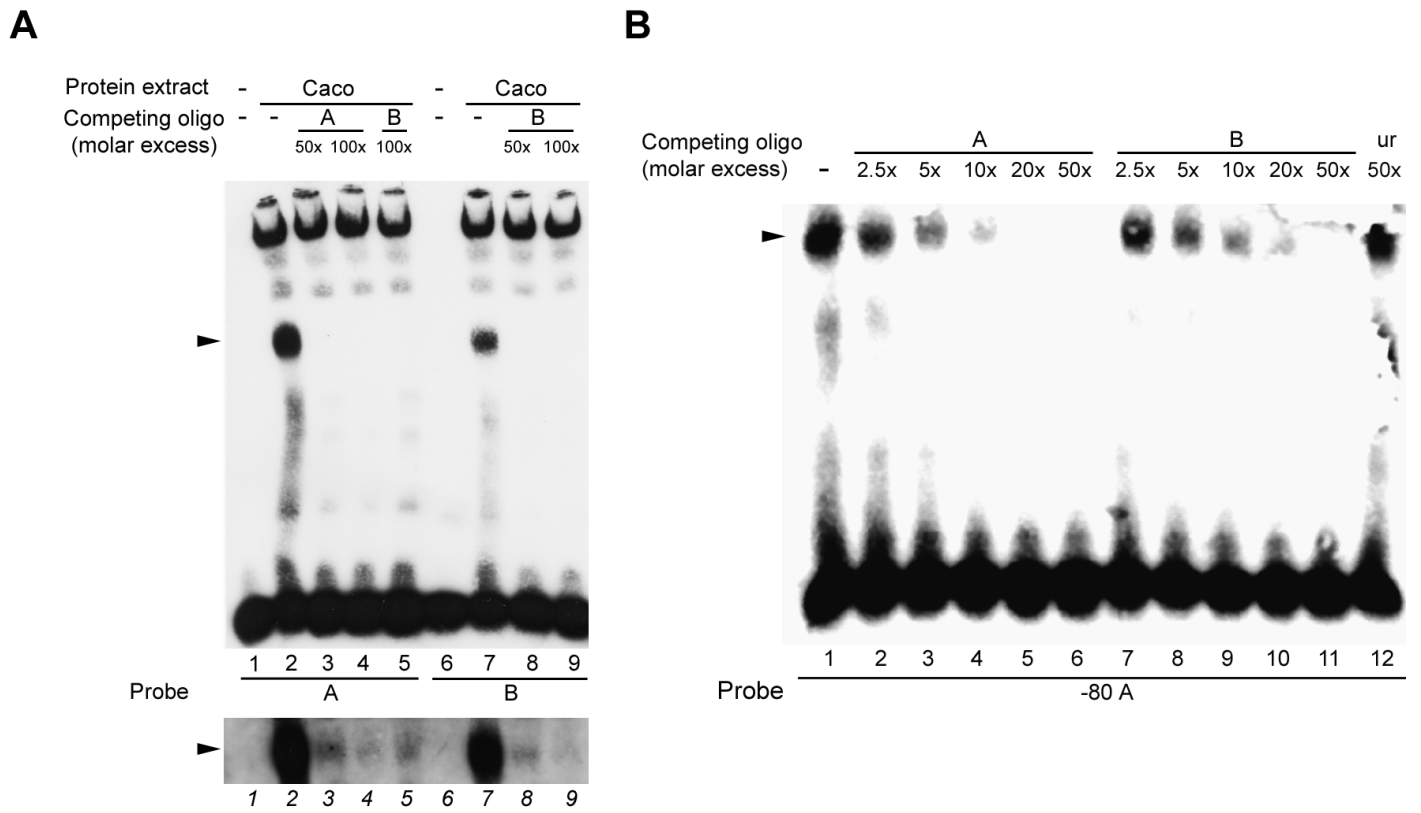


Figure 4

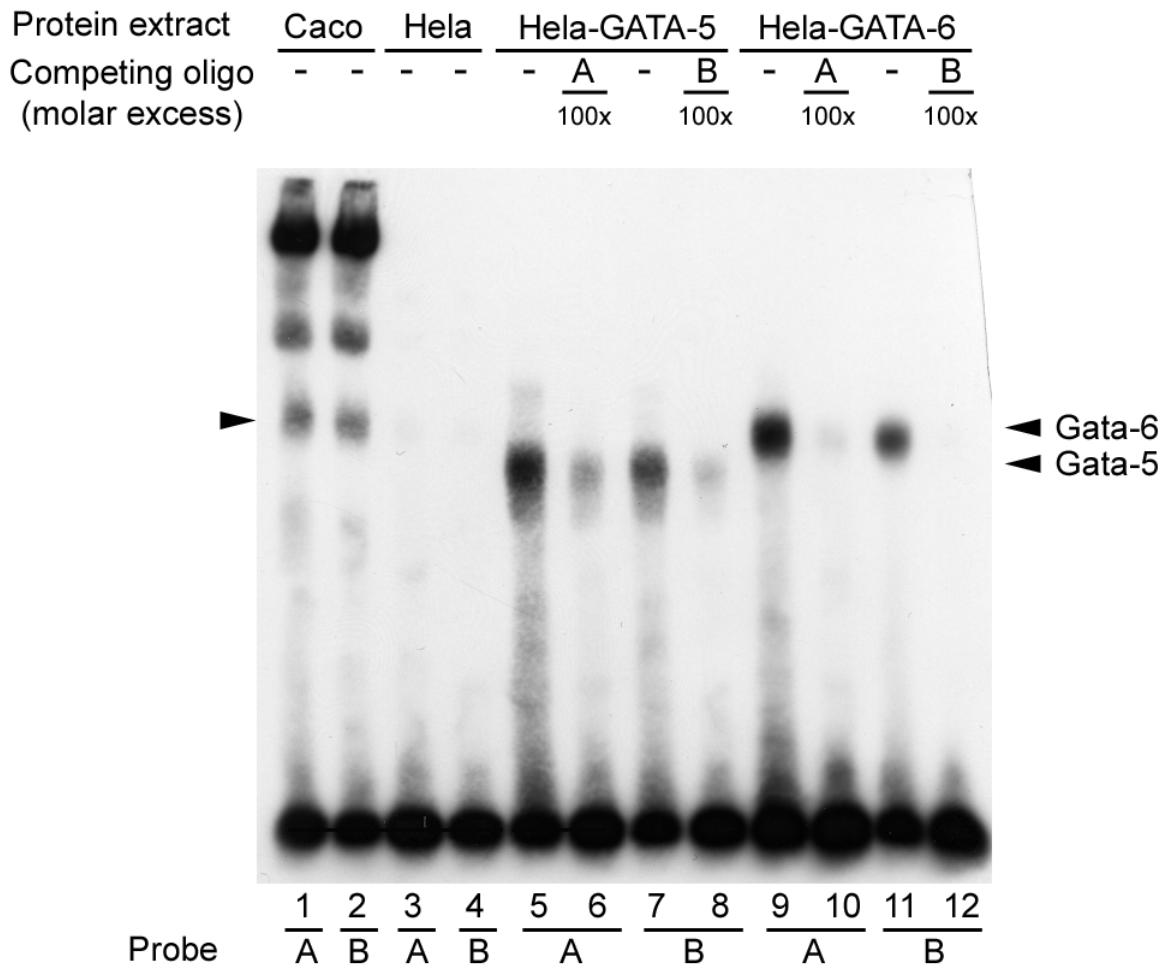
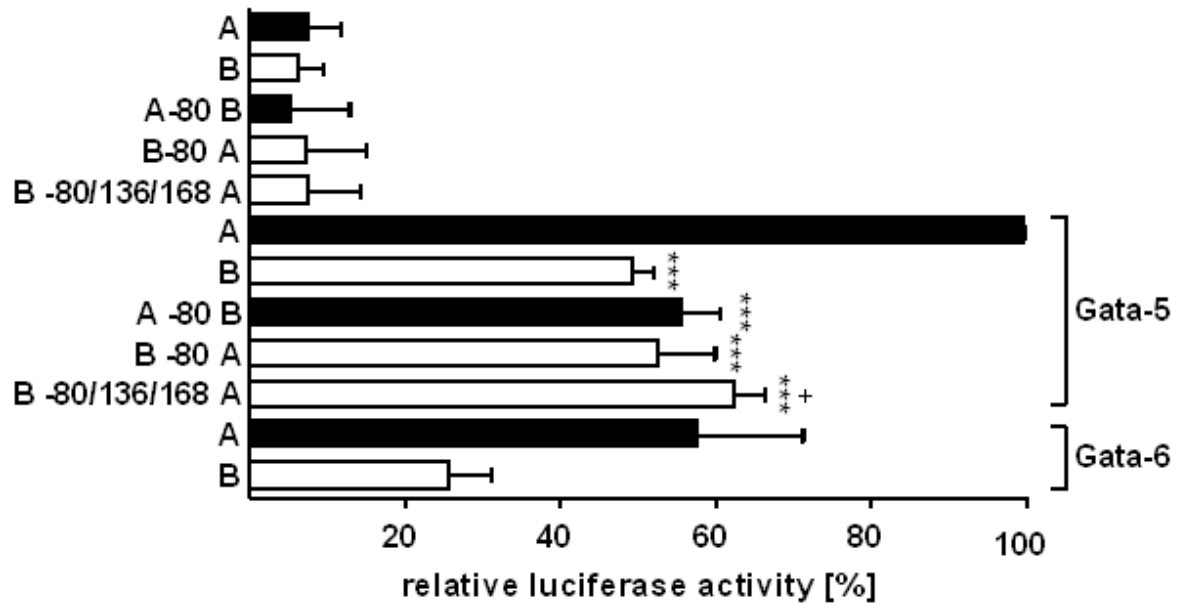


Figure 5



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

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 4 α (HNF-4 α), involved in regulation of genes of fatty acid metabolism and differentiation. Electromobility shift assays demonstrated that HNF-4 α 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-4 α 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-4 α , that are both candidate genes for diabetes type 2, may be a powerful approach.

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Keywords: hFABP2; HNF-4 α ; 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-4 α 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-4 α 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 meet the requirements in absorption of macro- and micro-nutrients. 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-4 α , originally considered as orphan nuclear receptor, was previously shown to bind fatty acid acyl-CoA esters as ligands. These were able to modulate HNF-4 α 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).

[14]. HNF-4 α 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-4 α 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-4 α . 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-4 α 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 *EcoRV* blunt-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-1 α and pRc/CMV-HNF-4 α 2 were a kind gift from Dr. G. Ryffel (University of Essen, Germany). Open reading frames of HNF-1 α and HNF-4 α 2 were amplified by PCR and subcloned in expression vector pDest40 using Gateway technology. Preparation of human PPAR γ 2 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-4 α 2, HNF-1 α , and PPAR γ /RXR α . 