ARTICLE
Journal of Cellular Biochemistry 111:1330–1336 (2010)

Journal of Cellular Biochemistry

Identification of a Functional Hepatocyte Nuclear Factor 4 Binding Site in the Neutral Ceramidase Promoter

H. R. Maltesen, Jesper T. Troelsen, and Jørgen Olsen*

Department of Cellular and Molecular Medicine, University of Copenhagen, The Panum Institute, Bldg 6.4, Blegdamsvej 3, DK2200N, Denmark

ABSTRACT

The brush border membrane of the differentiated small intestinal epithelial cell is studded with membrane bound hydrolytic ectoenzymes involved in digestion. Previous studies of the regulation of genes encoding brush border enzymes have especially implicated the transcription factors hepatocyte nuclear factor HNF-1 and Cdx2. Recent genome-wide studies have, however, also identified HNF-4α as a transcription factor with a high number of target genes in the differentiated small intestinal epithelial cell. The Asah2 gene encodes neutral ceramidase, which is a hydrolytic brush border enzyme involved in ceramide digestion. It was the purpose of the present work to experimentally verify the functional importance of a HNF-4α binding site predicted by bioinformatic analysis to be present in the Asah2 promoter. Using supershift analysis, HNF-4α overexpression, and HNF-4α knockdown experiments it was confirmed that the predicted HNF-4α binding site identified in the Asah2 promoter is functional. The results support the hypothesis that HNF-4α might be important for intestinal glycolipid metabolism. J. Cell. Biochem. 111: 1330–1336, 2010.

KEY WORDS: HNF-4α; INTESTINAL DIFFERENTIATION; ASAH2; PROMOTER REGULATION

The brush border membrane is a hallmark of the differentiated small intestinal enterocyte; the absorptive epithelial cell of the small intestinal mucosa. Membrane bound hydrolytic ectoenzymes are embedded in the brush border membrane where they carry out their digestive functions [for a review see Norén et al., 1986]. The disaccharidase ectoenzymes lactase–phlorizin hydrolase and sucrase–isomaltase are two well described brush border enzymes and the study of their genes have led to the understanding of the pivotal role of the transcription factors Cdx2 and hepatocyte nuclear factor-1 (HNF-1) for the expression of these genes in the differentiated enterocyte. Whereas binding sites for Cdx2 are typically found in the promoters of genes such as the disaccharidases with small intestinal specific gene expression, binding sites for HNF-1 are also found in the promoters of genes such as, for example, the aminopeptidase N gene [Olsen et al., 1991; Sjostrom et al., 2000] expressed in a variety of epithelial or epithelially derived tissues.

Using genome-wide gene expression analysis approaches, we have recently demonstrated the massive involvement of another transcription factor, the HNF-4α, in the regulation of genes expressed in the differentiated enterocyte [Stegmann et al., 2006; Boyd et al., 2009]. Moreover, we have experimentally validated the promoters of the genes encoding the brush border ectoenzymes alkaline phosphatase [Olsen et al., 2005] and trehalase [Boyd et al., 2009] as targets for HNF-4α.

HNF-4α belongs to the superfamily of nuclear receptors [Sladek et al., 1990] and is expressed in the liver, small intestine, kidney [Sladek et al., 1990], and pancreas [Miquerol et al., 1994]. It is found in the nucleus, binds to DNA as a homodimer [Jiang et al., 1995], and belongs to the family of orphan receptors. HNF-4α is important for normal gastrulation [Chen et al., 1994], hepatocyte differentiation [Hayhurst et al., 2001; Parviz et al., 2003], and colon development and physiology [Garrison et al., 2006]. In the liver HNF-4α is essential for lipid homeostasis by activating genes involved in lipid metabolism [for a review see Hayhurst et al., 2001]. By bioinformatic analysis of genome-wide gene expression data and ^{1}H NMR metabolome data, HNF-4α was predicted to also participate in lipid metabolism in the small intestine [Stegmann et al., 2006]. To provide empirical support for this prediction, it is therefore of interest to experimentally verify the presence of HNF-4α binding sites in promoters for genes encoding enzymes involved in lipid metabolism in the small intestine. The bioinformatic identification of promoters

Grant sponsor: Danish Medical Research Council, Alfred Nielsen and Wife's Foundation, The Lundbeck Foundation.
*Correspondence to: Jørgen Olsen, Department of Cellular and Molecular Medicine, University of Copenhagen, The Panum Institute, Bldg 6.4, Blegdamsvej 3, DK2200N, Denmark. E-mail: jolsen@sund.ku.dk
Received 20 April 2009; Accepted 17 August 2010 • DOI 10.1002/jcb.22862 • © 2010 Wiley-Liss, Inc.
Published online 27 August 2010 in Wiley Online Library (wileyonlinelibrary.com).

which contained predicted HNF-4α binding sites and which encoded genes with increased expression during enterocyte differentiation [Stegmann et al., 2006] revealed a potential HNF-4α binding site in the promoter for the gene encoding the brush border hydrolase neutral ceramidase. Neutral ceramidase catalyzes the hydrolysis of the *N*-acyl linkage in ceramides to yield sphingosine and a fatty acid [for a review see Duan and Nilsson, 2009]. Neutral ceramidase is encoded by the Asah2 gene which in the mouse is transcribed from two different promoters generating two transcripts differing in their 5′ ends but with identical coding sequences [Okino et al., 2002].

In the present work, we demonstrate that the predicted HNF-4α binding site in the promoter closest to the coding part of the Asah2 gene is functional. We thereby implicate HNF-4α in the regulation of the expression of an enzyme involved in small intestinal glycolipid metabolism.

