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**Regulation of the human mucin MUC4 by taurodeoxycholic and taurochenodeoxycholic
bile acids in oesophageal cancer cells is mediated by hepatocyte nuclear factor 1 α**

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Short title: HNF1 α mediates the regulation of human mucin MUC4 by bile acids

Keywords: MUC4, bile acids, oesophagus, Barrett, adenocarcinoma, HNF1 α , HNF4 α

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Abbreviations

ChIP chromatin immunoprecipitation

EMSA electrophoretic mobility shift assay

GC glycocholic acid

GNa sodium glycocholate

HNF hepatocyte nuclear factor

OA oesophageal adenocarcinoma

S.D. Standard deviation

siRNA small interfering RNA

TC taurocholic acid

TCDC taurochenodeoxycholic acid

TDC taurodeoxycholic acid

TF transcription factor

Synopsis

MUC4 is a membrane-bound mucin overexpressed in the early steps of oesophageal carcinogenesis and implicated in tumour progression. We previously showed that bile acids, main components of gastro-oesophageal reflux and tumour promoters, upregulate MUC4 expression. HNF1 α and HNF4 α transcription factors are known to mediate bile acids effects, and we previously identified *cis*-elements for these factors in *MUC4* distal promoter. Our aim was to demonstrate that these two transcription factors were directly involved in MUC4 activation by bile acids. MUC4, HNF1 α and HNF4 α expression was evaluated by immunohistochemistry in human oesophageal tissues. Our results indicate that MUC4, HNF1 α and HNF4 α were co-expressed in oesophageal metaplastic and adenocarcinomatous tissues. Studies at the mRNA, promoter and protein levels indicated that HNF1 α regulates endogenous *MUC4* expression by binding to two cognate *cis*-elements respectively located at -3332/-3327 and -3040/-3028 in the distal promoter. We also showed by siRNA approach, co-transfection and site-directed mutagenesis that HNF1 α mediates taurodeoxycholic and taurochenodeoxycholic bile acids activation of endogenous *MUC4* expression and transcription in a dose-dependent manner. In conclusion, these results describe a new mechanism of regulation of MUC4 expression by bile acids, in which HNF1 α is a key mediator. These results bring new insights in MUC4 up-regulation in oesophageal carcinoma associated with bile reflux.

Introduction

The incidence of oesophageal adenocarcinoma (OA) is increasing rapidly in Western populations [1]. Despite recent advances in multimodal therapy, the prognosis for invasive OA remains poor reflecting the natural history of this disease to disseminate early [2]. Most if not all of OA develop from Barrett's metaplasia, defined as the replacement of any portion of the normal squamous lining by a metaplastic columnar epithelium which is visible macroscopically [3]. Chronic duodeno-gastro-oesophageal reflux of acid and bile plays an important role in initiation and evolution of Barrett's metaplasia along the metaplasia-dysplasia-carcinoma sequence [4]. Although importance of bile acids in the development of adenocarcinoma in Barrett's metaplasia is still being discussed among authors, many studies suggest they are a main factor in initiating the process [1,5]. A better understanding of the molecular mechanisms triggered by bile acids that may contribute to development and evolution of Barrett's metaplasia into OA is thus necessary and will help identify them as key factors in oesophageal cancer pathogenesis associated with reflux.

In normal oesophagus, mucins protect the underlying mucosa against potential injuries such as reflux of gastroduodenal contents including bile acids [6]. Among the members that compose the family of mucins, membrane mucins are thought to play important roles in tumour cell biology, cell proliferation, tumour progression and metastasis [7]. MUC1 and MUC4 are the best characterized membrane mucins [8,9]. They are large O-glycoproteins with an extended heavily glycosylated extracellular domain that protrudes far away from cellular membrane and is involved in cell recognition processes. Consequently, overexpression of membrane mucins at the cancer cell surface will provide the cancer cell new biological properties by altering their interactions with immune cells, epithelial or endothelial cells or the extracellular matrix [7,9]. In the light of identifying new markers and

therapeutic targets in OA, MUC4, could be of particular interest because it is known to be a mediator of tumour growth and metastasis by acting as a ligand for the receptor tyrosine kinase ErbB2 [8,10,11]. MUC4 is expressed in the oesophageal stratified squamous epithelium and during the whole OA carcinogenetic sequence [12-14]. A recent study identified *MUC4* as a promising early diagnostic tumoural marker since it was overexpressed from the high grade dysplasia state and in adenocarcinomatous tissues [14]

In relation with (i) *MUC4* overexpression in oesophageal adenocarcinoma and (ii) the role of bile acids in induction and degeneration of Barrett's metaplasia, we recently showed that taurocholic (TC), taurodeoxycholic (TDC), taurochenodeoxycholic (TCDC) and glycocholic (GC) acids and sodium glycocholate (GNa) were strong activators of MUC4 expression [15]. We showed that this regulation occurred at the transcriptional level, and involved activation of phosphatidylinositol 3-kinase pathway. Bile acids may however mediate their effects using other pathways. Activation of hepatocyte nuclear factors (HNF) 1 α and HNF4 α was recently identified as a mechanism used by bile acids to activate transcription of their target genes [16,17]. The recent identification of HNF1 α *cis*-elements in the distal promoter of *MUC4* [18] was a positive argument in favour of a possible implication of that transcription factor (TF) in mediating *MUC4* regulation by bile acids. In this paper, we show for the first time that HNF1 α is a key mediator of MUC4 activation by TDC and TCDC bile acids.

Materials and methods

Tissue sample collection and immunochemistry

Surgical specimens were collected from 10 patients who underwent curative oesophagectomy for adenocarcinoma developed on Barrett's metaplasia without neoadjuvant treatment. Consent form was obtained from each patient. The surgical specimen was quickly immersed in 10% (v/v) neutral formaldehyde solution (pH 7.4) in phosphate buffer. Samples from normal mucosa, Barrett's metaplasia and adenocarcinoma were processed for paraffin embedding. The diagnosis was assessed by two pathologists after staining the section (4 μ m) with haematoxylin-eosin-saffron. Immunohistochemical studies for MUC4 expression were performed as described in [15]. Monoclonal anti-MUC4 antibody [19] was used at 1:20000 dilution. A positive control for MUC4 immunostaining was included in each set of experiments on human lung sections. Immunohistochemical studies for HNF1 α and HNF4 α expression were performed as described in [20]. The primary antibodies directed against human HNF1 α and human HNF4 α (Santa Cruz Biotechnology) were used at a 1:2000 dilution. A positive control for HNF1 α and HNF4 α immunostaining was included in each set of experiments on human small intestine sections.

Cell culture

The oesophageal adenocarcinoma cell line OE33 was purchased from ECACC (European Collection of Cell Cultures) and was cultured as previously described [15]. HNF1 α expressing KATO-III cells were cultured as previously described [21]. Cells were treated with bile acids and their conjugates for 24h as described before [15]. Optimal concentrations for bile acids were: taurocholic acid (TC): 0.5 mM, taurodeoxycholic acid (TDC): 1 mM, taurochenodeoxycholic acid (TCDC): 0.5 mM, glycocholic acid (GC): 0.5 mM, sodium glycocholate (GNa): 0.5 mM. For dose effect experiments, a third, a half and the full

concentration for each bile acid were used. To inhibit protein synthesis or transcription, cells were treated for 30 min with cycloheximide (20 $\mu\text{g/ml}$) or actinomycinD (ActD) (1 $\mu\text{g/ml}$), respectively. All reagents were from Sigma unless otherwise indicated.