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-4 α 2 and pDest40-HNF-1 α , with 33 ng of PPAR γ 2 and RXR α 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'-CATGGCAGATGTTTAAAGTCATTCTTTCTTT-3', -468/-480: 5'-GAATCTTATTAACCTTAGCTTTTCAAC-3', -336/-324: 5'-CAACATAGTCTGCACCTTGAACCTTAGAAAAAC-3', mutated -336/-324: 5'-CAACATAGTCTGCAAGGGTAACTTAGAAAAAC-3', -120/-102: 5'-GTGATTTCTGAACTTTAAGCTTCCACATCAC-3' and unrelated oligo: 5'-CAGAACGAGAATTAAGAATTAATAAGAATAAGAATTAATTAATTGCTTG-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 μ l) contained 1 mM Tris, 50 mM KCl, 1 mM DTT, 1 μ g 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 \times 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-4 α (C-19): sc-6556 and HNF-1 α (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 \pm 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

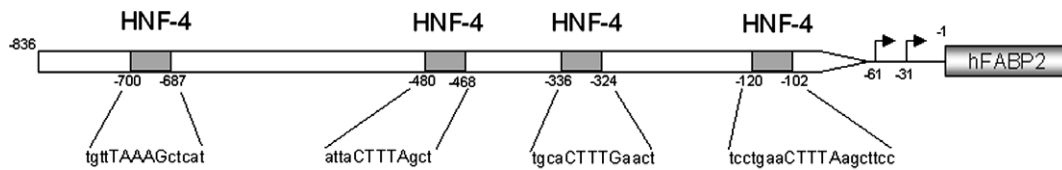


Fig. 1. Putative HNF-4 binding sites in the human intestinal fatty acid binding protein 2 (hFABP2) gene promoter. The nucleotide sequence -836 to -1 relative to translation start site is shown. The sequence was analysed with TRANSFAC database and compared with the HNF-4 factor matrix. An element, which 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-4 α 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-4 α to this promoter element at corresponding position $-120/102$ in human FABP2. However, promoter sequence is not fully conserved and binding affinity of HNF-4 α 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-4 α 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-4 α antibody or supershift signal may be located in unspecific signals. To assure that the effect was caused specifically by HNF-4 α 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-4 α binds

