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

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MUC4 (mucin 4) 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, up-regulate MUC4 expression [Mariette, Perrais, Leteurtre, Jonckheere, Hemon, Pigny, Batra, Aubert, Triboulet and Van Seuningen (2004) Biochem. J. **377**, 701–708]. HNF (hepatocyte nuclear factor) 1α and HNF4α transcription factors are known to mediate bile acid 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, HNF1α and HNF4α expressions were evaluated by immunohistochemistry in human oesophageal tissues. Our results indicate that MUC4, HNF1α and HNF4α were co-expressed in oesophageal metaplastic and adenocarcinomatous tissues. Studies at the

mRNA, promoter and protein levels indicated that HNF1α 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 (small interfering RNA) approach, co-transfection and site-directed mutagenesis that HNF1α mediates taurodeoxycholic and taurochenodeoxycholic bile acid 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 HNF1α is a key mediator. These results bring new insights into MUC4 up-regulation in oesophageal carcinoma associated with bile reflux.

Key words: adenocarcinoma, Barrett's metaplasia, bile acid, hepatocyte nuclear factor 1α (HNF1α), mucin 4 (MUC4), oesophagus.

INTRODUCTION

The incidence of OA (oesophageal adenocarcinoma) 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 the importance of bile acids in the development of adenocarcinoma in Barrett's metaplasia is still being discussed among authors, many studies suggest that they are the 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 (mucin 1) and MUC4 (mucin 4) 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 the 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 (erythroblastic leukaemia viral oncogene homologue 2) [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 in the high-grade dysplasia state and in adenocarcinomatous tissues [14].

In relation with (i) *MUC4* overexpression in OA and (ii) the role of bile acids in induction and degeneration of Barrett's metaplasia, we recently showed that TC (taurocholic acid), TDC (taurodeoxycholic acid), TCDC (taurochenodeoxycholic acid)

and glycocholic acids and GNa (sodium glycocholate) were strong activators of MUC4 expression [15]. We showed that this regulation occurred at the transcriptional level, and involved activation of phosphoinositide 3-kinase pathway. Bile acids may however mediate their effects using other pathways. Activation of HNF (hepatocyte nuclear factor) 1α and HNF4α was recently identified as a mechanism used by bile acids to activate transcription of their target genes [16,17]. The recent identification of HNF1α cis-elements in the distal promoter of *MUC4* [18] was a positive argument in favour of the possible implication of that transcription factor in mediating *MUC4* regulation by bile acids. In the present paper, we show for the first time that HNF1α 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 ten patients who underwent curative oesophagectomy for adenocarcinoma developed on Barrett's metaplasia without neoadjuvant treatment. Permission for removal of surgical samples was obtained from the Centre Hospitalier Régional et Universitaire de Lille Review Board. A consent form was obtained from each patient. The surgical specimen was quickly immersed in 10 % (v/v) neutral formaldehyde solution (pH 7.4) in PBS. 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 μm) with haematoxylin–eosin–saffron. Immunohistochemical studies for MUC4 expression were performed as described in [15]. Monoclonal anti-MUC4 antibody [19] was used at 1:20 000 dilution. A positive control for MUC4 immunostaining was included in each set of experiments on human lung sections. Immunohistochemical studies for HNF1α and HNF4α expression were performed as described in [20]. The primary antibodies directed against human HNF1α and human HNF4α (Santa Cruz Biotechnology) were used at a 1:2000 dilution. A positive control for HNF1α and HNF4α immunostaining on human small intestine sections was included in each set of experiments.