Small interfering RNA (siRNA) assays

KATO-III cells were seeded the day before transfection at a density of 20×10^3 cells/well in antibiotic free medium. Cells were transfected with 100nM of either TCF1 (HNF1 α) SMARTpool[®] or HNF4 α SMARTpool[®] siRNA, using 1 μl of DharmaFECT[™]4 transfection reagent according to the manufacturer's instructions (Dharmacon). Controls included mock transfected cells, cells transfected with siCONTROL[™] Non Targeting siRNA or siCONTROL[™] GAPD siRNA. Bile acid treatment was performed 24h after transfection and lasted 24h as described above. Total RNA was isolated 48h after transfection as described thereafter. Each siRNA was assayed in triplicate in at least three separate experiments. PCR was performed on 5 μl of cDNA as previously described. Densitometric analysis of DNA bands was carried out using the GelAnalyst-GelSmart software (Clara Vision). Results were expressed as MUC4/GAPDH ratio.

RT-PCR

Total RNAs from cultured cells were prepared using the RNeasy mini-kit from Qiagen. One μg of total RNA was used to prepare cDNA (Advantage[™] RT-for-PCR kit, Clontech) as described before [22]. PCR was performed on 5 μl of cDNA using specific pairs of primers for *MUC4*, *HNF1 α* , *HNF4 α* , and *18S* or *β -actin* as internal controls (MWG-Biotech). Primer information is given in table I. PCR reactions were carried out in 50 μl final solutions as described in [23]. PCR products were analysed on 1.5% agarose gels containing ethidium bromide run in 1X Tris-borate-EDTA buffer. 100 basepair (bp) DNA ladder was purchased from Amersham Biosciences. RT-PCR were carried out on cDNAs from four different sets of

experiments. Densitometric analysis of DNA bands was carried out as above. Results were expressed as ratio of MUC4/18S or MUC4/ β -actin.

Plasmids and expression vectors

The two pGL3-*MUC4* deletion mutants used in this study were previously described [24]. They cover the -3713/-3059 and the -3135/-2837 regions of the distal promoter. Two mutated versions of these constructs were made using the QuickChange Site-Directed Mutagenesis kit (Stratagene). The two HNF binding sites identified at -3332/-3327 and -3040/-3028 were mutated in the -3713/-3059 and -3135/-2837 deletion mutants, respectively. The oligonucleotides containing the mutation were designed according to the manufacturer's instructions and are shown in table I. pSG5-HNF1 α and pSG5-HNF4 α expression vectors were a kind gift of Dr J. K. Divine (Washington University, St Louis, MO). Plasmids used for transfection studies were prepared using the Endofree plasmid Maxi kit (Qiagen).

Transient transfections

Transient transfection experiments were performed using Effectene[®] reagent (Qiagen) as previously described [21]. Luciferase activity was corrected for transfection efficiency by co-transfecting cells with pRL-TK vector (Promega, Charbonnières, France). Total cell extracts were prepared after a 48h incubation time at 37°C using 1X Passive Lysis Buffer (Promega) as described in the manufacturer's instruction manual. Luciferase activities were measured on a TD 20/20 luminometer (Turner Design). Each plasmid was assayed in triplicate in at least three separate experiments. In bile acids experiments, relative luciferase activity was expressed as fold activation of luciferase activity in bile acid treated cells compared with untreated cells. In co-transfection studies, 1 μ g of the pGL3-*MUC4* deletion mutant was transfected with 0.25 μ g of the expression vectors pSG5-HNF1 α , pSG5-HNF4 α or the corresponding empty vector (pSG5) as the reference. Results were expressed as fold induction of luciferase activity in cells transfected with expression vectors compared with that obtained

with the empty vector. To study the effect of HNF1 α and HNF4 α overexpression on endogenous *MUC4* mRNA level, cells (0.5×10^6) were transfected with 4 μ g of the expression vector of interest and cultured for 48 h before being lysed and processed for total RNA extraction as previously described [20].

Western blotting

Total cellular extracts were prepared using standard procedures and kept at -80 °C before use. Briefly, cells were scraped in the medium and centrifuged for 5 min at 1600 x g. The pellet was washed twice with 1X PBS, centrifuged 1 min at 9000 x g and then resuspended in lysis buffer [50 mM Tris-HCl (pH 7.4), 1mM EDTA, 0.25% sodium deoxycholate, 150 mM NaCl, 1% NP-40] supplemented with protease inhibitors [aprotinin (1 μ g/ml), leupeptin (1 μ g/ml), 1 mM phenylmethanesulfonyl fluoride] and phosphatase inhibitors [5 mM sodium fluoride, 5 mM sodium orthovanadate]. The lysate was incubated for 30 min on ice, before being centrifuged for 10 min at 16000 x g at 4°C. The supernatant was collected for western blotting analysis. Protein content (2 μ l of total extracts) was measured in 96-well plates using the bicinchoninic acid method as described in the manufacturer's instruction manual (Pierce). For *MUC4* expression analysis, proteins (20 μ g) were separated on a 2 % SDS-agarose gel. For β -actin expression analysis, proteins were separated using a 10% SDS-polyacrylamide gel electrophoresis. Resolved proteins were transferred onto a 0.2 μ m nitrocellulose membrane (Schleicher and Schuell) and subjected to the standard immunodetection procedure using specific mouse monoclonal antibodies against β -actin (Sigma A5441) and *MUC4* [19]. Secondary antibodies consisted of alkaline phosphatase-conjugated IgG (Promega) (for β -actin) or horseradish peroxidase-conjugated anti-mouse (Pierce) (for *MUC4*). For *MUC4* detection, blots were processed with West[®] Pico chemiluminescent substrate (Pierce) and the signal was detected by exposing the processed blots to Hyperfilm[™] ECL (Amersham

Biosciences). For β -actin detection, the blots were processed with Nitro Blue Tetrazolium Chloride and 5-bromo-4-chloro-3-indolyl phosphate substrate (Life Technologies).

Nuclear extract preparation and electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared from KATO-III cells as previously described [25] and kept at -80°C until use. Protein content was measured in 96-well plates using bicinchoninic acid protein assay (Pierce). Identification of putative HNF binding sites was carried out by analysing the sequence of *MUC4* promoter with MatInspector V2.2 software (www.genomatix.de) [26]. The oligonucleotides (MWG-Biotech) were annealed before being phosphorylated at the 5'-end with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (Amersham Biosciences) and T4 polynucleotide kinase (Roche). Wild-type and mutated oligonucleotides used as probes and competitors in EMSAs are shown in table I. Nuclear protein incubation with radiolabelled probes and competitions with unlabelled probes were as described in [23]. For supershift analyses, 2 μl of anti-HNF1 α (sc-6547x) or anti-HNF4 α (sc-6556x) (Santa Cruz Biotechnology) antibodies were added to the proteins and left for 2 h at room temperature before adding the radiolabelled probe. Electrophoresis conditions and gel processing were as described before [27].