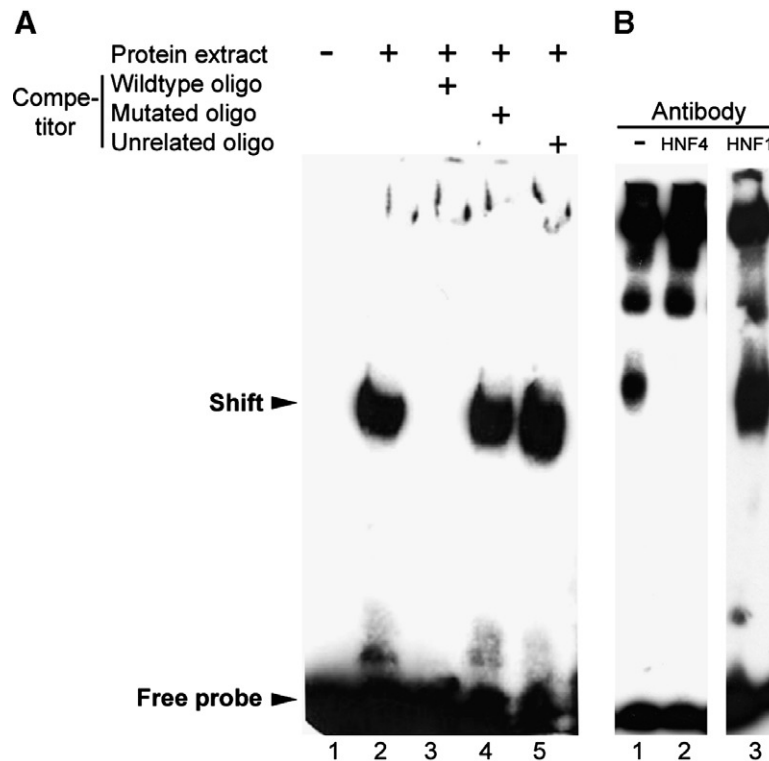


Fig. 2. Specific interaction of HNF-4 α to the human hFABP2 gene promoter at position $-336/-324$. Two microliters of nuclear extracts isolated from postconfluent Caco2 cells were used for each binding reaction with 20 fmol of biotinylated oligos (no extract was added in B, lane 1) in electromobility 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 μ g of HNF-4 α 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-4 α 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-4 α –/– mice [27]. Together these findings give rise to assumption that HNF-4 α is

essential for FABP2 expression. This dramatic effect may be explained by synergistic action of HNF-4 α with other transcription factors. HNF-4 α 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-4 α in HeLa cells

In order to study the influence of position –336/–324 on HNF-4 α induced hFABP2 promoter activity directly we used HeLa cells that do not express FABP2 [24] and HNF-4 α . As shown in Fig. 3B, hFABP2 promoter exhibits a 7.3-fold increase in luciferase activity when co-transfected with HNF-4 α plasmid. A mutation in the HNF-4 binding site at positions –336/–324 reduced the activation of the hFABP2 promoter by HNF-4 α 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-4 α 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-4 α but also by PPAR/RXR heterodimers [32,33] we tested the activation of FABP2 promoter

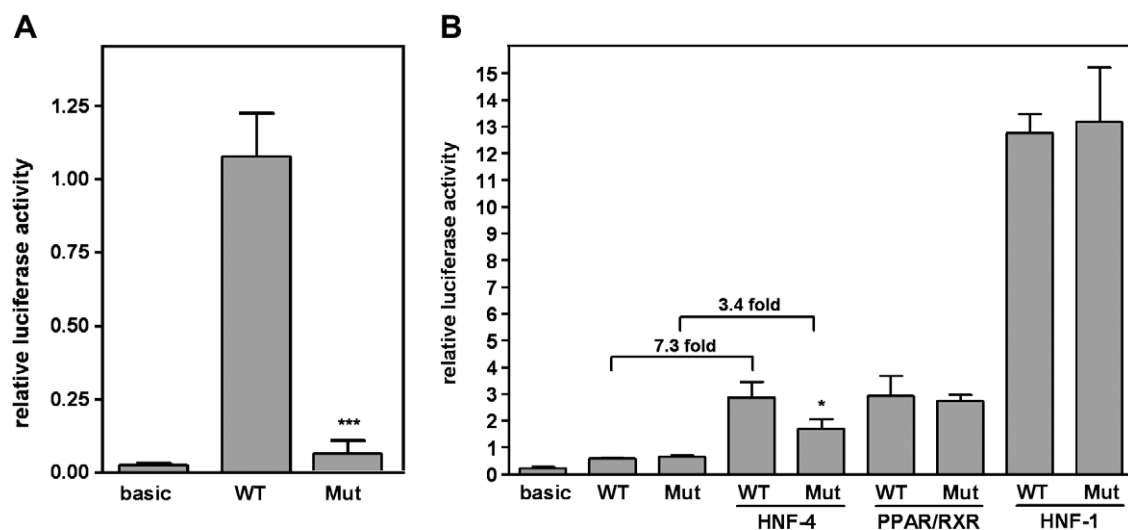


Fig. 3. Reporter assay of hFABP2 promoter wild type (WT) and HNF-4 mutant (Mut) constructs after transient transfection using dual luciferase assay. (A) Basal activities of FABP2 promoter constructs were assessed in postconfluent Caco-2 cells 120 h after transfection. *** $p < 0.001$, as compared with 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-4 α 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 \pm SD for two (A) or three (B) independent experiments.

constructs by PPAR γ /RXR α . No differences in activation of wild type and mutant with or without 2 μ M of PPAR γ 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-4 α is involved in transcriptional regulation of Apo A IV by lipid micelles, when micelles supplied to apical pole of Caco-2 cells. If HNF-4 α also plays a role in regulation of hFABP2 by dietary lipids needs to be proved.

Interestingly, HNF-4 α 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-4 α , 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-4 α 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-4 α binding sites in the human FABP2 promoter and identified a functional HNF-4 α 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-4 α binding site in regulation of FABP2 by dietary lipids should be in focus of future investigations.