Cell culture

The OA cell line OE33 was purchased from ECACC (European Collection of Cell Cultures) and was cultured as previously described [15]. HNF1α-expressing KATO-III cells were cultured as previously described [21]. Cells were treated with bile acids and their conjugates for 24 h as described previously [15]. Optimal concentrations for bile acids were: TC, 0.5 mM; TDC, 1 mM; TCDC, 0.5 mM; glycocholic acid, 0.5 mM; and GNa, 0.5 mM. For dose–effect experiments, a third, a half and the full concentration of each bile acid was used. To inhibit protein synthesis or transcription, cells were treated for 30 min with cycloheximide (20 μg/ml) or actinomycin D (1 μg/ml) respectively. All reagents were from Sigma unless otherwise indicated.

siRNA (small interfering RNA) 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 100 nM of either TCF1 (transcription factor 1; HNF1α) SMARTpool® or HNF4α 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 24 h after transfection and lasted 24 h as described above. Total RNA was isolated 48 h after transfection as described below. 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 (glyceraldehyde-3-phosphate dehydrogenase) ratio.

RT (reverse transcriptase)–PCR

Total RNAs from cultured cells were prepared using the RNeasy mini-kit from Qiagen. One microgram of total RNA was used to prepare cDNA (Advantage™ RT-for-PCR kit; Clontech) as described previously [22]. PCR was performed on 5 μl of cDNA using specific pairs of primers for *MUC4*, *HNF1α*, *HNF4α* and *18 S* or β-actin as internal controls (MWG-Biotech). Primer information is given in Supplementary Table 1 at http://www. BiochemJ.org/bj/402/bj4020081add.htm. PCRs were carried out in 50 μl final solutions as described in [23]. PCR products were analysed on 1.5 % (w/v) agarose gels containing ethidium bromide run in 1 × TBE (45 mM Tris/borate/1 mM EDTA) buffer. A 100 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 MUC4/18 S or MUC4/β-actin.

Plasmids and expression vectors

The two pGL3-*MUC4* deletion mutants used in the present 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 QuikChange® 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 Supplementary Table 1. pSG5-HNF1α and pSG5-HNF4α expression vectors were a gift from Dr J. K. Divine (Washington University, St Louis, MO, U.S.A.). 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 48 h incubation time at 37°C using 1 × 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 acid 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 μg of the pGL3-*MUC4* deletion mutant was transfected with 0.25 μg of the expression vectors pSG5-HNF1α, pSG5-HNF4α 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 HNF1α

and HNF4α overexpressions on endogenous *MUC4* mRNA level, cells (0.5×10^6) were transfected with 4 µg of the expression vector of interest and cultured for 48 h before being lysed and processed for total RNA extraction as described previously [20].

Western blotting

Total cellular extracts were prepared using standard procedures and kept at $-80\,°C$ before use. Briefly, cells were scraped into the medium and centrifuged for 5 min at 1600 *g*. The pellet was washed twice with $1 \times$ PBS, centrifuged for 1 min at 9000 *g* and then resuspended in lysis buffer (50 mM Tris/HCl, pH 7.4, 1 mM EDTA, 0.25 % sodium deoxycholate, 150 mM NaCl and 1 % Nonidet P40) supplemented with protease inhibitors (1 µg/ml aprotinin, 1 µg/ml leupeptin and 1 mM PMSF) and phosphatase inhibitors (5 mM sodium fluoride and 5 mM sodium orthovanadate). The lysate was incubated for 30 min on ice, before being centrifuged at 16 000 *g* for 10 min at 4 °C. The supernatant was collected for Western blotting analysis. Protein content (2 µl 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 µg) were separated on a SDS (0.1 %)/agarose [2 % (w/v)] gel. For β-actin expression analysis, proteins were separated using SDS/10 % PAGE. Resolved proteins were transferred on to a 0.2 µm 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® (enhanced chemiluminescence; Amersham Biosciences). For β-actin detection, the blots were processed with Nitro Blue Tetrazolium and BCIP (5-bromo-4-chloroindol-3-yl phosphate) substrate (Life Technologies).