MATERIALS AND METHODS

PREPARATION OF NUCLEAR EXTRACTS

Mouse intestinal epithelium was isolated from mouse ileum by a previously described procedure [Stegmann et al., 2006]. In brief, C57Bl/6j mice were euthanized by cervical dislocation and the terminal 10–15 cm of the ileum was removed and placed in ice cold phosphate buffered saline (PBS). The following procedures were all performed at 4°C. The intestine was flushed with ice cold PBS so that no mucus was left and was then inverted on an 11.5 mm long plastic rod. The inverted intestine was submerged in a chelating buffer (27 mM Na_3-citrate, 5 mM Na_2HPO_4, 96 mM NaCl, 8 mM KH_2PO_4, 1.5 mM KCl, 55 mM D-sorbitol, 44 mM sucrose, 0.5 mM DTT) in a capped plastic centrifuge tube. The tube was rotated mechanically. After 4 h of rotation fresh buffer was added and the intestine was incubated for 14 h followed by 1 h of rotation. Remaining adherent epithelial cells were released by shaking (50 s) the intestine with a modified electrical milk whip. Isolation of nuclei and extraction of nuclear proteins followed the procedure described in Olsen et al. [1991]. The released epithelial cells were pelleted and resuspended in 300 ml buffer A (10 mM Hepes, pH 7.9, 0.88 M sucrose, 2.5 mM magnesium acetate, 1 mM EDTA, 1.5 mM $CaCl_2$) containing 0.05% (v/v) Nonidet P-40. The suspension was transferred to a modified food processor allowing blending under air free conditions thereby avoiding air bubbles to form during homogenization. Cell lysis was followed by microscopy and when more than 90% of the cells were lysed, the homogenate was pelleted (Sorval GSA rotor, 3,500*g*, 30 min). The pellet was resuspended in buffer B (10 mM Hepes, pH 7.9, 25 mM KCl, 2.5 mM magnesium acetate, 1 mM EDTA, 2 M sucrose, 10% (v/v) glycerol), layered over 10-ml cushions of buffer B and centrifuged in a Beckmann SW 28 rotor (55,000*g*, 1 h). The nuclear pellet was washed once by resuspension in buffer A containing 50 mM KCl. The purified nuclei were lysed for 20 min in buffer C (20 mM Hepes, pH 7.9, 25% (v/v) glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA). The lysate was cleared (25,000*g*, 30 min) and dialysed for 4 h against buffer D (20 mM Hepes, pH 7.9, 0.1 M KCl, 20% (v/v) glycerol, 0.2 mM EDTA). The dialysate was cleared (20,000*g*, 20 min). All buffers contained 1 mM DTT and protease inhibitor cocktail (P8340; Sigma) added just prior to use.

ANALYSIS OF THE ASAH2 TRANSCRIPTS IN THE MOUSE BRAIN, LIVER, AND SMALL INTESTINE

Four C57Bl/6j mice were euthanized by cervical dislocation and pieces (75 mg) of the liver, the small intestinal mucosa and the brain were immediately removed from each mouse and homogenized in 750 μl lysis buffer (Ambion) with 10 mM DTT (Sigma–Aldrich). Total RNA was extracted using the mirVana Total RNA isolation kit (Ambion).

The RNA concentration and quality was determined with the RNA 6,000 nano assay (Agilent Technologies) using the 2100 bioanalyzer (Agilent Technologies).

First strand cDNA was synthesized with 1 μg total RNA extracted from mouse liver, brain or small intestinal mucosa in a total volume of 20 μl using RevertAid^{TM} H Minus First Strand cDNA Synthesis Kit (Fermentas) following the manufacturers protocol with minor modifications. Poly (dT) oligomer (Fermentas) was used as primer.

Several transcripts have been reported for Asah2 [Okino et al., 2002]. We chose to discriminate between the downstream "brain type" transcript that corresponds to the reference sequence with accession number NM_018830, and the upstream "liver type" transcript with accession number AB037111 (see Fig. 1). Primers were planned using DNAstar software (DNASTAR). The forward primers lie in each transcript's first exon (ccgggcagaggatacacaag and tcaatcgcggagccttttct, NM_018830 and AB037111, respectively). The reverse primers lie in the second exon for NM_018830 (gcctctaagcacaccagcactc) and in the first exon of AB037111 (ggccgctggtctcccgtctcc). Mouse small intestinal cDNA and mouse genomic DNA were used as templates for the NM_018830 and AB037111 amplicons, respectively. PCR was conducted and amplified fragments were isolated by agarose gel electrophoresis. Gel purified DNA was sequenced with a Bigdye terminator cycle sequencing kit on a 3100 Avant Genetic Analyzer (both Applied Biosystems) and used as standard in the quantitative real time RT-PCR analysis.

Real time PCR was done with the LightCycler 480 SYBR Green I Master kit (Roche). The reactions were run on a LightCycler 480 II PCR machine (Roche) with the following programme: An initial denaturation at 95°C for 10 min (1 cycle) and amplification where each cycle consists of denaturation at 95°C for 10 s, annealing for 10 s (at 59°C for NM_018830 and 61°C for AB037111), and extension at 72°C for 20 s (40 cycles). The fluorescence was measured at the end of the extension. Finally, a melting curve was performed by increasing the temperature stepwise, while continually measuring the fluorescence. The melting curve was inspected to ensure that the fluorescence signal stemmed from the correct PCR product. In each run, a standard sample with 10^5 copies of the relevant amplicon was included.

ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

A potential HNF-4α binding site has previously been identified in the Asah2 promoter [see Supplementary table 10 in Stegmann et al., 2006]. Two complementary oligonucleotides corresponding to the predicted HNF-4α binding site (5′-ggatatctgacctgtgacccctgt-3′ and 5′- tcacaggggtcacaggtcagatat-3′) were annealed and 5′ end labeled with [γ-^{32}P]ATP (GE Healthcare) using T4 polynucleotide kinase (Fermentas). The product was purified on a G-25 spin column (GE

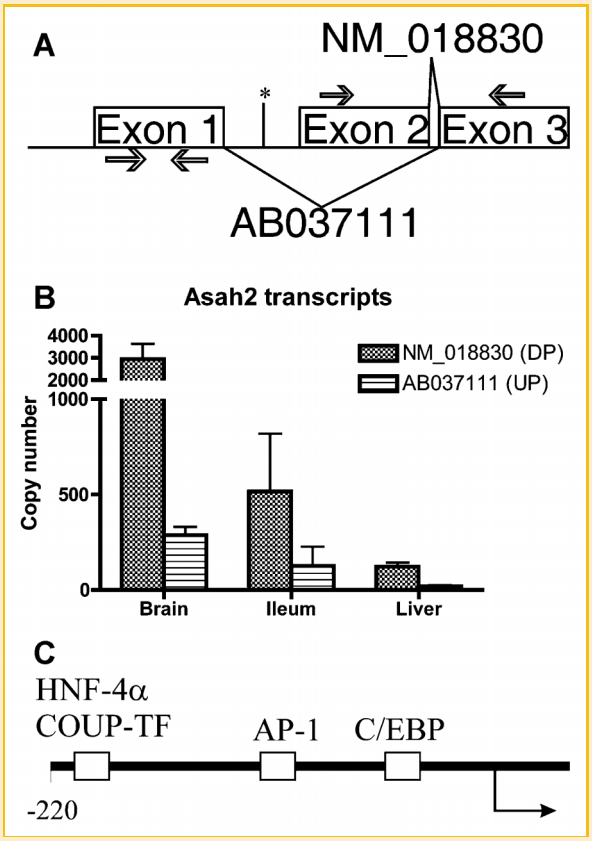

Fig. 1. The genomic structure of the Asah2 gene. A: Exon 1 is transcribed from the UP. Splicing of the exon 1 containing transcript results in the joining of exon 1 to exon 3 [Okino et al., 2002]. This transcript is represented in the Genbank entry with the accession number AB037111. Exon 2 is transcribed from the DP and splicing of the transcript containing exon 2 results in the joining of exon 2 to exon 3 [Okino et al., 2002]. This transcript is represented in the Genbank entry with the accession number NM_018830. The primers used in the RT-PCR are marked as arrows. The predicted HNF-4α lies in the DP upstream of exon 2 and is marked by an *. A schematic structure of this promoter is shown in C. B: Quantification of the numbers of NM_018830 transcripts (transcribed from the DP) and AB037111 transcripts (transcribed from the UP) in total RNA extracted from mouse brain small intestine and liver. It can be observed that approximately threefold more of the NM_018830 transcript is present in all three tissue compared with the AB0371111 transcript. This indicates that the DP upstream of exon 2 is the most active promoter in all three tissues. RNA from four different mice were analyzed (N = 4). C: A schematic representation of the DP upstream of exon 2 is shown together with some of the potential transcription-factor binding sites (COUP-TF, AP-1, and C/EBP) previously predicted by bioinformatic analysis [Okino et al., 2002]. The predicted HNF-4α binding site is the focus of the present work.

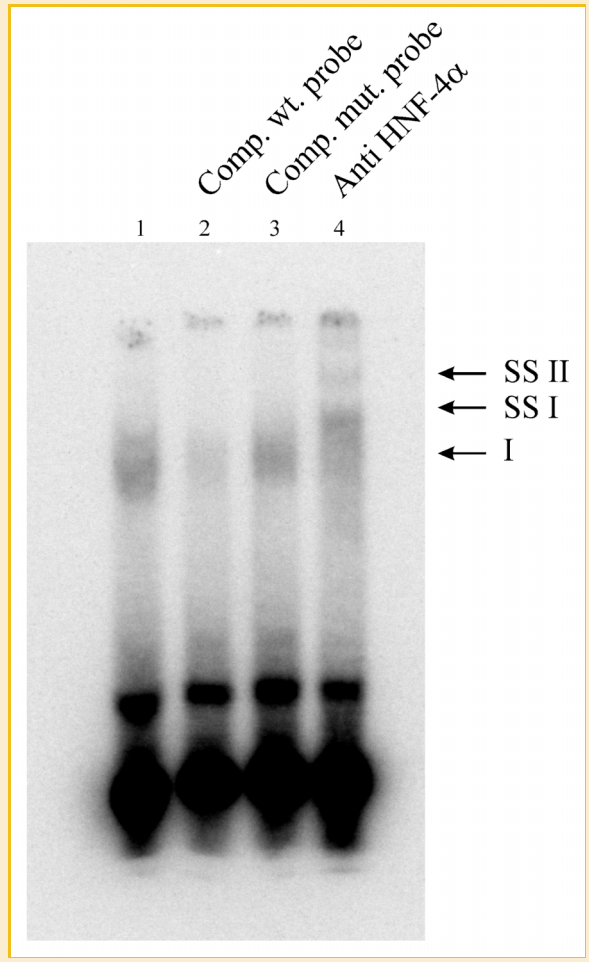

Fig. 2. EMSA analysis of the Asah2 gene HNF-4α binding site. Interactions between nuclear proteins and a ^{32}P-labeled double stranded oligonucleotide (position −202 to −227 [Okino et al., 2002]) were analyzed by EMSA. Nuclear extract from mouse small intestinal epithelial cells was added to the binding reactions (lanes 1–4), where indicated in the figure, the unlabeled competitors (Comp.) were added in a 250-fold molar excess. Anti HNF-4α IgG was added to lane 4. WT, wild type; mut, mutated. Arrows point to: I, shifted probe, SS I, fast migrating supershift, and SS II, slowly migrating supershift.