Acknowledgments

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**Transcriptional regulation of the Fatty Acid Binding Protein 2 (FABP2) gene by
the hepatic nuclear factor 1 alpha (HNF-1 α)**

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

***Corresponding author**

Maja Klapper, Molecular Nutrition, Institute of Human Nutrition and Food Science,
Christian-Albrechts-University of Kiel, Heinrich-Hecht-Platz 10, 24118 Kiel, Germany.
Tel.: 049 (0) 431-8805661; Fax: 049 (0) 431-8805658; E-mail: klapper@molnut.uni-
kiel.de

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Abstract

The human fatty acid binding protein (FABP2) is involved in intestinal absorption and intracellular trafficking of long-chain fatty acids. *FABP2* knock-out mice and association analysis of functional promoter haplotypes revealed that *FABP2* is a susceptibility gene for insulin resistance and related traits. Here we investigate transcriptional regulation of *FABP2* by the endodermal hepatic nuclear factor 1 alpha (HNF-1 α). In electromobility shift and supershift assays we show the presence of two overlapping HNF-1 α binding sites within the *FABP2* promoter regions -185 to -165 and -169 to -149. Reporter gene analysis in Caco-2 and Hela cells demonstrated that HNF-1 α activates an 836 bp *FABP2* promoter construct by 3.5 and 20-fold, respectively. This induction is identical between *FABP2* promoter haplotypes. Mutational analysis of HNF-1 α element -185 to -165 resulted in about 50 % reduction of basal activity of the *FABP2* promoter construct in Caco-2 cells, whereas disruption of the -169 to -149 element had marginal effect. Co-transfection experiments in Hela cells with HNF-1 α revealed similar effects on activity of *FABP2* promoter mutants. In conclusion, our data suggest a major role of HNF-1 α in control of *FABP2* expression in intestine via a functional HNF-1 α recognition element within *FABP2* promoter region -185 to -165.

Introduction

The human intestinal fatty acid binding protein FABP2 belongs to a family of small 15 kDa cytosolic proteins that have multiple functions in metabolism and regulation. They bind and transport free fatty acids to metabolic enzymes and specific subcellular compartments including the nucleus [reviewed in (Hauerland and Spener, 2004)]. Furthermore they seem to be involved in regulation of growth and differentiation (Hauerland and Spener, 2004). Although the specific function of FABP2 is still not fully elucidated, it is proposed to be involved in fat absorption by binding and intracellular transport of newly absorbed non-esterified long-chain free fatty acids (Baier et al., 1996; Levy et al., 2001; Lowe et al., 1987). Hereby, fatty acid transfer from membranes to FABP2 and vice versa occurs by protein-membrane collisional interaction (Thumser and Storch, 2000). The gene encoding human FABP2 contains four exons and is localized on chromosome 4q28-q31 (Sweetser et al., 1987). *FABP2* is specifically expressed in the intestine and is up-regulated during development and cell differentiation (Cohn et al., 1992; Gordon et al., 1985; Sweetser et al., 1988). The highest levels of the FABP2 protein were found in absorptive cells of the upper villus region (Haegebarth et al., 2006; Shields et al., 1986; Sweetser et al., 1988).

FABP2 is a candidate gene for diabetes type 2. The rare allele T54 of the functional *FABP2* Ala54Thr polymorphism is associated risk parameters of insulin resistance (Baier et al., 1996; Weiss et al., 2002). More recently, *FABP2* promoter polymorphisms -80Del>T, -136Ins>Del (AGTAG>Del), -168AAG>T, -260C>G, -471G>A, and -778G>T were identified. They are in complete linkage disequilibrium. The resulting *FABP2* promoter haplotypes A>B were found to be associated with postprandial triglyceride levels (Geschonke et al., 2002), BMI (Damcott et al., 2003)

and type 2 diabetes (Li et al., 2006). Reporter analysis with *FABP2* promoter constructs demonstrated that the two promoter haplotypes differ in their transcriptional activity by factor of two to three. Male *FABP2* knock-out mice showed increased insulin and triglyceride plasma levels, increased body weight, and changes in liver histology, maybe resulting from impaired lipid metabolism (Agellon et al., 2006; Vassileva et al., 2000). Thus, the expression level of *FABP2* turns out to be an important determinant for insulin sensitivity and triglyceride metabolism.

The regulation of *FABP2* has been studied in some extent with other mammalian promoters, e.g. rat and mouse. In these studies, a direct involvement of HNF-4, ARP-1 (Rottman and Gordon, 1993) and GATA-4/TGF β -SMAD signaling (Belaguli et al., 2007) in the transcriptional regulation of *FABP2* was shown. Recently, we have demonstrated that the human *FABP2* is regulated by the hepatocyte nuclear factor HNF-4 (Klapper et al., 2007), an endodermal transcription factor that binds fatty acids as ligands. As well as HNF-4, HNF-1 α is also a key regulator of gene expression in pancreatic beta-cells, intestine, kidney, and liver. HNF-1 α is abundantly expressed in enterocytes and bind as homo- or heterodimers with HNF-1 β to the same consensus site (Rey-Campos et al., 1991). Heterozygous mutations in HNF-1 α cause maturity-onset diabetes of the young (MODY) type 3, which is the most prevalent MODY subtype (Yamagata et al., 1996). It is characterized by pancreatic beta-cell-deficient insulin secretion. Furthermore, HNF-1 α is required for expression of specific intestinal genes during development (Bosse et al., 2007). It activates intestine-specific expressed genes such as sucrose isomaltase (SI) (Wu et al., 1994) and lactase-phlorizin hydrolase (LPH) (Mitchelmore et al., 1998). Interaction of HNF-1 α with other semi-restricted transcription factors such as in intestine abundantly expressed CDX-2 and GATA-4 have been already described and are responsible for tissue-specific

high expression of intestinal genes (Boudreau et al., 2002; Divine et al., 2004; Krasinski et al., 2001; Mitchelmore et al., 2000; van Wering et al., 2004; van Wering et al., 2002).

In the present study we aimed to investigate if the human *FABP2* promoter and its variants are regulated by endodermal HNF-1 α . By use of electromobility shift and super shift assays, reporter analysis, promoter mutants and cotransfection experiments we provide evidence that HNF-1 α plays a major role in the control of the human *FABP2* 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.

Human FABP2 promoter luciferase constructs. The dual luciferase system was used (Promega, Madison, WI). Cloning procedures were performed using Gateway Technology (Invitrogen), described previously (Klapper et al., 2007; Li et al., 2006). 836 bp fragments upstream from initiation codon of the *FABP2* promoter haplotype A and B were subcloned into pGL4.10[luc2] resulting in final pGL4.10[luc2]-hFABP2 (haplotype A, WT) and pGL4.10[luc2]-hFABP2-B (haplotype B, HT B) promoter reporter constructs, respectively. The empty pGL4.10[luc2] vector served as a negative control. Introduction of mutations into HNF-1 α consensus sites of pGL4.10[luc2]-hFABP2 resulting in pGL4.10[luc2]-hFABP2-M1 (M1) pGL4.10[luc2]-hFABP2-M2 (M2), pGL4.10[luc2]-hFABP2-M1/M2 (M1/M2) were performed using Quick ChangeTM 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 vector pRc/CMV-HNF-1 α was a kind gift from Dr. G. Ryffel (University of Essen, Germany). Open reading frame of HNF-1 α was amplified by PCR and subcloned in expression vector pDest40 using Gateway technology.