Nuclear extract preparation and EMSA (electrophoretic mobility-shift assay)

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 the 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 (http://www.genomatix.de) [26]. The oligonucleotides (MWG-Biotech) were annealed before being phosphorylated at the 5′-end with [γ-^{32}P]ATP (Amersham Biosciences) and T4 polynucleotide kinase (Roche). Wild-type and mutated oligonucleotides used as probes and competitors in EMSAs are shown in Supplementary Table 1. Nuclear protein incubation with radiolabelled probes and competitions with unlabelled probes were as described in [23]. For supershift analyses, 2 µl of anti-HNF1α (sc-6547x) or anti-HNF4α (sc-6556x) (Santa Cruz Biotechnology) antibodies were added to the proteins and left for 2 h at room temperature (22 °C) before adding the radiolabelled probe. Electrophoresis conditions and gel processing were as described previously [27].

ChIP (chromatin immunoprecipitation)

KATO-III cells (15×10^6 cells) were treated with 1 % (v/v) formaldehyde for 10 min at room temperature and cross-links were quenched with glycine at a final concentration of 0.125 M

for 5 min. Cells were rapidly rinsed with ice-cold $1\times$ D-PBS (Dulbecco-PBS), with D-PBS containing protease inhibitors (10 µg/ml leupeptin, 10 µg/ml aprotinin, 0.2 mM EDTA and 0.5 mM PMSF), and scraped off and collected by centrifugation at 700 *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$_2$ and 0.1 % Nonidet P40) 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 200 W. After clearing by centrifugation at 10 000 *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 and 1.2 mM EDTA). The chromatin solution was precleared by incubation with sonicated salmon sperm DNA/BSA/Protein G–agarose gel slurry (Upstate Biotechnology) for 1 h at 4 °C with rotation followed by centrifugation at 1500 *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 Biotechnology). 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 2 h. Agarose beads were collected and washed sequentially for 4 min in Low Salt Immune Complex wash buffer, High Salt buffer and LiCl buffer (Upstate) and twice with TE buffer [10 mM Tris/HCl (pH 8.0) and 1 mM EDTA]. The complexes were eluted with 2×0.25 ml of 1 % SDS/0.1 M NaHCO$_3$ after a 15 min incubation at room temperature. Formaldehyde cross-links were reversed with 0.3 M 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 Supplementary Table 1. PCR was performed in a 30 µl final volume consisting of $1 \times$ PCR buffer II, 2.5 mM MgCl$_2$, 1.5 units of AmpliTaq Gold polymerase (Applied Biosystems) and 5 pmol of each primer. The temperature-cycling protocol consisted of 12 min of preheating at 94 °C followed by 35 cycles of 45 s 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 the present paper are means $\pm$ S.D. When indicated, data were analysed by Mann–Whitney *U* test with differences $P \leqslant 0.05$ considered significant.