healthcare) and gel shift buffer (25 mM Tris–HCl pH 7.5, 5 mM MgCl, 60 mM KCl, 0.5 mM EDTA; 5% Ficoll 400; and 2,5% glycerol all from Merck) was added to a final concentration of 10 fmol/μl. Nuclear extract (3 μl; 1 μg/ml) was added to 15 μl buffer II (gel shift buffer; Protease inhibitor mix (Sigma), 1 mM DTT, 1 μg dIdC). Next, 2.5 pmol of cold competitor was added to the samples specified in the legend to Figure 2. The competitor added was either the WT oligonucleotide, or an oligonucleotide where the predicted HNF-4α binding site had been mutated (5′-ggatatcttctagatgacccctgt-3′, the underscored nucleotides had been changed to an *XbaI* site). The samples were incubated on ice for 10 min, 10 fmol of the ^{32}P labeled probe was added to each sample, and the samples were incubated for another 20 min on ice. Two micrograms of anti HNF-4α (SC-8987; Santa Cruz Biotechnology) was added to one of the samples, and it was incubated for another 10 min at 37°C. Loading buffer (3 μl; 0.2% (w/v) bromphenol blue, 10% glycerol (v/v), 45 mM Tris–borate pH 8.3, 1 mM EDTA) was added and the samples were run on a 5% non-denaturating polyacrylamide gel in TBE buffer at 100 V and 25 mA for approximately 1.5 h. The gel was used to expose a PhosphorImager screen, which was scanned with a Storm 820 (Molecular Dynamics). The HNF-4α polyclonal antibody used is raised against an epitope from human HNF-4α and cross-reacts with HNF-4α of mouse origin. The EMSA supershift experiment was replicated, also with nuclear extract from the enterocyte-like

human colon carcinoma cell line Caco-2, with similar results (not shown).

PLASMID CONSTRUCTS AND LUCIFERASE ASSAYS

The Asah2, AB037111, upstream promoter (UP) was amplified from mouse genomic DNA using the LPF1 and LPR1 primers used in Okino et al. [2002] with the modification that *Nhe*I and *Hin*dIII restrictions sites were added to 5′ end of the primers. The Asah2, NM_018830, downstream promoter (DP) from −967 to +57 bp with transcription start site as +1 was amplified from genomic mouse DNA with the following sense (S1) and antisense (AS1) primer pair: 5′-atatgctagcagggcatggctgtgagtcg-3′ and 5′-ataaagcttgtgcctgctgg-gatgcttgtgtat-3′, respectively, where the underlined nucleotides are *Nhe*I and *Hin*dIII sites, respectively. Both the upstream and the downstream Asah2 promoter PCR products were then cloned into the *Nhe*I and *Hin*dIII sites in front of the firefly luciferase reporter gene in the pGL3 basic plasmid (Promega Corporation). Site directed mutagenesis against the presumed HNF-4α binding site (ggggtca-caggtcag) was made by overlap PCR as originally described by [Higuchi et al., 1988] using the downstream Asah2 promoter construct as a template. In the first two separate PCR reactions, the following primers were used (5′-ctagcaaaataggctgtccc-3′with 5′-tttcacaggggtcatctagaagatatcctg-3′ and 5′-caggatatcttctaga-gacccctgtgaaa-3′ with 5′-gccttatgcagttgctctcc-3′). The underlined nucleotides had been changed into an *Xba*I site. In the second PCR reaction, the primers 5′-ctagcaaaataggctgtccc-3′ and 5′-gccttatg-cagttgctctcc-3′ were used with the two products from the first PCR reactions. The product was cloned into the *Nhe*I and *Hin*dIII sites of the pGL3 basic vector. The region surrounding the HNF-4α site from position −37 to −254 in the cloned Asah2 DP was amplified using the primers 5′-tgggtcgactgaatcctatcaatatcag-3′ and 5′-aacgtcgacc-caaggctctgtaacttaacc-3′ (introduced *Sal*I sites are underlined) and cloned into the *Sal*I site downstream of the luciferase gene in the Asah2 UP pGL3 construct, thus placing the HNF-4α site in a downstream enhancer position relative to the Asah2 UP. An additional construct was made using the mutated version of the HNF-4α site. The constructs were verified by sequencing.

Cos-7 cells were grown in Dulbecco's modified eagle medium with 10% FBS, penicillin (100 U/ml), and streptomycin (100 μg/ml). 5×10^4 Cos-7 cells were seeded per well, and 24 h after seeding, transfection with 0.67 μg Asah2 promoter/luciferase construct, 83.3 ng CMV LACZ, and 0.417 μg of either pCDNA3.1 HNF-4α (encoding rat HNF-4α) or pCDNA3.1. Exgen 500 (Fermentas) was used as the transfection agent and 15.6 equivalents were used. The medium was changed 6 h after transfection. For each transfection series, six wells were transfected. Four transfection series were performed: (1) The Asah2 DP with and without co-transfection with HNF-4α expression plasmid (2) The Asah2 DP with mutated HNF-4α binding site with and without co-transfection with HNF-4α expression construct (3) The Asah2 UP construct with the HNF-4α site in enhancer position with and without co-transfection with the HNF-4α expression plasmid (4) The Asah2 UP construct with the mutated HNF-4α site in enhancer position with and without co-transfection with the HNF-4α expression plasmid. The luciferase and beta-galactosidase activity was measured with Dual-light system (Applied Biosystems) 48 h after transfection. Luciferase activity was normalized to β-galactosidase activity.

For HNF-4α knockdown experiments, HepG2 cells were co-transfected with Asah2 promoter luciferase constructs and HNF-4α shRNA expression constructs (catalog number KH05540P, SureSi-lencing shRNA plasmids;SABiosciences). Four different HNF-4α shRNA expression plasmids targeting different regions of the human HNF-4α mRNA (GenBank entry NM_178849) are provided with the kit together with a control plasmid expressing shRNA with a sequence unrelated to HNF-4α. A mixture with equal amounts (0.1 μg each) of the four HNF-4α shRNA expression plasmids was used in the experiment.