Transient transfections and reporter assays. Transient transfections were performed as described recently with FuGene6 (Roche, Basel, Switzerland) according to the manufacturer's instructions (Klapper et al., 2007). 1×10^4 Caco cells or 4×10^3 Hela cells were plated per 96-well. Caco-2 cells were co-transfected with 47,5 ng pGL4.10[luc2]-hFABP2, pGL4.10[luc2]-hFABP2-M1 (M1), pGL4.10[luc2]-hFABP2-M2 (M2), pGL4.10[luc2]-hFABP2-M1/M2 (M1/M2) or pGL4.10[luc2] as negative control and 2,5 ng pGL4.74[hRluc/TK] vector encoding Renilla luciferase as internal control. For co-transfections with HNF-1 α in Caco-2 and Hela cells 30 ng of pGL4.10[luc2]-hFABP2 promoter constructs and 66 ng of the expression vector pDest40-HNF-1 α or 66 ng empty pDest40 vector and 3 ng pGL4.74[hRluc/TK] vector were used. Luciferase activities were measured at indicated time points by Dual-Luciferase® Reporter Assay System (Promega).

For preparation of nuclear extracts containing HNF-1 α Hela cells were used. 4×10^5 cells were seeded in T25 flasks, transfected with 4 μ g expression plasmid pDest40-HNF-1 α or empty expression vector pDest40 and extracts were prepared 48 h after transfection.

Electromobility shift assay (EMSA). The Electromobility shift assay were essentially performed as described previously (Klapper et al., 2007). In supershift reactions the

following antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were used: HNF-1 α (C-19): sc-6547 and HNF-1 β (C-20): sc-7411.

Statistical analyses. Statistical analyses were performed in GraphPad Prism 4 (Graphpad Software, Inc., San Diego, CA) using ANOVA and unpaired two-tailed t-test. Significant differences were considered for P values less than 0.05.

Database identifier for investigated polymorphisms. For mentioned polymorphisms of human *FABP2* promoter haplotypes A and B the rs-numbers are: -80Del>T: rs5861422; -136AGTAG>Del: rs5861423; -168AAG>T: rs1973598; -260G/A: rs6857641; -471G/A: rs2282688; -778G/T: rs10034579.

Results

HNF-1 α activates FABP2 promoter in Caco-2 and Hela cells

In order to study transcriptional regulation of human *FABP2* promoter by HNF-1 α , we initially expressed HNF-1 α together with a luciferase reporter construct contained a functional 836 bp segment of the *FABP2* promoter (Klapper et al., 2007) in intestinal Caco-2 cells. We measured reporter activities at different time points after transient transfection (24-120 h) by dual luciferase assay. As shown in figure 1, *FABP2* promoter reporter activity was increased up to 3.5-fold by HNF-1 α compared to non-HNF-1 α transfected cells. Since Caco-2 cells express endogenously HNF-1 α (Boudreau et al., 2001; Hu and Perlmutter, 1999), we additionally studied the activation of *FABP2* promoter by HNF-1 α in Hela cells that do express neither HNF-1 α (Hu and Perlmutter, 1999) nor *FABP2* (Rottman and Gordon, 1993). The *FABP2* promoter activity was induced 20-fold by HNF-1 α in Hela cells (Fig. 3A), compared to relative low activation in Caco-2 cells. These data indicate that HNF-1 α is a strong activator of *FABP2* expression.

EMSA confirms specific interaction of HNF-1 α with FABP2 promoter regions -185 to -165 and -169 to -149

To identify potential HNF-1 α binding sites within the *FABP2* promoter we performed *in-silico* search using TRANSFAC Professional database (Kel et al., 2003; Matys et al., 2006). Hereby, two potential HNF-1 α binding sites within the promoter regions -185 to -165 and -169 to -149 were found. These elements are of interest because their overlapping nature suggested strong interaction with HNF-1 α . To test this hypothesis, we performed electromobility shift and super shift assays with a 49 bp oligonucleotide containing the two elements of the *FABP2* promoter (Fig 2A). As

shown in figure 2B, we obtained two specific complexes (indicated as A and B) with nuclear protein extracts from post-confluent and differentiated Caco-2 cells (lane 1, 2). Addition of an HNF-1 α antibody abolished the specific shift A completely (lane 3), whereas an antibody against HNF-1 β lead not to retardation of the specific complexes A or B (lane 4). Next, we used nuclear extracts from Hela cells that were transfected with HNF-1 α . As shown in figure 2B, we could detect strong binding of HNF-1 α (lane 7) to the oligonucleotide that were supershifted by addition of an HNF-1 α antibody (lane 8). No HNF-1 α -DNA complexes and supershifted signals were formed with Hela extracts that were transfected with empty vector (lanes 5, 6).

To investigate the specificity and binding characteristics of the two HNF-1 α binding sites we performed EMSAs as competition experiments with nuclear protein extract from Caco-2 cells. As shown in figure 2C, the shifts A and B (lane 2) were competed-away by 100 molar excess of unlabelled probe (lane 3). In contrast, an excess of an unrelated oligonucleotide (lane 4) could not interfere with binding of proteins. Next, we performed competition experiments with oligonucleotides (Fig. 2A) containing a 4-bp mutation ATTA→CGGC in either of the binding sites -185 to -165 (M1) and -169 to -149 (M2), or in both sites simultaneously (M1/M2). As shown in figure 2 C, excess of unlabelled M1 (lane 5) or M2 (lane 6) eliminated the shifts A and B partially indicating that HNF-1 α is still able to bind the mutated unlabelled oligonucleotides. When both HNF-1 α consensus sites were mutated simultaneously, the appropriate oligonucleotide M1/M2 did not bind any transcription factor and the specific shifts A and B were restored completely (lane 7). These data demonstrate that HNF-1 α binds to both consensus sites within the *FABP2* promoter regions -185 to -165 and -169 to -149. However, as evidenced by the supershift data (Fig. 2 B) only complex A is caused by HNF-1 α binding.

The HNF-1 α binding sites within FABP2 promoter region -185 to -165 and -169 to -149 are responsible for 50 % of the FABP2 promoter activity

To investigate the functional importance of HNF-1 α binding sites at promoter regions -185 to -165 and -169 to -149 for *FABP2* expression, promoter-reporter assays were performed in Caco-2 cells. Within the *FABP2* promoter construct, we introduced a 4-bp mutation ATTA \rightarrow CGGC into the core sequences of either HNF-1 α consensus sites (Fig. 2A) at positions -185 to -165 (M1), -169 to -149 (M2) or into both consensus sites simultaneously (M1/M2). We measured luciferase activities at 120 h after transfection because *FABP2* mRNA as well as protein are considerably expressed in Caco-2 cells under these conditions (Li et al., 2006). As shown in figure 3A, the mutation in HNF-1 α binding site at position -185 to -165 (M1) decreases the relative reporter activity to 50% in comparison to the *FABP2* promoter wild type construct. In contrast, mutation of HNF-1 α binding site at position -169 to -149 (M2) had only marginal effect onto *FABP2* promoter activity. Simultaneous disruption of both HNF-1 α binding elements could not further reduce the promoter activity of *FABP2* promoter mutant M1/M2 in comparison to single mutant M1.