Chromatin Immunoprecipitation (ChIP)

KATO-III cells (15×10^6 cells) were treated with 1% (v/v) formaldehyde for 10 min at room temperature and crosslinks were quenched with glycine at a final concentration of 0.125 M for 5 min. Cells were rapidly rinsed with ice-cold 1X Dubelcco-Phosphate-Buffered saline (D-PBS), with D-PBS containing protease inhibitors (10 $\mu\text{g}/\text{ml}$ leupeptin, 10 $\mu\text{g}/\text{ml}$ aprotinin, 0.2 mM EDTA and 0.5 mM PMSF) and scraped and collected by centrifugation at 700 x g for 5 min at 4°C , before being resuspended in lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl_2 , 0.1% NP-40) plus protease inhibitors and incubated for 10 min on ice. Chromatin was sheared with the Bioruptor system (Diagenode). The extracts were sonicated

twice for 5 pulses of 30-s each with a 30-s rest between each pulse at 200W. After clearing by centrifugation at 10 000 x g for 10 min at 4°C, the supernatant was diluted in dilution buffer (16.7 mM Tris-HCl, pH 8.0, 167 mM NaCl, 0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA). The chromatin solution was precleared by incubation with sonicated salmon sperm DNA/bovine serum albumin/Protein-G-agarose gel slurry (v/v) (Upstate) for 1 h at 4°C with rotation and centrifugation at 1500 x g for 1 min at 4°C. An aliquot of the total supernatant was removed as input control and the rest was fractionated and precipitated with either 4 µg of the specific antibody or normal rabbit IgGs (Upstate). Immunoprecipitation was performed overnight on a rotating platform at 4°C, protein G-agarose gel slurry was then added (v/v) and left for another 2h. Agarose beads were collected and washed sequentially for 4 min in Low Salt Immune Complex Wash Buffer, High Salt buffer, LiCl buffer (Upstate) and twice with TE buffer (pH 8.0). The complexes were eluted with 2 x 0.25 ml of 1% SDS-0.1 M NaHCO₃ after a 15 min incubation at room temperature. Formaldehyde crosslinks were reversed with NaCl at 65°C overnight. Chromatin associated proteins were digested with Qiagen Protease at 50°C for 1 h and the DNA was purified with the Wizard[®] DNA Clean-Up System (Promega). Samples (50 ng) were then subjected to PCR analysis. Primers are indicated in table I. PCR was performed in a 30 µl final volume consisting of 1x PCR buffer II, 2.5 mM MgCl₂, 1.5U AmpliTaq Gold polymerase (Applied Biosystems) and 5 pmoles of each primer. The temperature-cycling protocol consisted of 12 min of preheating at 94°C followed by 35 cycles of 45 sec of denaturation at 94°C, 1 min of primer annealing at 50°C, and 1 min of primer extension at 72°C followed by a 10 min final extension at 72°C. PCR products (15 µl) were analysed on a 2% agarose gel.

Statistics

All values in this article are means values ± standard deviation (S.D.). When indicated, data were analysed by Mann-Whitney *U* test with differences $P \leq 0.05$ considered significant.

Results

Expression of MUC4, HNF1 α and HNF4 α in human oesophageal tissues

Study of MUC4 expression in human normal, metaplastic and adenocarcinomatous oesophageal tissues indicated that MUC4 was expressed in the cytoplasm of superficial epithelial cells of the normal mucosa (Figure 1). MUC4 cytoplasmic expression was found in Barrett's metaplastic tissue both in non-goblet columnar cells and goblet cells. In adenocarcinoma, MUC4 expression was diffuse and heterogeneous, independently of tumour differentiation. HNF1 α and HNF4 α were not expressed in normal oesophageal mucosa. They were both expressed in Barrett's metaplasia with strong nuclear staining in all cell types on the surface and glands (including goblet cells). Expression of both factors was visualized independently of tumour differentiation in well-differentiated (inset) and undifferentiated adenocarcinomatous tissues.

Regulation of MUC4 expression by HNF1 α and HNF4 α

To investigate whether HNF1 α and HNF4 α TF participate in *MUC4* mRNA regulation, we combined knockdown assays with specific siRNA (Figure 2A) and overexpression experiments (Figure 2B and 2C). HNF1 α and HNF4 α knockdown in gastric KATO-III cells, which express these two TF, led to a substantial decrease of *MUC4* mRNA ($65\pm 8\%$ and $70\pm 6\%$, respectively), indicating that both TF regulate endogenous *MUC4* expression (Figure 2A). Results from four experiments in oesophageal OE33 cells in which HNF1 α and HNF4 α were overexpressed showed that overexpression of HNF1 α led to a 3.2-fold increase (3.2 ± 0.4) of *MUC4* mRNA (Figure 2B, lane 2) whereas overexpression of HNF4 α had a significant weaker effect (1.2 ± 0.1 -fold increase, $P = 0.02$) (lane 3). At the protein level, we also observed an increase of MUC4 apomucin expression (x 2.1) when HNF1 α was

overexpressed (Figure 2C, lane 2) whereas no variation was seen in HNF4 α transfected cells (lane 3).

Activation of *MUC4* transcription at the promoter level by HNF1 α was confirmed by performing co-transfection studies in OE33 cells with the -3135/-2837 and the -3713/-3059 constructs that cover the *MUC4* distal promoter. The results shown in figure 2D indicate that HNF1 α strongly transactivated (21.2 ± 3.4 -fold activation) the -3135/-2837 region and to a lower extent the -3713/-3059 region (7.9 ± 1.6 -fold activation). The effect of HNF4 α on both constructs was significantly weaker (3.0 ± 0.6 - and 4.7 ± 0.4 -fold activation, $P = 0.001$ and $P = 0.03$, respectively).

Identification of two HNF1 α cis-elements in MUC4 distal promoter

Having shown that HNF1 α and HNF4 α were transactivating the *MUC4* distal promoter, we undertook to identify their binding sites using EMSA (Figure 3A). Three putative HNF binding sites are present in the *MUC4* distal promoter (Figure 2C). When radiolabelled probes T144 (-3040/-3028) and T106 (-3332/-3327) were incubated with nuclear extracts, two shifted bands were visualized with T144 probe (lane 2) whereas only one major retarded band was seen with T106 probe (lane 11). Specificity of the protein-DNA complexes was confirmed by the loss of the shifted bands when cold competition was performed with a 50x excess of either unlabelled T144 (lane 3) or T106 (lane 12) DNA probes. As expected, competition with a 50x excess of unlabelled mutated T144 (lane 4) or T106 (lanes 13) probes did not modify the pattern of the shift. We further confirmed the direct involvement of HNF DNA motifs in the complex formation by using radiolabelled mutated T144 and T106 probes in which the HNF binding sequence was mutated (see table I). In that case, no binding with nuclear proteins was observed indicating that these nucleotides are directly mediating HNF binding to the *MUC4* promoter (lanes 8 and 17, respectively). Finally, direct implication of HNF1 α in the binding

to the T144 and T106 probes was proven by the supershift obtained when a specific anti-HNF1 α antibody was added to the reaction mixture (lane 5 and 14). No supershift was observed, when anti-HNF4 α antibody was added (lanes 6 and 15). The third putative HNF binding site T91 (-3461/-3457) did not bind HNF1 α or HNF4 α (not shown). *In vivo* binding of HNF1 α to the chromatin region covering the HNF1 α *cis*-elements identified at -3040/-3028 (T144) and -3332/-3327 (T106) was confirmed by ChIP analysis (Figure 3B, lanes 2 and 5). The direct implication of these two HNF *cis*-elements in mediating HNF1 α and HNF4 α activation of the *MUC4* promoter was then assessed by carrying out transfections in OE33 cells in the presence of mutated forms of the -3135/-2837 and -3713/-3059 pGL3-*MUC4* promoter constructs (Figure 3C). Mutation of the T144 HNF *cis*-element in the -3135/-2837 construct abolished transactivating effects of HNF1 α and HNF4 α . Mutation of the T106 site in the -3713/-3059 region led to a 75 \pm 4% (HNF1 α) and 50 \pm 5% (HNF4 α) decrease of luciferase activity.

MUC4 regulation by bile acids occurs at the transcriptional level

Before studying the role of HNF1 α in mediating *MUC4* activation by TC, TDC, TCDC, GC and GNa bile acids, previously identified as activators of *MUC4* expression [15], we checked whether that process occurred at the transcriptional level. To this aim, oesophageal cancer cells were pretreated with actinomycinD or cycloheximide before bile acid exposure. *MUC4* mRNA expression was then assessed by RT-PCR. The results indicate that activation of *MUC4* expression by bile acids occurred at the transcriptional level since *MUC4* mRNA level returned to basal level when cells were pre-treated with actinomycinD (Figure 4). This process did not require *de novo* protein synthesis since pre-treatment of OE33 cells with cycloheximide did not modify the level of *MUC4* mRNA (not shown).