RESULTS

THE DOWNSTREAM ASAH2 PROMOTER IS ACTIVE IN THE SMALL INTESTINAL EPITHELIUM

Two different promoters placed in tandem upstream of the coding part of Asah2 have been described [Okino et al., 2002]. The promoter defined by sequencing of cDNAs derived from liver mRNA is located upstream of a promoter defined by sequencing of cDNAs derived from brain mRNA. In the remaining part of this article we will refer to the two promoters as the UP and DP, respectively. Transcription from the UP begins at exon 1 (Fig. 1 A). Exon 1 ends in an exon/intron splice site which during processing is joined to the next intron/exon junction found in the 5′ end of exon 3. The mature transcript from the UP is described in the Genbank nucleotide sequence entry with the accession number AB037111 (Fig. 1). Transcription from the DP begins with exon 2, which during processing is joined to exon 3. The mature transcript from the DP is described in the Genbank nucleotide sequence entry with the accession number NM_018830. The mature transcripts from both promoters contain identical coding sequences for neutral ceramidase. RNA was extracted from mouse small intestinal mucosa, liver, and brain followed by quantification (qRT-PCR) of the relative abundance of Asah2 transcripts containing exon 1 or spliced exon 2, and 3, respectively (Fig. 1 B). The result shows that the transcripts containing spliced exon 2 and exon 3 (the NM_018830 transcripts) and thus originating from the DP is expressed at approximately three times higher levels than transcripts containing exon 1 in all examined tissues. Moreover, highest abundances of both types of transcripts were found in the brain, whereas the liver contained lowest amounts of the two types of Asah2 transcripts. The DP is the one containing the predicted HNF-4α binding site [Stegmann et al., 2006].

THE PREDICTED HNF-4α BINDING SITE IN THE DOWNSTREAM ASAH2 PROMOTER BINDS HNF-4α PROTEIN

The predicted HNF-4α binding site is found in position −207 to −221 relative to the transcription start site of the Asah2 DP. It overlaps with a previously predicted [Okino et al., 2002] COUP-TF binding site (Fig. 1C). The binding of nuclear proteins to a ^{32}P labeled double stranded oligo nucleotide containing the predicted HNF-4α binding site was analyzed by EMSA. The added nuclear proteins were extracted from nuclei originating from

fractionated mouse ileal epithelium. The experiment shows that the probe is shifted when nuclear extract is added and supershifted when an HNF-4α antibody is included in the binding reaction (Fig. 2, lanes 1 and 4). Excess unlabeled competitor with the same nucleotide sequence as the Asah2 HNF-4α probe efficiently competes with the binding. Contra wise excess unlabeled probe with a mutation in the predicted Asah2 HNF-4α binding site does not compete for binding (Fig. 2, lanes 2 and 3). Moreover, addition of the HNF-4α antibody results in the generation of two more slowly migrating complexes (SS I and SS II). The faster migrating supershifted complex (SS I) migrates just above the shifted probe (complex I) whereas the more slowly migrating complex (SS II) migrates more slowly. Almost no protein/DNA complex remains at the position corresponding to the protein/DNA complex I observed in the absence of HNF-4α antibody. This suggests that most of the probe is occupied by protein complexes containing HNF-4α.

THE HNF-4α BINDING SITE ONLY STIMULATES THE DP ASAH2 PROMOTER

The predicted HNF-4α binding site is placed as a proximal promoter element 207 bp upstream of the DP. Seen from the UP, the predicted HNF-4α binding site is placed as an enhancer element 4,777 bp downstream of exon 1 (Fig. 1). We therefore wanted to investigate whether the predicted HNF-4α element would be able to affect both the UP and the DP.

When a construct where the Asah2 DP drives the firefly luciferase reporter gene is co-transfected into Cos-7 cells with a HNF-4α expression vector, the reporter gene activity is about 2.5 times higher (95% confidence limits: 1.8–3.1) than when cells were co-transfected with the empty expression vector. The activation by HNF-4α co-expression is abolished when a mutation is introduced into the predicted HNF-4α site (Fig. 3 A).

To analyze the HNF-4α site in an enhancer position relative to the UP, the region from position −37 to −254 of the DP was cloned and placed downstream of the luciferase gene driven by the UP. No stimulation (1.1-fold, 95% confidence limits: 1.0–1.1) is observed following co-transfection into Cos-7 cells with an HNF-4α expression vector (Fig. 3 B).

To analyze the importance of endogenous HNF-4α protein for the activity of the Asah2 promoters siRNA knockdown experiments were conducted (Fig. 4). No intestinal cell lines which can be transiently transfected during their differentiated state are available and we therefore choose the human hepatoma cell line HepG2 for this experiment. As a positive control promoter, the intestinal alkaline phosphatase promoter (Alpi) was chosen since it has a well characterized HNF-4α binding site [Olsen et al., 2005]. siRNAs targeting the HNF-4α transcript were generated from expression plasmids encoding short hairpin RNA (shRNA) molecules with sequences derived from the HNF-4α coding sequence. Four different HNF-4α shRNA expression plasmids were obtained from a commercial supplier and used in the experiment. As a negative control, a plasmid encoding a shRNA with a sequence unrelated to HNF-4α was used. When the HNF-4α shRNA expression plasmids are co-transfected with the Alpi luciferase construct, the luciferase activity is strongly reduced (Fig. 4A). Only a minor reduction is observed with a Alpi promoter construct containing a mutated