RESULTS

Expression of MUC4, HNF1α and HNF4α 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. HNF1α and HNF4α 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
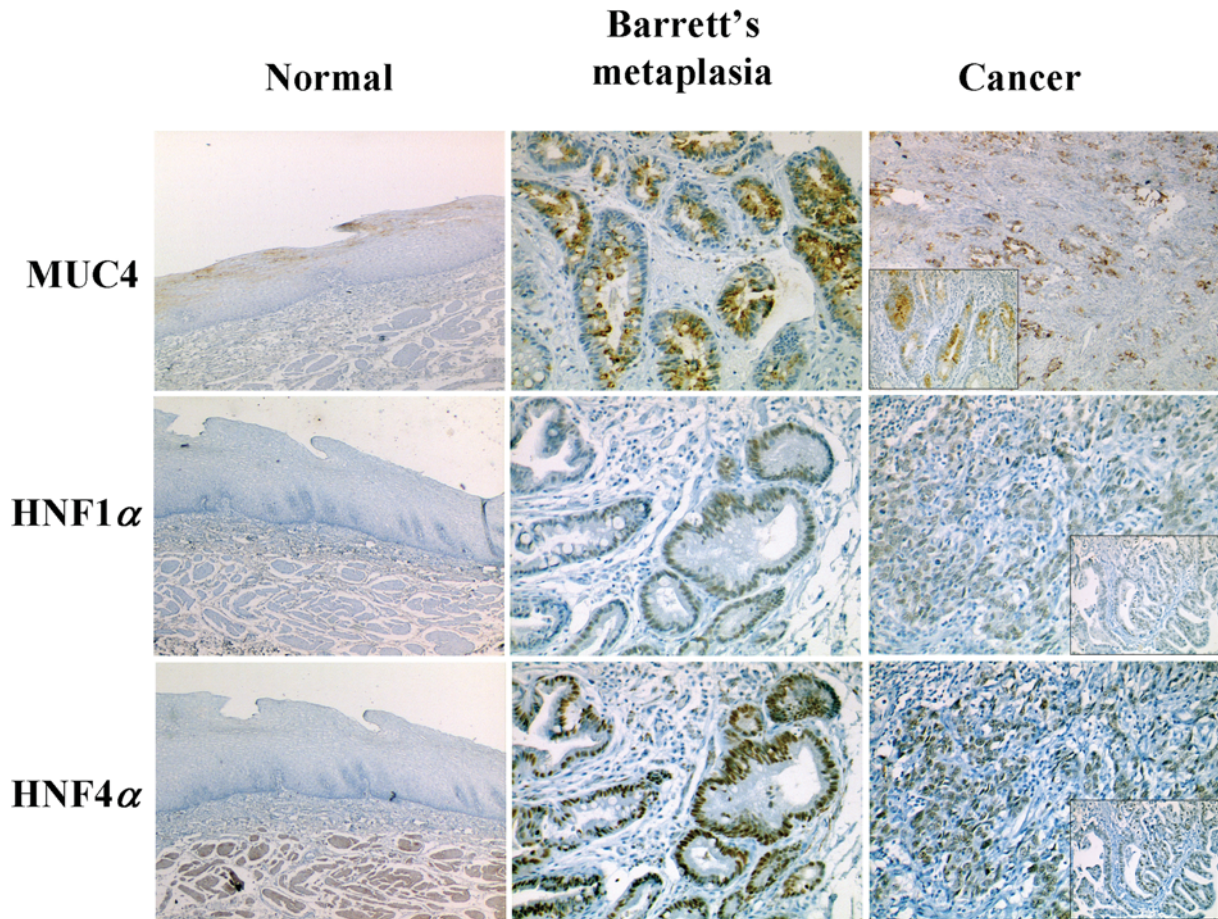
Figure 1   Expression of MUC4, HNF1α and HNF4α in human normal, metaplastic and adenocarcinomatous oesophageal tissue

Immunohistochemical studies of human normal oesophageal mucosa, Barrett's metaplasia and OA, with anti-HNF1α, anti-HNF4α and anti-MUC4 antibodies. Inset: expression in well-differentiated areas of adenocarcinomatous tissues. Magnification: ×150 for normal tissues; ×250 for Barrett's neoplasia and cancer tissues.

visualized independently of tumour differentiation in well-differentiated (inset) and undifferentiated adenocarcinomatous tissues.

Regulation of MUC4 expression by HNF1α and HNF4α

To investigate whether HNF1α and HNF4α transcription factors participate in *MUC4* mRNA regulation, we combined knockdown assays with specific siRNA (Figure 2A) and overexpression experiments (Figures 2B and 2C). HNF1α and HNF4α knockdown in gastric KATO-III cells, which express these two transcription factors, led to a substantial decrease in *MUC4* mRNA (65 ± 8 % and 70 ± 6 % respectively), indicating that both transcription factors regulate endogenous *MUC4* expression (Figure 2A). Results from four experiments in oesophageal OE33 cells in which HNF1α and HNF4α were overexpressed showed that overexpression of HNF1α led to a 3.2-fold increase (3.2 ± 0.4) of *MUC4* mRNA (Figure 2B, lane 2), whereas overexpression of HNF4α had a significantly weaker effect (1.2 ± 0.1-fold increase, $P = 0.02$) (lane 3). At the protein level, we also observed an increase in MUC4 apomucin expression (2.1 times) when HNF1α was overexpressed (Figure 2C, lane 2), whereas no variation was seen in HNF4α-transfected cells (lane 3).