HNF1 α mediates MUC4 up-regulation by TDC and TCDC bile acids

To demonstrate that HNF1 α was involved in bile acid regulation of *MUC4* endogenous expression and promoter activity we combined knockdown assays (Figure 5A) and co-transfection experiments (Figure 5B). HNF1 α knockdown led to a substantial decrease of *MUC4* mRNA ($66\pm 5\%$ decrease, Figure 5A). We then focused our studies on TDC and TCDC since overexpression of HNF1 α did not influence *MUC4* activation by TC, GC or GNa (not shown). Treatment of cells with TDC and TCDC enhanced the basal expression level of *MUC4* mRNA ($\times 1.8\pm 0.2$ and 2.4 ± 0.6 , respectively) (Figure 5A). Knockdown of HNF1 α strongly inhibited TDC- and TCDC-mediated increase of *MUC4* mRNA level ($91\pm 2\%$ and $94\pm 3\%$, respectively). This indicates that HNF1 α mediates endogenous *MUC4* expression in response to these bile acids. At the promoter level, we found that overexpression of HNF1 α followed by cell treatment with full dose of TDC and TCDC resulted in a 3.8 ± 0.8 -fold and a 6.1 ± 0.3 -fold activation of the -3135/-2837 deletion fragment (Figure 5B, left panel). Studies on the -3713/-3059 region of the *MUC4* distal promoter (Figure 5B, right panel) again indicated that overexpression of HNF1 α followed by full dose of TDC or TCDC induced a strong activation of *MUC4* promoter (9.0 ± 0.6 -fold and 4.7 ± 0.5 -fold, respectively). Moreover, studies performed with different doses of bile acids indicated that HNF1 α mediation of TDC and TCDC effects on the two *MUC4* promoter fragments were dose-dependent (Figure 5B).

Finally, we demonstrated that the two HNF *cis*-elements identified in Figure 3 were necessary for HNF1 α -mediated up-regulation of *MUC4* promoter by TDC and TCDC. To this end, we compared *MUC4* promoter activity in cells co-transfected with HNF1 α expression vector and wild-type pGL3-*MUC4* constructs followed by TDC or TCDC treatment to cells transfected under the same conditions with the mutated forms of *MUC4* promoter (Figure 6). The mediation by HNF1 α was completely abolished when co-transfecting the mutated form

of the -3135/-2837 construct (grey bars). When using the -3713/-3059 mutated construct, promoter transactivation mediated by HNF1 α decreased for more than a half (70 \pm 6% for TDC and 60 \pm 9% for TCDC).

Discussion

In this paper we demonstrate that activation of MUC4 expression by TDC and TCDC bile acids is dose-dependent and is mediated *via* binding of HNF1 α to two cognate *cis*-elements in the *MUC4* distal promoter. Bile acids are a component of duodeno-gastro-oesophageal reflux often associated with development and/or promotion of OA on Barrett's metaplasia [4]. Thus, our data identify these two bile acids as potent inducers of MUC4 expression during oesophageal carcinogenesis associated with bile reflux. Since these two bile acids were previously shown to be especially toxic in *in vivo* conditions [28] and that TDC is a secondary bile acid found in higher concentration in patients with Barrett's oesophagus compared to asymptomatic patients [28], they may be considered as tumour promoters and potential therapeutic targets.

In a previous study, we had demonstrated that MUC4 regulation by TC, TDC, TCDC, GC and GNa bile acids in oesophageal adenocarcinomatous cells involved phosphatidylinositol 3-kinase signalling pathway and to a lower extent mitogen-activated protein kinase, protein kinase A and protein kinase C pathways [15]. In this report we show that induction of MUC4 expression may occur *via* an alternative pathway involving HNF1 α , this mechanism being restricted to TDC and TCDC. The fact that bile acids are able to activate MUC4 expression using different pathways indicates that it is an important mechanism, that necessitates alternative pathways, in order to increase the capacity of the oesophageal cancer cell to respond to bile acids. We could not show HNF1 α mediation for DC, GC and GNa suggesting that other mechanisms are involved. Nuclear factor- κ B and TFs of the activating transcription factor-1/cAMP-responsive element-binding protein family have been described to mediate phosphatidylinositol 3-kinase effects [29]. Further investigations will be needed to answer that question. Although HNF4 α is able to regulate endogenous *MUC4* expression, we could

not identify an HNF4 binding element, which suggests that the regulation is indirect, most likely involving participation of co-factors as it has already been shown for HNF4 [30]. Moreover, HNF4 α has no functional role in mediating *MUC4* regulation by TDC and TCDC. Taken together, our *in vitro* and *ex vivo* data showing co-expression of HNF1 α and MUC4 in Barrett's metaplasia and adenocarcinoma, strongly suggests that HNF1 α plays a major role in controlling MUC4 expression during oesophageal carcinogenesis.

HNF1 α is a homeodomain-containing TF expressed in the liver, kidney, intestine, stomach and pancreas [31]. Inactivation of HNF1 α has been associated with the development of human liver adenomas, and some hepatocarcinoma, suggesting a potential role for HNF1 α as a tumour suppressor gene in these diseases [32]. In our study, we did not find loss of HNF1 α expression during oesophageal carcinogenetic sequence. On the contrary, expression was induced in Barrett's metaplasia and adenocarcinoma. This suggests that HNF1 α plays a fundamental role in the progression of oesophageal carcinogenesis. Moreover, HNF1 α is known to regulate the expression of several intestine-specific genes (lactase, sucrase isomaltase) [33,34], and to control intestinal cell differentiation [35], a mucosa in which MUC4 is expressed [36]. Since intestinal differentiation of oesophageal mucosa, secondary to bile acid reflux, is a characteristic step toward development of OA derived from Barrett's metaplasia, it may explain the strong HNF1 α expression in Barrett's metaplasia mucosa with concomitant activation of MUC4. Quantitative study of HNF1 α expression during the carcinogenetic sequence leading to oesophagus adenocarcinoma in a higher number of patients could be interesting to investigate its potential diagnostic value.

HNF1 α binds the consensus sequence GGTTAATNATTAAC(A/C) [30,37]. The two *cis*-elements identified in *MUC4* promoter are very similar to that consensus sequence (CTTAATAAACATC at -3332/-3327 and GTGGAATATTAAC at -3040/-3028). Mutation of some nucleotides in the -3040/-3028 element completely abolished binding and

transactivation of MUC4 by HNF1 α indicating that HNF1 α alone is sufficient to convey MUC4 activation *via* this site. Mutation of the other *cis*-element at -3332/-3327 inhibited HNF1 α binding but transactivation was partially affected, suggesting probable involvement of a co-factor. Among TFs known to synergize with HNF1 α [34] and for which we found putative binding sites in the distal promoter of *MUC4* [18] are Cdx-2 and GATA-4. Moreover Cdx TF are also associated with OA developed on Barrett's metaplasia [38,39]. Preliminary data, however, did not suggest additive or synergistic effect between these three TF in oesophageal OE33 cancer cells (unpublished work). These synergistic activities may thus be restricted to intestinal cells [34].