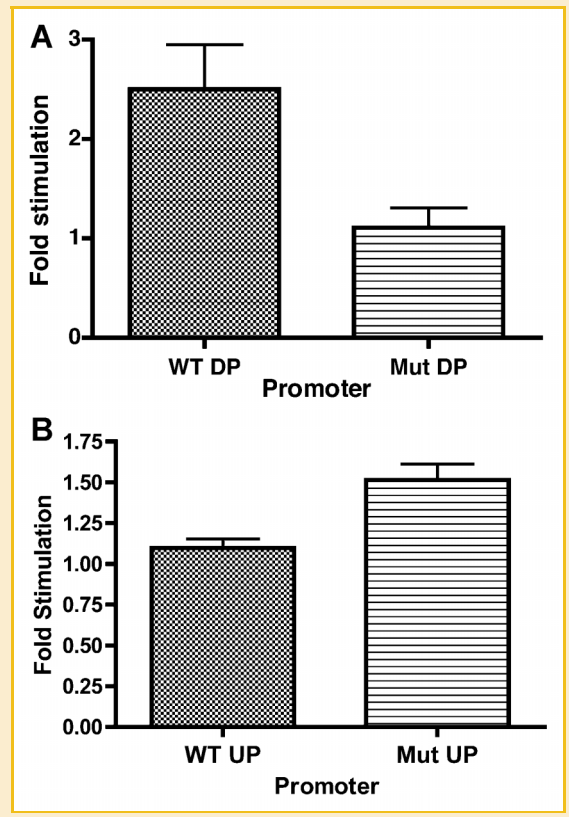

Fig. 3. HNF-4α only stimulates the Asah2 DP. A: The Asah2 DP was cloned in front of the firefly luciferase gene. An Asah2 DP construct with mutation in the HNF-4α binding site was also constructed. Luciferase activity was measured after the cells were co-transfected with a HNF-4α expression vector or an empty expression vector. The luciferase activity following HNF-4α expression is expressed relative to the activity measured following co-transfection with the empty expression vector. Six independent transfections were performed (N = 6). B: The Asah2 UP was cloned in front of the firefly luciferase gene. The region from position −37 to −254 from the Asah2 DP and surrounding the HNF-4α binding site was subsequently placed downstream of the luciferase reporter gene in enhancer position relative to the Asah2 UP. A construct with mutated HNF-4α binding sites was also constructed. Luciferase activity was measured after the cells were co-transfected with a HNF-4α expression vector or an empty expression vector. The luciferase activity following HNF-4α expression is expressed relative to the activity measured following co-transfection with the empty expression vector. Six independent transfections were performed (N = 6).

HNF-4α binding site. None of the two Alpi promoter constructs are affected when the negative control shRNA expression plasmid is used. Thus knock down of HNF-4α in HepG2 cells strongly reduces Alpi promoter activity and the full reduction requires an intact HNF-4α binding site. When the same experiment is conducted with the Asah2 DP luciferase construct (Fig. 4B), similar findings are observed although the reduction in reporter gene activity following HNF-4α knockdown is smaller than the reduction observed for the Alpi promoter. The reduction in the Asah2 DP activity also, however, depends on the presence of an intact HNF-4α binding site. The Asah2 UP with the HNF-4α site at an enhancer position is not, however, affected by the expression of HNF-4α siRNA (Fig. 4C).

DISCUSSION

In the initial mapping of the mouse Asah2 gene, three different Asah2 5′ ends were described [Okino et al., 2002]. The 5′ end of Asah2 mRNA was identical in mouse liver and Swiss 3T3 cells. These transcripts were dubbed "liver type" [Okino et al., 2002] and originated from the promoter which in the present article is termed the UP signifying its upstream position relative to the other DP responsible for the "brain type" of Asah2 transcripts found in the mouse brain. In addition, another "brain type" mRNA originating further downstream was reported but this transcript only constituted 30% of the Asah2 mRNA found in brain [Okino et al., 2002]. In the present work, we have made a quantitative determination of the amounts of the "liver type" and the major "brain type" Asah2 transcripts found in mouse brain, liver, and small intestine. We find that transcripts previously referred to, as "liver type" Asah2 transcripts and originating from the DP are in fact the dominating transcript found in all three tissues. The Asah2 DP has previously been analyzed for the presence of potential transcription factor binding sites [Okino et al., 2002] but the HNF-4α binding sites was not uncovered, presumably because a position weight matrix for HNF-4α was not included in the bioinformatic analysis. A potential binding site for the COUP-TF transcription factor was however pointed out previously in a region overlapping the HNF-4α binding site. This is in accordance with the fact that the COUP-TF and HNF-4α binding sites have almost identical consensus binding sequences [Kimura et al., 1993]. In the present work, we have focused on the HNF-4α binding site in the Asah2 DP and demonstrated the binding of HNF-4α. A functional role of HNF-4α binding to this site was also demonstrated by HNF-4α over-expression and knockdown experiments. We therefore conclude that substantial evidence supports the presence of a functionally important HNF-4α binding site in the Asah2 DP. The HNF-4α knockdown experiment only resulted in approximately 30% decrease in Asah2 DP activity, which indicates the presence of other functionally important binding sites in the promoter. Transcription factors binding to these yet uncovered *cis*-elements might be responsible for the transcription from the Asah2 DP in the brain where HNF-4α is not expressed.