Activation of *MUC4* transcription at the promoter level by HNF1α 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 2(D) indicate that HNF1α 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 HNF4α 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 HNF1α cis-elements in MUC4 distal promoter

Having shown that HNF1α and HNF4α were transactivating the *MUC4* distal promoter, we undertook to identify their binding sites using EMSA. Three putative HNF-binding sites are present in the *MUC4* distal promoter (Figure 2D). When radiolabelled probes T144 (−3040/−3028) and T106 (−3332/−3327) were incubated with nuclear extracts, two shifted bands were visualized with T144 probe (Figure 3A, 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 unlabelled competition was performed with a 50 times excess of either unlabelled T144 (lane 3) or T106 (lane 12) DNA probes. As expected, competition with a 50 times 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
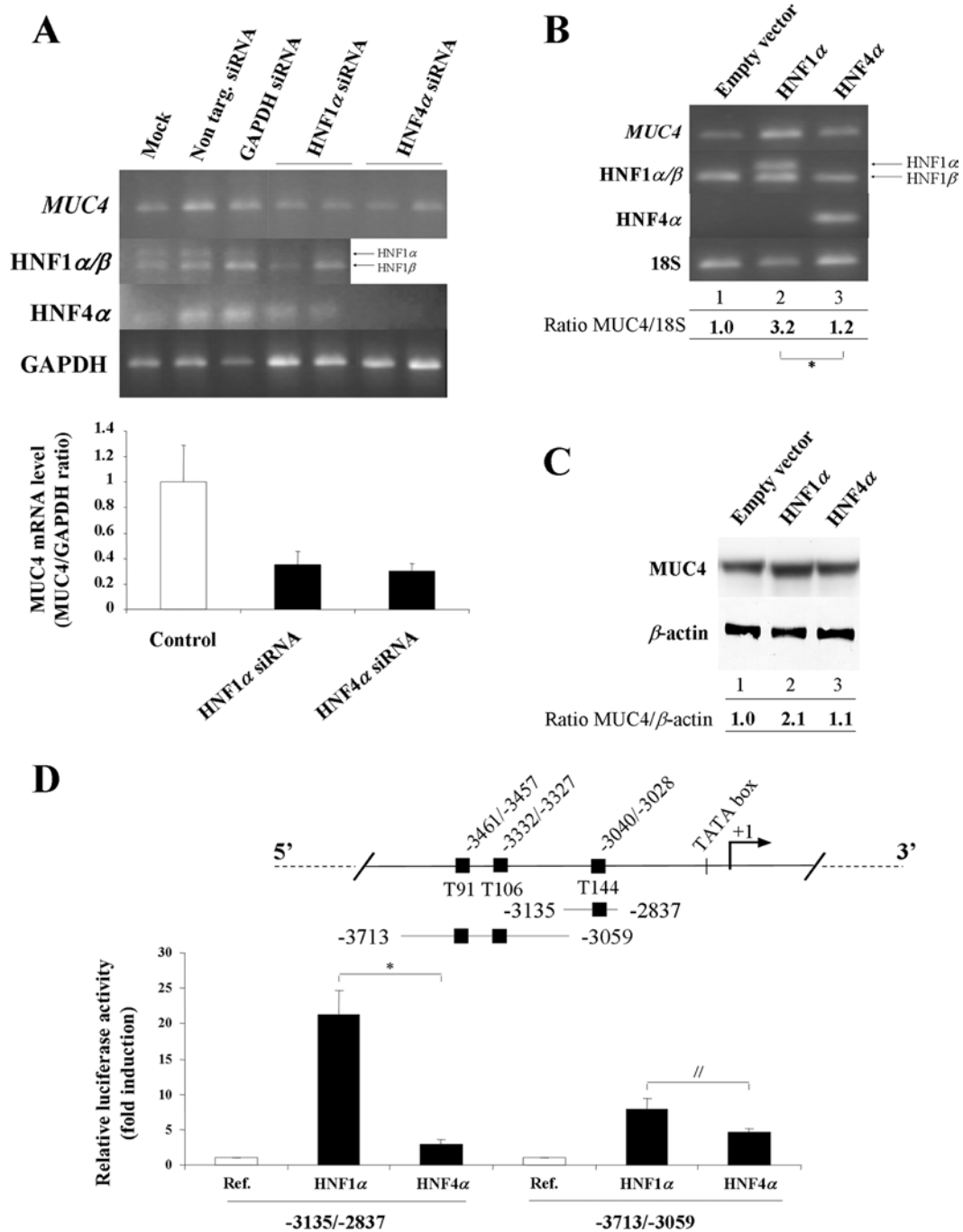
Figure 2   Transcriptional regulation of *MUC4* by HNF1α and HNF4α in epithelial cancer cells