The pattern of mucin expression is tissue-specific and changes during neoplastic progression suggest an important role for mucins in tumour growth and progression and have recently justify their use as reliable phenotypic markers [40,41]. During the different steps of the OA carcinogenetic sequence, MUC4, a high molecular weight membrane-bound mucin, is overexpressed from the high grade dysplasia state and in adenocarcinomatous tissues and is presently evaluated by us and other groups as a promising early diagnostic tumoural marker [12-14]. Moreover, membrane-bound mucins have been associated with both steric protection of epithelial surfaces and cellular signalling functions [7,41,42]. MUC4 is a mediator of tumour growth and metastatic properties of cancer cells by acting as a ligand for the receptor tyrosine kinase ErbB2 [8,10,11]. ErbB2 is an oncogene over-expressed in 11 to 35% of OA derived from Barrett's metaplasia [43,44] and tumours overexpressing ErbB2 have a poorer survival prognosis [43]. Recently, we showed that MUC4-ErbB2 interaction may play a role in the progression/differentiation balance of pancreatic tumour cells [11]. Obviously, this duality of function could be of interest in terms of therapeutic application with MUC4 in OA and could also explain the variations of MUC4 prognostic value published in the literature in the upper aerodigestive tract (good prognosis) [41] or in pancreatic adenocarcinoma (poor

prognosis) [45]. Dual biological activity has been proposed for the MUC4 mucin since it may be expressed either as a membrane-associated protein or as a soluble mucin [7,8,46]. The soluble form, which may be produced by either alternative splicing or proteolysis, contributes to maintain a pool of secreted MUC4 that participates in epithelial protection [46]. Interestingly, in our study MUC4 expression was found both at the apical surface and in the cytoplasm of oesophageal cancer cells. This pattern of expression has already been described for MUC4 in physiological fluids [46] as well as in other tumour locations [47] and may thus represent a more general pattern of expression of MUC4 that translates its dual role in epithelial cell biology.

In conclusion, we have demonstrated that HNF1 α is a strong activator of *MUC4* expression, and that it specifically mediates *MUC4* up-regulation by TDC and TCDC bile acids. This novel mechanism of regulation of *MUC4* may account for its up-regulation during OA associated with bile reflux. In the future, the development of an animal model of induced oesophageal carcinogenesis will allow us to evaluate the exact role of MUC4 overexpression in the development of oesophagus adenocarcinoma on Barrett's metaplasia associated with reflux, the consequences on the biological properties of oesophageal cancer cells and identify potential molecular targets that could have a therapeutic value.

Acknowledgements

This paper is dedicated to Dr Jean-Pierre Aubert, Director of our Laboratory, who died in September 2005. He initiated the collaboration between surgeons and scientists of the unit in order to stimulate translational research and promote potential clinical implications.

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Figure legends

Figure 1: Expression of MUC4, HNF1 α and HNF4 α in human normal, metaplastic and adenocarcinomatous oesophageal tissue. Immunohistochemical studies of human normal oesophageal mucosa, Barrett's metaplasia, and oesophageal adenocarcinoma, with anti-HNF1 α , anti-HNF4 α , and anti-MUC4 antibodies. Inset: expression in well-differentiated areas of adenocarcinomatous tissues.

Figure 2: Transcriptional regulation of MUC4 by HNF1 α and HNF4 α in epithelial cancer cells. (A) siRNA experiments were carried out as described in the material and methods section. MUC4, HNF1 α , HNF4 α , and GAPDH mRNA levels were assessed by RT-PCR. PCR products (10 μ l for MUC4 and 5 μ l for HNF1 α , HNF4 α and GAPDH) were analysed on a 1.5% agarose gel. Diagram showing data expressed as MUC4/GAPDH ratio. Control corresponds to the means value from mock cells and cells transfected with non targeting siRNA; this value was arbitrarily set to 1. (B) RT-PCR analysis of MUC4, HNF1 α , HNF4 α mRNA levels in OE33 cells transfected with 4 μ g of pSG5 empty vector (lane 1), pSG5-HNF1 α (lane 2), or pSG5-HNF4 α (lane 3) expression vectors. PCR products (15 μ l for MUC4 and 4 μ l for HNF1 α , HNF4 α and 18S) were analysed on a 1.5% agarose gel. The value corresponding to cells transfected with empty vector was arbitrarily set to 1. *, $P = 0.02$. (C) Western blotting analysis of MUC4 protein expression in total cellular extracts prepared from OE33 cells transfected under the same conditions as above. (D) Schematic representation of MUC4 distal promoter with the three HNF putative binding sites (black squares). Co-transfection experiments in OE33 cells were performed in the presence of 1 μ g of MUC4 pGL3-deletion constructs -3135/-2837 or -3713/-3059 and 0.25 μ g of pSG5-HNF1 α or pSG5-HNF4 α . Ref. refers to the normalized luciferase activity of pGL3-MUC4

constructs transfected with the empty vector pSG5 (white bar); this value was arbitrarily set to 1. *, $P = 0.001$. //; $P = 0.03$.

Figure 3: Identification of two HNF1 α cis-elements and study of their functionality in regulating MUC4 distal promoter. (A) Identification of HNF cis-elements by EMSA. 8 μ g of nuclear extracts from HNF1 α expressing KATO-III cells were incubated with radiolabelled T144 (lanes 1-6), mutated T144 (lanes 7-9), T106 (lanes 10-15), or mutated T106 (lanes 16-18) DNA probes. Radiolabelled probe alone (lanes 1, 7, 10 and 16). Incubation of T144, Mut. T144, T106 or Mut. T106 probes with nuclear proteins (lanes 2, 8, 11 and 17). Cold competition with 50x excess of unlabelled T144 (lane 3), unlabelled Mut. T144 (lanes 4 and 9), unlabelled T106 (lane 12) or unlabelled Mut. T106 (lanes 13 and 18) probes, respectively. Supershift analyses with anti-HNF1 α (lanes 5 and 14) or anti-HNF4 α (lanes 6 and 15) antibody, respectively. DNA protein complexes (HNF) and supershifts (ssHNF1 α) are indicated by arrows on the side of the autoradiograms. n.s.: non specific. (B) ChIP analysis of HNF1 α binding to chromatin covering the T144 (left panel) and T106 (right panel) binding sites using specific primers (Table I). Input (lanes 1 and 6), anti-HNF1 α (lanes 2 and 5), rabbit IgGs (lanes 3 and 4). PCR products (15 μ l) were analysed on 2% agarose gels. (C) Site-directed mutagenesis of the T144 and T106 HNF cis-elements in the -3135/-2837 or -3713/-3059 constructs and their effects on their regulation by HNF1 α or HNF4 α . Ref. refers to the normalized luciferase activity of wild type or mutated pGL3-MUC4 constructs co-transfected with the empty vector pSG5 (white bar). This value was arbitrarily set to 1.

Figure 4: Effect of actinomycinD pretreatment on MUC4 regulation by bile acids in OE33 cells. Cells were pretreated with actinomycinD (ActD) for 30 min before a 24h bile acid incubation. MUC4 and β -actin mRNA levels were studied by RT-PCR and compared to

cells only incubated with bile acids. PCR products (15 μ l and 4 μ l, respectively) were analysed on a 1.5% agarose gel.

Figure 5: HNF1 α -mediated up-regulation of *MUC4* transcription by TDC and TCDC is dose-dependent. (A) siRNA experiments were carried out as described in the material and methods section. *MUC4*, *HNF1 α* , and *GAPDH* mRNA levels were assessed by RT-PCR. PCR products (10 μ l for *MUC4* and 5 μ l for *HNF1 α* and *GAPDH*) were analysed on a 1.5% agarose gel. Diagram showing data expressed as *MUC4*/*GAPDH* ratio. Control corresponds to the means value from mock cells and cells transfected with non targeting siRNA; this value was arbitrarily set to 1. (B) Co-transfection experiments in OE33 cells were performed in the presence of 1 μ g of pGL3-*MUC4* constructs -3135/-2837 or -3713/-3059 and 0.25 μ g of pSG5-HNF1 α expression vector. Cells were then incubated for 24h with $\frac{1}{2}$ dose, $\frac{3}{4}$ dose and full dose of TDC or TCDC. Ref. refers to the normalized luciferase activity of pGL3-*MUC4* constructs co-transfected with HNF1 α expression vector without bile acid treatment; this value was arbitrarily set to 1.