The importance of the HNF-4α binding site for the Asah2 UP was also analyzed. It was found that the HNF-4α binding site did not stimulate the Asah2 UP when placed at an enhancer position

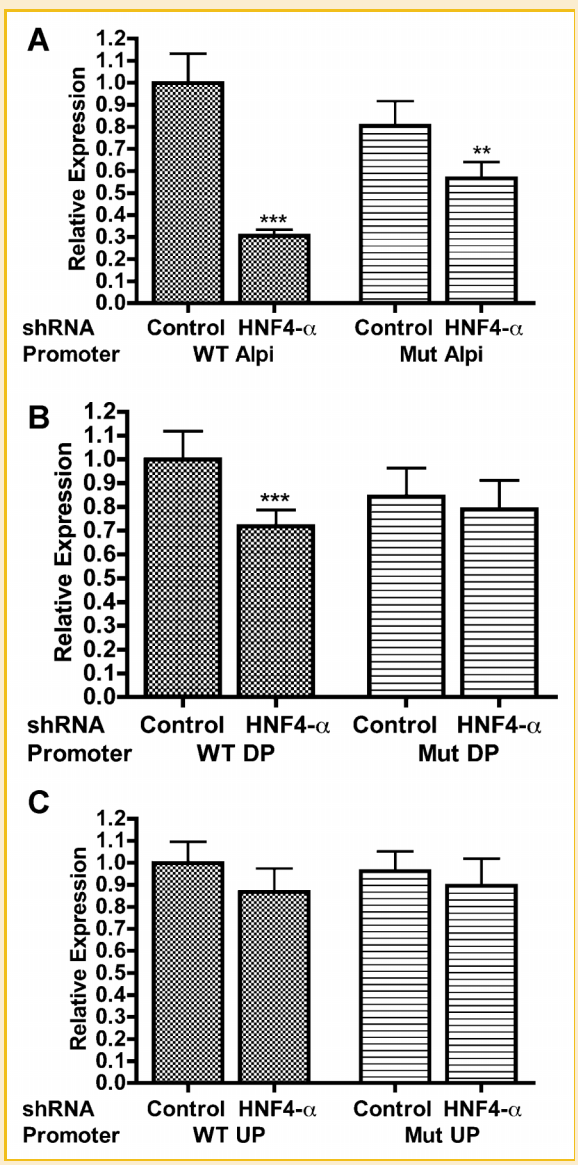

Fig. 4. Effect of knockdown of HNF-4α in HepG2 cells on the two Asah2 promoters. A: As a positive control promoter for the effect of HNF-4α knockdown, the Alpi promoter, which contains a well characterized HNF-4α binding site [Olsen et al., 2005] was placed in front of the luciferase reporter gene and transfected into HepG2 cells. A mixture of four plasmids expressing shRNA with sequences derived from the HNF-4α coding sequence was co-transfected with the Alpi promoter construct. As a control, a plasmid expressing a shRNA with a sequence unrelated to HNF-4α was used. Co-transfection of a HNF-4α shRNA expression constructs results in reduced reporter gene activity with the wild type Alpi promoter whereas only a minor decrease is seen if the Alpi HNF-4α binding site is mutated. The luciferase activity is expressed relative to the activity observed for the WT Alpi promoter with control shRNA co-expression. Six independent transfections were performed ($N = 6$), $^{***}P < 10^{-4}$, $^{**}P < 10^{-3}$. B: The Asah2 DP was placed in front of the luciferase reporter gene and transfected into HepG2 cells. A mixture of four plasmids expressing shRNA with sequences derived from the HNF-4α coding sequence was co-transfected with the Asah2 promoter construct. As a control a plasmid expressing a shRNA with a sequence unrelated to HNF-4α was used. Co-transfection of HNF-4α shRNA expression constructs results in reduced reporter gene activity with the wild type Asah2 DP whereas no significant decrease is seen if the Asah2 HNF-4 binding site is mutated. The luciferase activity is expressed relative to the activity observed for the WT Asah2 DP with control shRNA co-expression. Six independent transfections were performed ($N = 6$), $^{***}P < 10^{-3}$. C: The Asah2 UP was cloned in front of the firefly luciferase gene. The region from position −37 to −254 from the DP and surrounding the HNF-4α binding site was subsequently placed downstream of the luciferase reporter gene in enhancer position relative to the UP. A mixture of four plasmids expressing shRNA with sequences derived from the HNF-4α coding sequence was co-transfected with the Asah2 UP construct. As a control a plasmid expressing a shRNA with a sequence unrelated to HNF-4α was used. Co-transfection of HNF-4α shRNA expression constructs did not result in altered reporter gene activity from the UP Asah2 promoter construct. The luciferase activity is expressed relative to the activity observed for the WT Asah2 UP/HNF-4 enhancer construct with control shRNA co-expression. Six independent transfections were performed ($N = 6$).

downstream of the Asah2 UP. Thus the experiments indicate that the HNF-4α binding site is only important for the Asah2 DP.

The neutral ceramidase encoded by Asah2 is a typical brush border hydrolase [Norén et al., 1986] having its active site at exterior of the enterocyte and cleaving ceramide at the plasma membrane [Tani et al., 2007]. An Asah2 knockout mouse has been made, and when these mice were fed sphingomyelin, their feces had a significantly higher ceramide content compared to the WT mice [Kono et al., 2006], demonstrating that the neutral ceramidase is necessary for the digestion of ceramides in the small intestine. No change was found in intestinal ceramide content in the Asah2 knock out mice [Kono et al., 2006], but a decreased level of sphingosine was found in the jejunum and ileum although the difference was not significant for the latter. It has been previously shown that absorbed sphingosine can be rebuilt into the membrane as ceramide [Nilsson, 1968]. Kono et al. [2006] presented a model where sphingosine was either degraded into palmitic acid and then built into triglyceride, or conserved and built into sphingolipids.

We and others have previously shown that there is a significant difference in the lipid content between the immature small intestinal epithelial crypt cells and the mature differentiated villus epithelial cells [Bouhours and Glickman, 1976; Stegmann et al., 2006]. Moreover, this difference has been shown to be due to the presence of higher amounts of glucosylceramide in villus epithelial cells [Bouhours and Glickman, 1976]. Given the well established role described above of neutral ceramidase in ceramide metabolism the present work therefore points to a hypothesis linking HNF-4α expression with the high glycolipid content of the differentiated enterocyte. This hypothesis, though, still has to be formally proven in future experiments.