(**A**) siRNA experiments were carried out as described in the Materials and methods section. *MUC4*, HNF1α, HNF4α and *GAPDH* mRNA levels were assessed by RT–PCR. PCR products (10 μl for *MUC4* and 5 μl for *HNF1α*, *HNF4α* and *GAPDH*) were analysed on a 1.5% agarose gel. The lower panel shows results expressed as MUC4/GAPDH ratio. Control corresponds to the mean values from mock cells and cells transfected with non-targeting siRNA; this value was arbitrarily set to 1. (**B**) RT–PCR analysis of *MUC4*, HNF1α and HNF4α mRNA levels in OE33 cells transfected with 4 μg of pSG5 empty vector (lane 1), pSG5-HNF1α (lane 2), or pSG5-HNF4α (lane 3) expression vectors. PCR products (15 μl for *MUC4* and 4 μl for *HNF1α*, *HNF4α* and *18 S*) 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 μg of *MUC4* pGL3-deletion constructs $-3135/-2837$ or $-3713/-3059$ and 0.25 μg of pSG5-HNF1α or pSG5-HNF4α. '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. *$P = 0.001$. //$P = 0.03$.

in which the HNF-binding sequence was mutated (see Supplementary Table 1). In this 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 HNF1α in the binding

to the T144 and T106 probes was proved by the supershift obtained when a specific anti-HNF1α antibody was added to the reaction mixture (lanes 5 and 14). No supershift was observed when anti-HNF4α antibody was added (lanes 6 and 15). The third putative HNF-binding site T91 ($-3461/-3457$) did not bind HNF1α
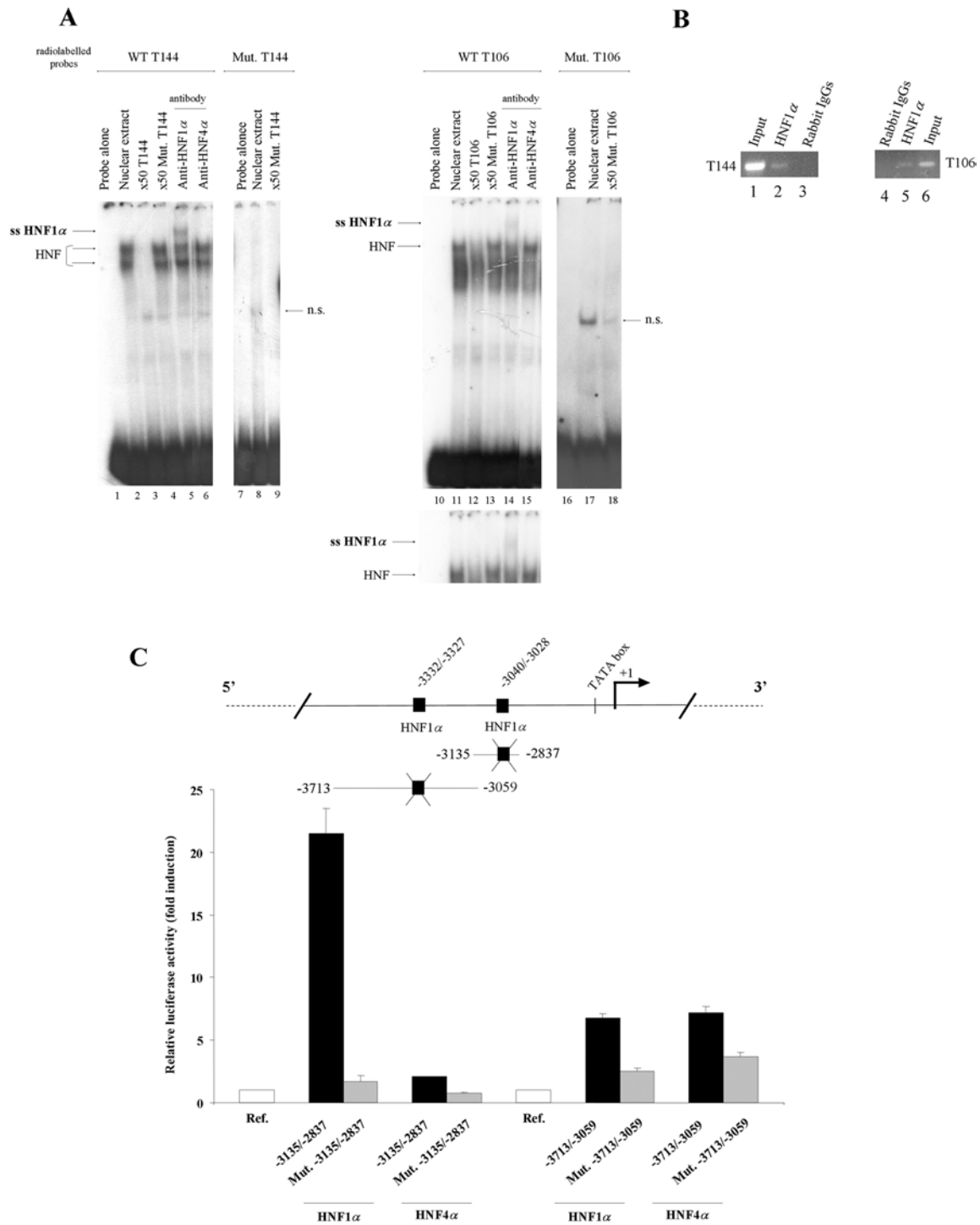