Figure 6: HNF1 α cis-elements are directly implicated in *MUC4* regulation by TDC and TCDC. Co-transfection experiments in OE33 cells were performed in the presence of 1 μ g of pGL3-*MUC4* constructs -3135/-2837, Mut. -3135/-2837, -3713/-3059 or Mut. -3713/-3059 and 0.25 μ g of pSG5-HNF1 α expression vector. Cells were then treated for 24h with TDC or TCDC. The values obtained in cells transfected with wild-type pGL3-*MUC4* constructs were arbitrarily set to 1.

Figure 1 : Piessen *et al.*

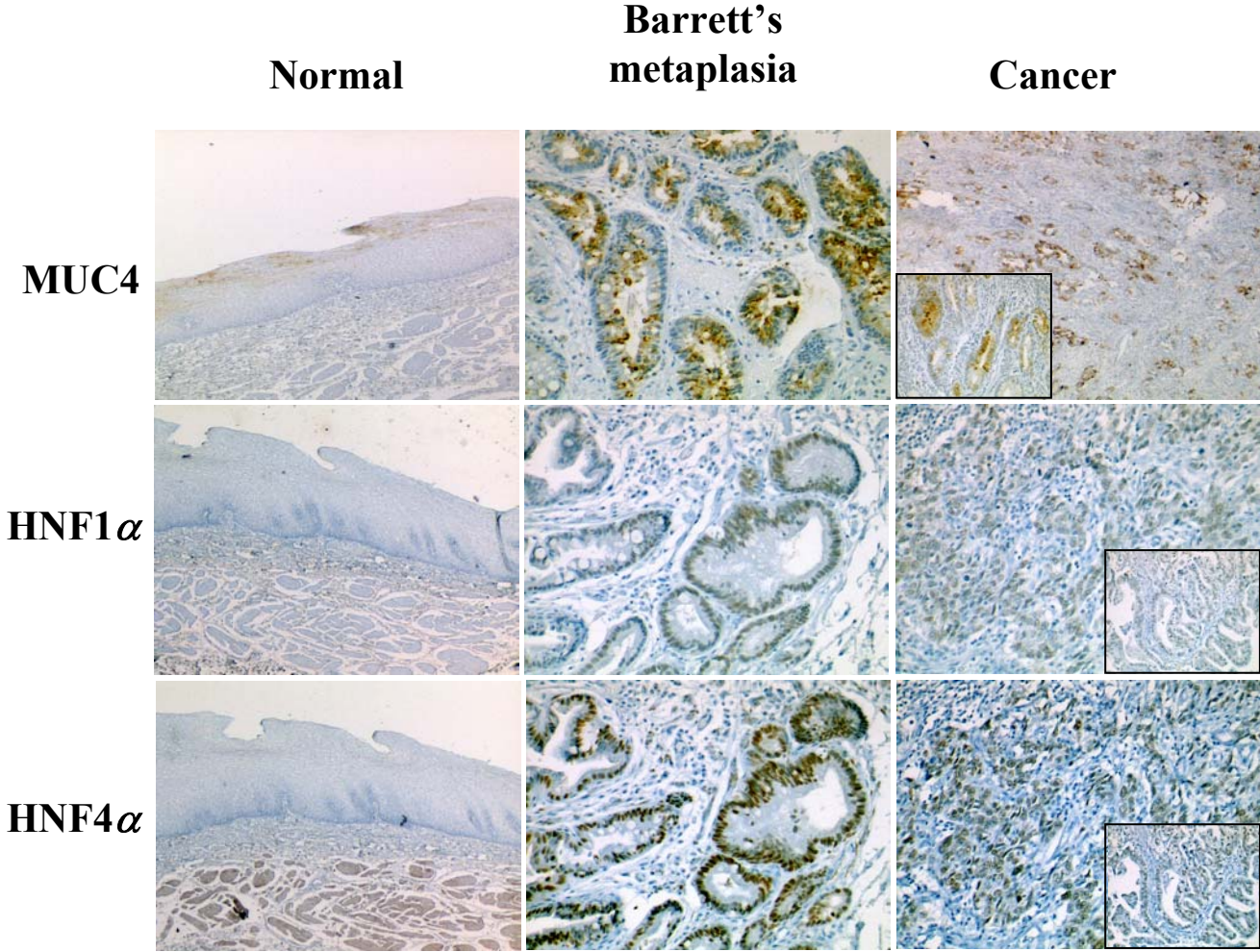


Figure 2 : Piessen *et al.*

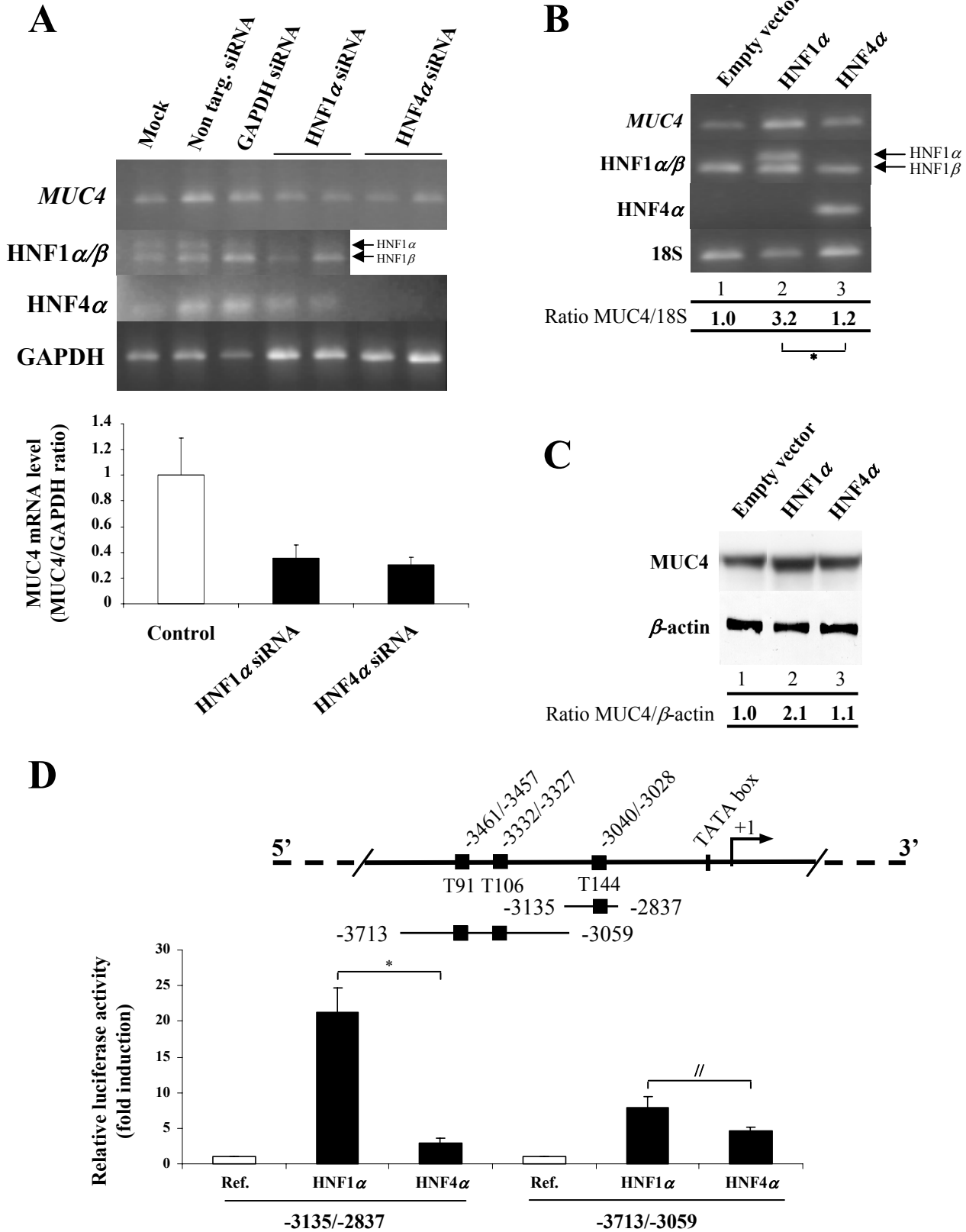
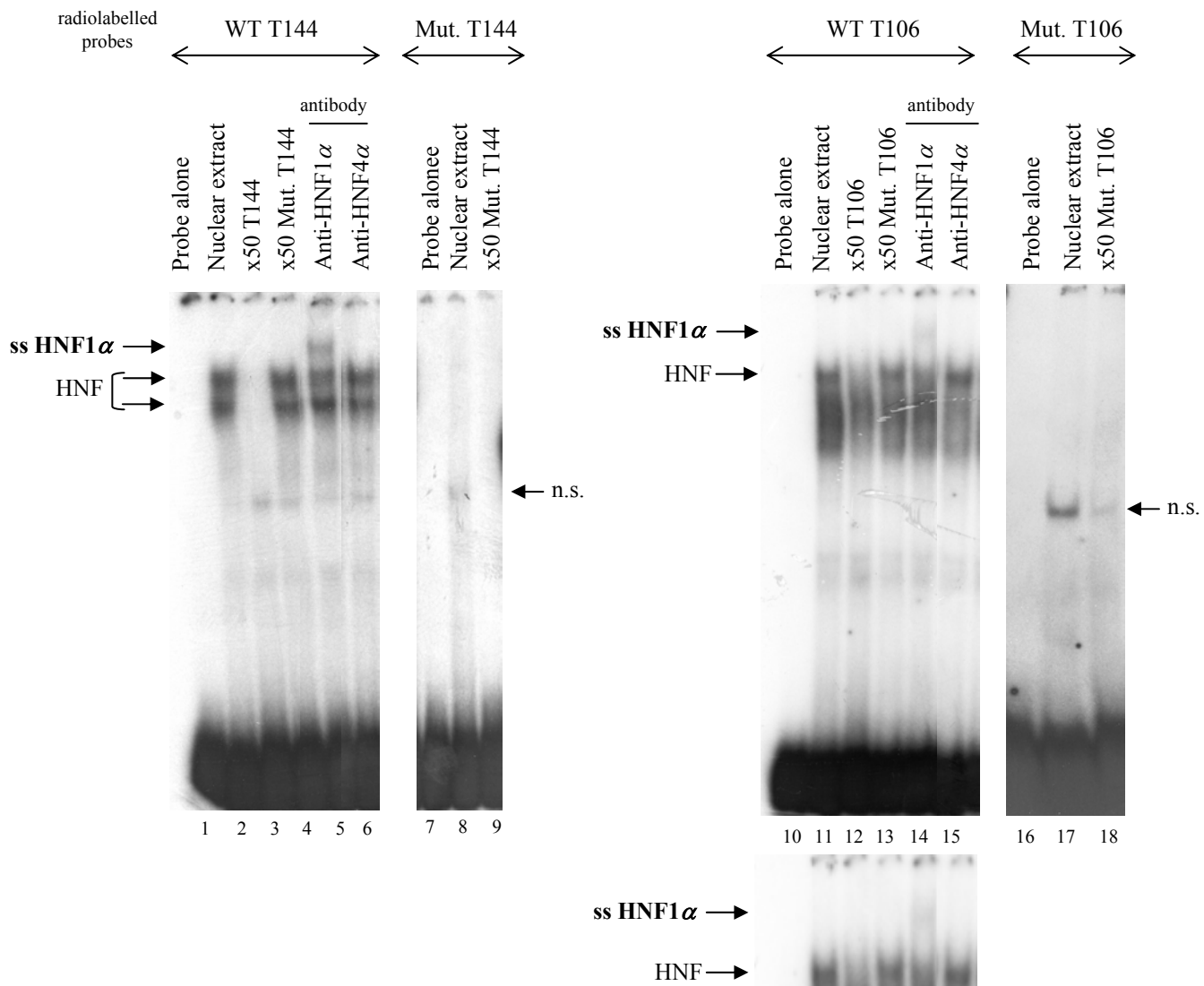


Figure 3 : Piessen et al.

A



B

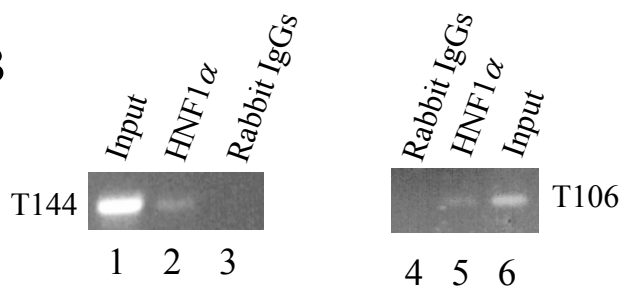


Figure 3 : Piessen et al.