ACKNOWLEDGMENTS

We thank Liselotte Laustsen for excellent technical assistance. This work was supported by grants from The Alfred Nielsen and Wife's Foundation, The Danish Medical Research Council, The Novo Nordic Foundation and the Lundbeck Foundation.

REFERENCES

Bouhours JF, Glickman RM. 1976. Rat intestinal glycolipids. II. Distribution and biosynthesis of glycolipids and ceramide in villus and crypt cells. Biochim Biophys Acta 441:123–133.

Boyd M, Bressendorff S, Moller J, Olsen J, Troelsen JT. 2009. Mapping of HNF4alpha target genes in intestinal epithelial cells. BMC Gastroenterol 9:68.

Chen WS, Manova K, Weinstein DC, Duncan SA, Plump AS, Prezioso VR, Bachvarova RF, Darnell JE, Jr. 1994. Disruption of the HNF-4 gene, expressed in visceral endoderm, leads to cell death in embryonic ectoderm and impaired gastrulation of mouse embryos. Genes Dev 8:2466–2477.

Duan RD, Nilsson A. 2009. Metabolism of sphingolipids in the gut and its relation to inflammation and cancer development. Prog Lipid Res 48:62–72.

Garrison WD, Battle MA, Yang C, Kaestner KH, Sladek FM, Duncan SA. 2006. Hepatocyte nuclear factor 4alpha is essential for embryonic development of the mouse colon. Gastroenterology 130:1207–1220.

Hayhurst GP, Lee YH, Lambert G, Ward JM, Gonzalez FJ. 2001. Hepatocyte nuclear factor 4alpha (nuclear receptor 2A1) is essential for maintenance of hepatic gene expression and lipid homeostasis. Mol Cell Biol 21:1393–1403.

Higuchi R, Krummel B, Saiki RK. 1988. A general method of in vitro preparation and specific mutagenesis of DNA fragments: Study of protein and DNA interactions. Nucleic Acids Res 16:7351–7367.

Jiang G, Nepomuceno L, Hopkins K, Sladek FM. 1995. Exclusive homodimerization of the orphan receptor hepatocyte nuclear factor 4 defines a new subclass of nuclear receptors. Mol Cell Biol 15:5131–5143.

Kimura A, Nishiyori A, Murakami T, Tsukamoto T, Hata S, Osumi T, Okamura R, Mori M, Takiguchi M. 1993. Chicken ovalbumin upstream promoter-transcription factor (COUP-TF) represses transcription from the promoter of the gene for ornithine transcarbamylase in a manner antagonistic to hepatocyte nuclear factor-4 (HNF-4). J Biol Chem 268:11125–11133.

Kono M, Dreier JL, Ellis JM, Allende ML, Kalkofen DN, Sanders KM, Bielawski J, Bielawska A, Hannun YA, Proia RL. 2006. Neutral ceramidase encoded by the Asah2 gene is essential for the intestinal degradation of sphingolipids. J Biol Chem 281:7324–7331.

Miquerol L, Lopez S, Cartier N, Tulliez M, Raymondjean M, Kahn A. 1994. Expression of the L-type pyruvate kinase gene and the hepatocyte nuclear factor 4 transcription factor in exocrine and endocrine pancreas. J Biol Chem 269:8944–8951.

Nilsson A. 1968. Metabolism of sphingomyelin in the intestinal tract of the rat. Biochim Biophys Acta 164:575–584.

Norén O, Sjöström H, Danielsen EM, Cowell GM, Skovbjerg H. 1986. The enzymes of the enterocyte plasma membrane. In: Desnuelle P, Sjöström H, Norén O, editors. *Molecular and cellular basis of digestion*. Amsterdam: Elsevier Science Publishers B.V. pp 335–365.

Okino N, Mori K, Ito M. 2002. Genomic structure and promoter analysis of the mouse neutral ceramidase gene. Biochem Biophys Res Commun 299:160–166.

Olsen J, Laustsen L, Karnstrom U, Sjostrom H, Noren O. 1991. Tissue-specific interactions between nuclear proteins and the aminopeptidase N promoter. J Biol Chem 266:18089–18096.

Olsen L, Bressendorff S, Troelsen JT, Olsen J, 2005. Differentiation-dependent activation of the human intestinal alkaline phosphatase promoter by HNF-4 in intestinal cells. Am J Physiol Gastrointest Liver Physiol 289:G220–G226.

Parviz F, Matullo C, Garrison WD, Savatski L, Adamson JW, Ning G, Kaestner KH, Rossi JM, Zaret KS, Duncan SA. 2003. Hepatocyte nuclear factor 4alpha controls the development of a hepatic epithelium and liver morphogenesis. Nat Genet 34:292–296.

Sjostrom H, Noren O, Olsen J. 2000. Structure and function of aminopeptidase N. Adv Exp Med Biol 477:25–34.

Sladek FM, Zhong WM, Lai E, Darnell JE, Jr. 1990. Liver-enriched transcription factor HNF-4 is a novel member of the steroid hormone receptor superfamily. Genes Dev 4:2353–2365.

Stegmann A, Hansen M, Wang Y, Larsen JB, Lund LR, Ritie L, Nicholson JK, Quistorff B, Simon-Assmann P, Troelsen JT, Olsen J, 2006. Metabolome, transcriptome, and bioinformatic cis-element analyses point to HNF-4 as a central regulator of gene expression during enterocyte differentiation. Physiol Genomics 27:141–155.

Tani M, Ito M, Igarashi Y. 2007. Ceramide/sphingosine/sphingosine 1-phosphate metabolism on the cell surface and in the extracellular space. Cell Signal 19:229–237.