EMSA data showed two shifts with a HNF-1 α binding sites containing oligonucleotide and nuclear protein extracts from Caco-2 cells. Thus, we cannot completely rule out that abolishing of binding of other transcription factors to these consensus sites may also be responsible for reduction of *FABP2* promoter mutants in Caco-2 cells. For this reason we compared the activation of *FABP2* promoter wild type and mutants by HNF-1 α in HeLa cells after transfection with a HNF-1 α plasmid. As shown in figure 3B, mutation of the HNF-1 α binding site at position -185 to -165 lead to about 35% reduction of the HNF-1 α induced *FABP2* promoter-reporter activity. Mutation of the other HNF-1 α element has essentially no effect. Disruption of both sites resulted in

nearly 50% decrease of HNF-1 α mediated activation of *FABP2* promoter mutant M1/M2. The results obtained in HeLa cells are consistent with those obtained in post-confluent Caco-2 cells, where only eliminating of HNF-1 α element within *FABP2* promoter region -185 to -165 had significant influence onto basal activity of promoter constructs. In both cell lines elimination of both HNF-1 α consensus sites results in about 50% reduction of *FABP2* promoter basal activity and HNF-1 α induced activity, respectively.

The polymorphism -168AAG>T has no impact onto the activation of the FABP2 promoter by HNF-1 α

Because *FABP2* is a susceptibility gene for diabetes type 2 and related traits, its variant specific transcriptional regulation is of special interest. Two *FABP2* promoter haplotypes which contain polymorphic sites as positions -80, -136, -168, -260, -471, and -778 were identified. As described previously by us and other groups, the resulting minor *FABP2* promoter haplotype B has 2-3 fold lower basal transcriptional activity than major haplotype A in Caco-2 cells (Damcott et al., 2003; Formanack and Baier, 2004; Geschonke et al., 2002; Li et al., 2006). Interestingly, the two investigated HNF-1 α binding sites harbour the polymorphic site -168AAG>T (rs1973598) within their matrices (Fig. 2A). Therefore we questioned if this polymorphic site influences the activation of *FABP2* promoter by HNF-1 α . As shown in Fig. 3B, there is no difference in HNF-1 α induced activation of *FABP2* promoter haplotype A (WT) and B. Accordingly, EMSAs revealed no difference in HNF-1 α binding between the two haplotypes (not shown). Therefore, the *FABP2* promoter polymorphism -168AAG>T has no impact onto the HNF-1 α mediated transcriptional regulation of *FABP2*.

Discussion

In the present study we have investigated the presence of HNF-1 α binding sites in the human *FABP2* promoter. By using electromobility shift and super shift assays we identified two overlapping HNF-1 α elements within *FABP2* promoter regions -185 to -165 and -169 to -149. Inactivation of -185 to -165 HNF-1 α site resulted in reduction of basal and HNF-1 α induced *FABP2* promoter activity of about 50%. The disruption of -169 to -149 site had only marginal effect. Mutation of both elements simultaneously decreased basal and HNF-1 α induced promoter activity to 50% of wildtype activity in Caco-2 and Hela cells. Together, these data emphasize the crucial role of HNF-1 α as activator of *FABP2* promoter activity mediating the effect predominantly by binding to the more distal consensus site within *FABP2* promoter region -185 to -165. Interestingly this binding site has no corresponding element in *FABP2* mouse, rat or bovine promoter sequence at similar position but is conserved within a nearly conserved region in primates. This suggests a unique intestinal regulation of *FABP2* by HNF-1 α in primates.

Studies regarding transcriptional regulation of human *FABP2* were mainly done to characterize the basal activities of the two *FABP2* promoter haplotypes (Damcott et al., 2003; Formanack and Baier, 2004; Geschonke et al., 2002; Li et al., 2006). More recently, we have shown that *FABP2* is regulated by HNF-4 α (Klapper et al., 2007) and GATA factors (unpublished data), that are key regulators of intestinal differentiation processes. As described on liver and intestinal expressed fatty acid binding protein *FABP1*, *LPH* and *SI* promoters, HNF-1 α co-operates with these factors (Boudreau et al., 2002; Divine et al., 2004; Krasinski et al., 2001; Rowley et al., 2006; van Wering et al., 2004; van Wering et al., 2002) and is also involved in developmental expression of intestinal genes (Bosse et al., 2007). Therefore, HNF-

1 α may be the third important regulator for expression of intestinal *FABP2* during development and intestinal differentiation so far identified.

An influence of dietary factors on HNF-1 α and HNF-4 α by modulating signalling pathways was provided by several observations and mechanisms. Firstly, HNF-1 α is involved in the glucose-dependent transcriptional regulation of the sucrose-isomaltase (Gu et al., 2007; Rodolosse et al., 1998; Rodolosse et al., 1996) and sodium-dependent glucose transporter (Vayro et al., 2001). Secondly, expression and DNA binding of HNF-1 α in livers of protein restricted rats were increased (Marten et al., 1996). Thirdly, glucose as well fatty acids stimulated insulin secretion resulting in decreased activity of cAMP-dependent protein kinase A (PKA). Phosphorylation of HNF-4 α by PKA results in decreased binding affinity to DNA (Viollet et al., 1997). Fourthly, an increased ATP level, that reflect the energy status of a cell, inhibit 5'-AMP dependent kinase (AMPK) (Hardie, 2003). AMPK was shown to suppress the expression of several transcription factors including HNF-1 α and HNF-4 α (Leclerc et al., 2001) and phosphorylates HNF-4 α which leads to reduced protein stability and dimer formation/DNA binding (Hong et al., 2003). Expression and stability of HNF-1 α and binding of HNF-1 β to DNA, as previously shown with sucrose-isomaltase promoter, is also dependent on protein phosphatase 1a/2a activity (Carriere et al., 2001). Together, diet induced changes in ratio of cellular protein kinase and phosphatase activity seem to regulate transcription factor activities, including HNFs. Therefore, HNF-1 α may be a general mediator of nutritional signals and energy status for transcriptional adaptations of *FABP2* expression in enterocytes. Based on this hypothesis it seems to be interesting to study the influence of *FABP2* promoter haplotypes with respect to nutrients, such as fat intake in an epidemiological approach.