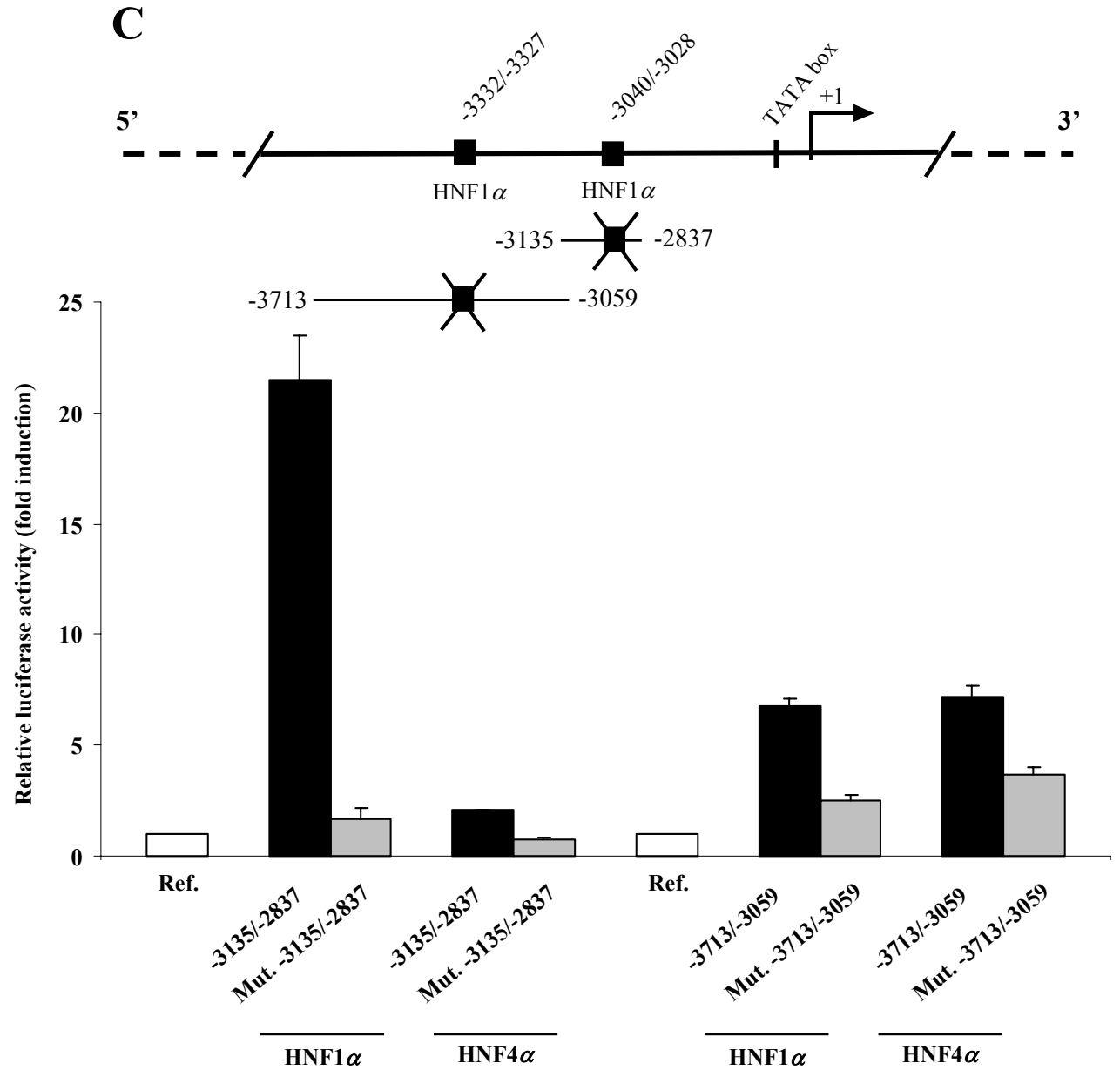


Figure 4 : Piessen et al.

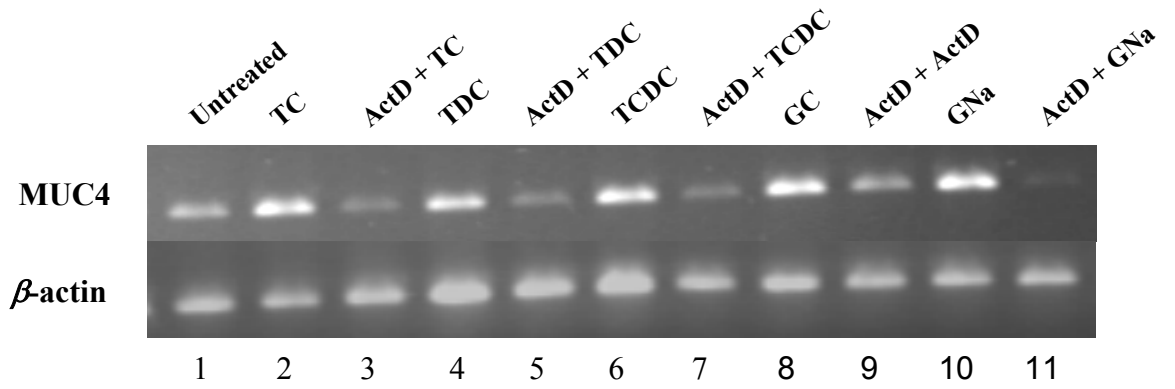
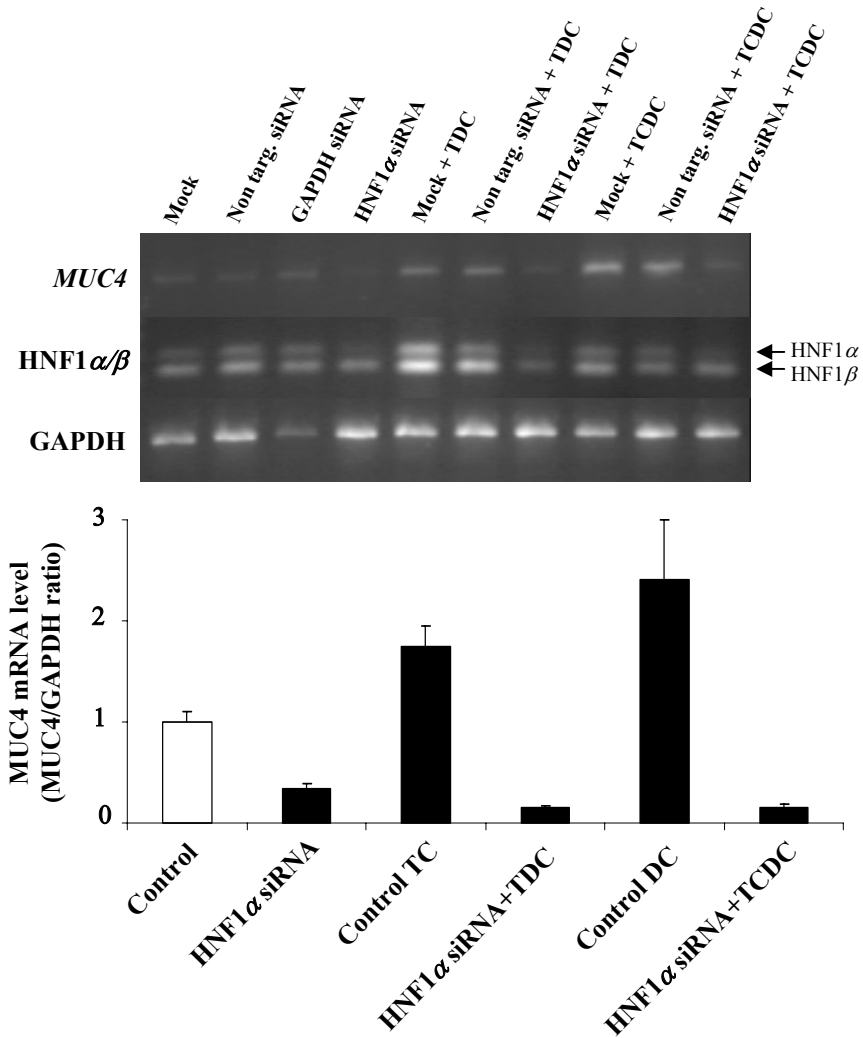


Figure 5 : Piessen et al.

A



B

-3135/-2837 and HNF1 α

-3713/-3059 and HNF1 α

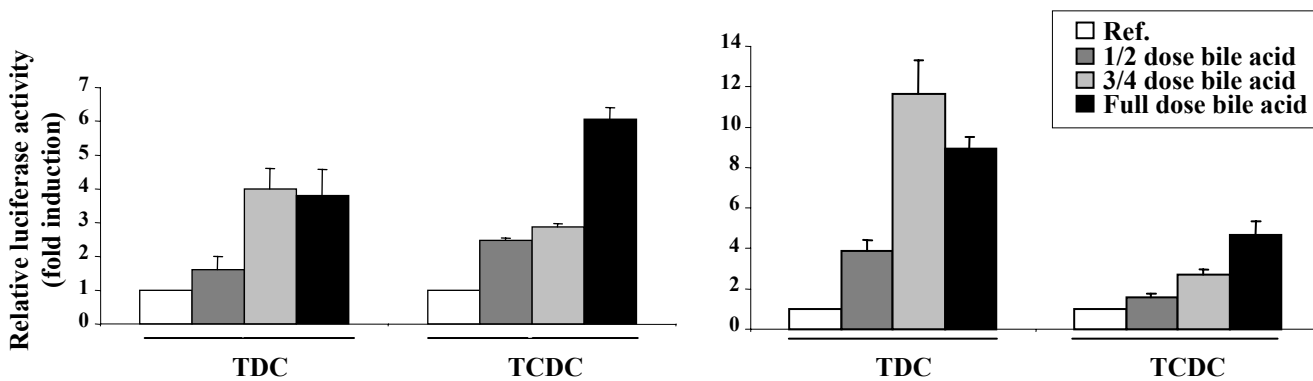


Figure 6 : Piessen et al.