Figure 3   **Identification of two HNF1α *cis*-elements and study of their functionality in regulating *MUC4* distal promoter**

(**A**) Identification of HNF *cis*-elements by EMSA. Nuclear extracts (8 μg) from HNF1α-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. Lanes 1, 7, 10 and 16, radiolabelled probe alone. Lanes 2, 8, 11 and 17, incubation of T144, mutated T144, T106 or mutated T106 probes with nuclear proteins. Lane 3, cold competition with 50 times excess of unlabelled T144. Lanes 4 and 9, cold competition with 50-fold excess of unlabelled Mut. T144. Lane 12, cold competition with 50-fold excess of unlabelled T106. Lanes 13 and 18, cold competition with 50 times excess of unlabelled Mut. T106 probe. Lanes 5 and 14, supershift analyses with anti-HNF1α. Lanes 6 and 15, supershift analysis with anti-HNF4α. DNA–protein complexes (HNF) and supershifts (ssHNF1α) are indicated by arrows on the left side of the autoradiograms. n.s., non-specific. (**B**) ChIP analysis of HNF1α binding to chromatin covering the T144 (left panel) and T106 (right panel) binding sites using specific primers (Supplementary Table 1; see http://www.BiochemJ.org/bj/402/bj4020081add.htm). Input (lanes 1 and 6), anti-HNF1α (lanes 2 and 5) and rabbit IgGs (lanes 3 and 4). PCR products (15 μl) 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 HNF1α or HNF4α. '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.
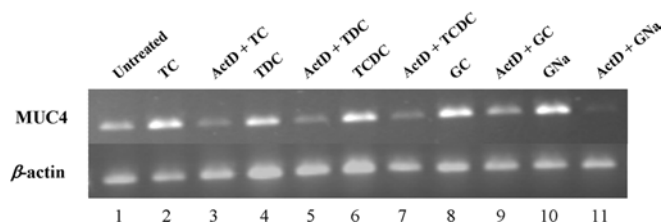
Figure 4   Effect of actinomycin D pretreatment on *MUC4* regulation by bile acids in OE33 cells