Interestingly, as well as *FABP2* also HNF-1 α is discussed to be associated with late onset form of diabetes type 2 (Holmkvist et al., 2006; Rissanen et al., 2000; Winckler et al., 2005). The identified coding SNPs of HNF-1 α I27L and A98V alter its transcriptional activity (Holmkvist et al., 2006). Although there is no differential regulation of *FABP2* promoter haplotypes by HNF-1 α , the combination of particular *FABP2* and *HNF-1 α* genotypes in individuals may be of importance for differences in expression level of *FABP2* *in-vivo*. It should be also noted, that there is no evidence *in-vivo* for genotype specific expression and function of *FABP2* promoter haplotypes, since it is very difficult to obtain healthy probes from the intestine. Therefore it seems worthwhile to determine the relative importance of these HNF-1 α genotypes on *FABP2* expression level *ex-vivo* in future studies.

In conclusion, in the present study we demonstrated that HNF-1 α is a strong activator of human *FABP2* promoter. This activation is essentially mediated by the *FABP2* promoter region -185 to -165. In future studies, the involvement of HNF-1 α in differentiation and nutrition dependent regulation of *FABP2* should be considered.

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Figure legends

Figure 1. HNF-1 α induced activity of human *FABP2* promoter construct was assessed in Caco-2 cells 24h, 48h, 72h, 96h or 120h after transfection using dual luciferase assay. The *Firefly* luciferase activities were normalized to *Renilla* luciferase activities and activities of negative control plasmid were subtracted. Resulting relative luciferase activities are expressed as fold activation of basal *FABP2* promoter activities. Experiment was performed in triplicate for each sample. The results are expressed as mean \pm SD.

Figure 2. Specific binding of HNF-1 α to human *FABP2* promoter regions -185 to -165 and -169 to -149. **A**, Sequences of oligonucleotides used in EMSA are presented. Underlined and bold nucleotides indicate the matrices and core sequence, respectively, of putative HNF-1 α binding sites. Polymorphism -168 AAG>T is marked. Sequence is given in 5'→3' direction. Sequence numbering is indicated relative to translational start site. **B**, EMSAs were performed with biotinylated oligonucleotide (WT) and without (lane 1) or with nuclear extracts from postconfluent Caco-2 cells (2-4) or HeLa cells (5-8), which were transfected with control plasmid (5, 6) or HNF-1 α plasmid (7, 8). Supershifts were performed with antibodies against HNF-1 α (3, 6, 8) or HNF-1 β (4). Specific shifts (A, B) are indicated with an arrowhead. Supershift was indicated with an asterisk. **C**, EMSAs were performed with biotinylated oligonucleotide (WT) and without (lane 1) or with of nuclear extracts from postconfluent Caco2 cells (2-7). Competition experiments were carried with 100-fold excess of unlabelled oligonucleotides as indicated and described in A. Specific shifts (A, B) are indicated with an arrowhead.

Figure 3. Reporter activity of human *FABP2* promoter wild type (WT) and mutants (M1, M2, M1/M2) was assessed in Caco-2 cells (A) and in HNF-1 α transfected HeLa cells (B) using dual luciferase assay. A 4-bp mutation ATTA→CGGC was introduced into the core sequences of either HNF-1 α consensus sites within *FABP2* promoter regions -185 to -165 (M1), -169 to -149 (M2) or into both consensus sites simultaneously (M1/M2). Constructs M1, M2 and M1/M2 contain mutations that are shown in Fig. 1. The *Firefly* luciferase activities were normalized to *Renilla* luciferase activities and negative control was subtracted. Results are given as relative luciferase activities. Each experiment was performed in triplicate for each sample. The results are expressed as mean \pm SEM for at least three independent experiments. **A**, Basal *FABP2* promoter activities were determined in Caco-2 cells 120 after transfection. The relative reporter activity of *FABP2* promoter WT was set to 100%. ***, $p < 0.001$ as compared with basal *FABP2* promoter activity **B**, Induction of *FABP2* promoter activity of promoter constructs by HNF-1 α was assessed in HeLa cells 48 h after transfection. The relative reporter activity obtained in HeLa cells transfected with a control plasmid was set to 1-fold. HT B, *FABP2* promoter haplotype B. In comparison to the wild type *FABP2* promoter construct (= WT, haplotype A), HT B contains the minor allele of polymorphism -168AAG>T. **, $p < 0.01$, ***, $p < 0.001$ as compared with HNF-1 α induced *FABP2* promoter activity.

Figure 1

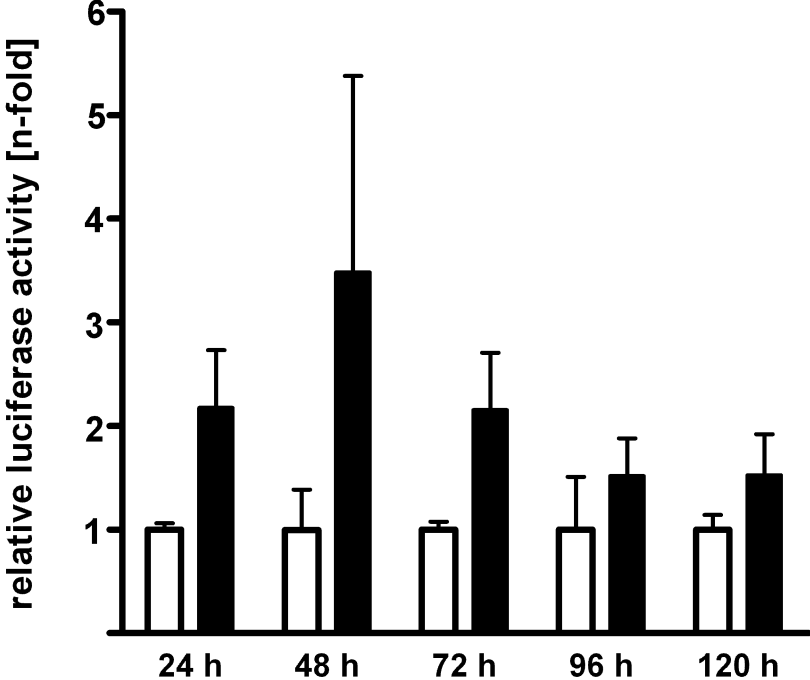


Figure 2

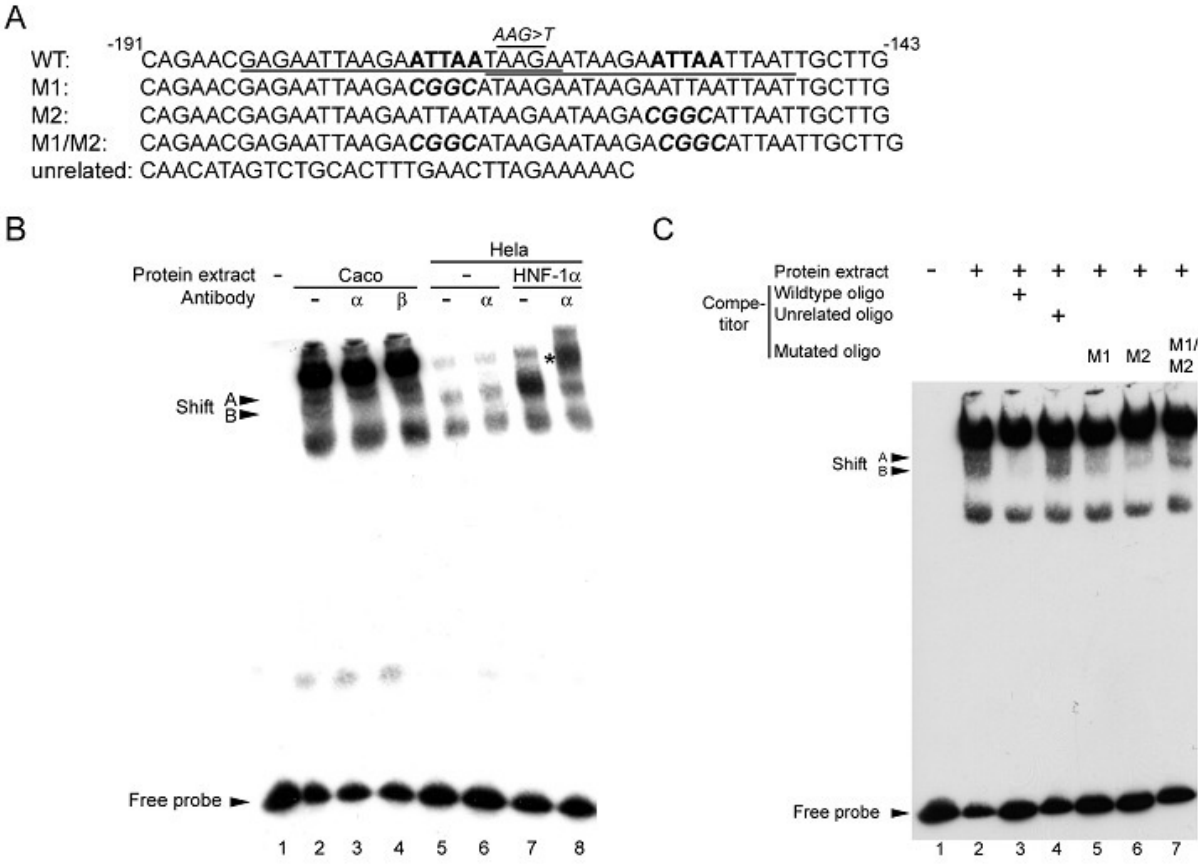
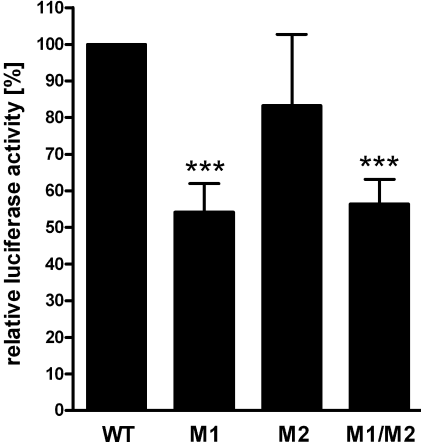
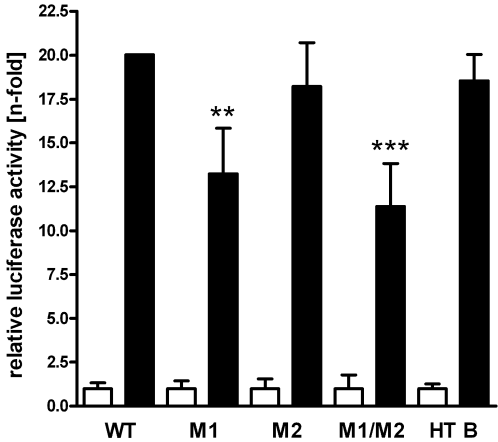


Figure 3

A



B



Lebenslauf

Maja Klapper

geboren am 24.01.1975 in Jena

Staatsangehörigkeit: deutsch

- seit 10/2003 Wissenschaftliche Mitarbeiterin an der Christian-Albrechts-Universität Kiel, Institut für Tierernährung und Stoffwechselfysiologie (Prof. Dr. S. Wolfram) bzw. Institut für Humanernährung und Lebensmittelkunde, Abteilung Molekulare Ernährung (Prof. Dr. F. Döring); Promotion: „Promoter variants and transcriptional regulation of the intestinal fatty acid binding protein gene (*FABP2*)“
- 02/2000 – Wissenschaftlicher Mitarbeiter an der Technischen Universität München, Lehrstuhl für Ernährungsphysiologie (Prof. Dr. H. Daniel); Schwerpunkt: Subzelluläre Lokalisation und Regulation des Peptidtransporters PEPT2
09/2003
- 12/1999 – Assoziiertes Mitglied des Graduiertenkollegs „Proteinfunktion auf atomarer Ebene“ an der Philipps-Universität Marburg, Institut für Physiologische Chemie
02/2000
- 1995 –1999 Studium der Oecotrophologie an der Justus-Liebig-Universität in Giessen
10/1999 Diplom
- 09/1997 – Diplomarbeit am Lehrstuhl Biochemie der Ernährung des Menschen (Prof. Dr. H. Daniel): „Konstitutive Expression epithelialer Peptidtransporter in der Hefe *Pichia pastoris* nach Zufallsmutagenese“
10/1998
- 03/1997 – Erasmus-Student an der University of Greenwich, School of Chemical and Life Sciences, London
08/1997
- 1993 - 1995 Studium der Trophologie an der Friedrich-Schiller-Universität in Jena
- 06/1993 Allgemeine Hochschulreife (Jena)
- 1983 – 1993 Polytechnische Oberschule mit erweitertem Russischunterricht Adolf Reichwein (seit 1991 Gymnasium), Jena
- 1981- 1983 Polytechnische Oberschule Clara Zetkin, Jena

Erklärung

Hiermit erkläre ich an Eides statt, dass ich die vorlegte Dissertation mit dem Titel

„Promoter variants and transcriptional regulation of the intestinal fatty acid binding protein gene (*FABP2*)“

selbständig und ohne unerlaubte Hilfe angefertigt habe und dass ich die Arbeit noch keinem anderen Fachbereich bzw. noch keiner anderen Fakultät vorlegt habe.

Maja Klapper

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