Cells were pretreated with actinomycin D (ActD) for 30 min before a 24 h bile acid incubation. *MUC4* and β-actin mRNA levels were studied by RT–PCR and compared with cells incubated with bile acids only. PCR products (15 and 4 μl respectively) were analysed on a 1.5 % agarose gel. GC, glycocholic acid.

or HNF4α (results not shown). *In vivo* binding of HNF1α to the chromatin region covering the HNF1α *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 HNF1α and HNF4α 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 HNF1α and HNF4α. Mutation of the T106 site in the − 3713/− 3059 region led to a 75 ± 4 % (HNF1α) and 50 ± 5 % (HNF4α) decrease in luciferase activity.

MUC4 regulation by bile acids occurs at the transcriptional level

Before studying the role of HNF1α in mediating *MUC4* activation by TC, TDC, TCDC, glycocholic acid 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 actinomycin D 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 pretreated with actinomycin D (Figure 4). This process did not require *de novo* protein synthesis since pretreatment of OE33 cells with cycloheximide did not modify the level of *MUC4* mRNA (results not shown).

HNF1α mediates MUC4 up-regulation by TDC and TCDC bile acids

To demonstrate that HNF1α 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). HNF1α knockdown led to a substantial decrease in *MUC4* mRNA (66 ± 5 % decrease, Figure 5A). We then focused our studies on TDC and TCDC since overexpression of HNF1α did not influence *MUC4* activation by TC, glycocholic acid or GNa (results not shown). Treatment of cells with TDC and TCDC enhanced the basal expression level of *MUC4* mRNA (1.8 ± 0.2 and 2.4 ± 0.6 times respectively) (Figure 5A). Knockdown of HNF1α strongly inhibited TDC- and TCDC-mediated increase in *MUC4* mRNA level (91 ± 2 % and 94 ± 3 % respectively). This indicates that HNF1α mediates endogenous *MUC4* expression in response to these bile acids. At the promoter level, we found that overexpression of HNF1α 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 HNF1α followed by full concentration of TDC (1 mM) or TCDC (0.05 mM) induced a strong activation of *MUC4* promoter (9.0 ± 0.6- and 4.7 ± 0.5-fold respectively). Moreover, studies performed with different concentrations of bile acids indicated that HNF1α 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 HNF1α-mediated up-regulation of *MUC4* promoter by TDC and TCDC. To this end, we compared *MUC4* promoter activity in cells co-transfected with HNF1α expression vector and wild-type pGL3-*MUC4* constructs followed by TDC or TCDC treatment with that in cells transfected under the same conditions with the mutated forms of *MUC4* promoter (Figure 6). The mediation by HNF1α 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 HNF1α decreased for more than a half (70 ± 6 % for TDC and 60 ± 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 HNF1α 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 results 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 under *in vivo* conditions [28] and that TDC is a secondary bile acid found in higher concentration in patients with Barrett's oesophagus compared with 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, glycocholic acid and GNa bile acids in OA cells involved phosphoinositide 3-kinase signalling pathway and to a lower extent mitogen-activated protein kinase, protein kinase A and protein kinase C pathways [15]. In the present study, we show that induction of MUC4 expression may occur via an alternative pathway involving HNF1α, 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 which necessitates alternative pathways in order to increase the capacity of the oesophageal cancer cell to respond to bile acids. We could not show HNF1α mediation for DC, glycocholic acid and GNa, suggesting that other mechanisms are involved. Nuclear factor κB and transcription factors of the activating transcription factor-1/cAMP-response-element-binding protein family have been described to mediate phosphoinositide 3-kinase effects [29]. Further investigations will be needed to answer that question. Although HNF4α 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 cofactors as has already been shown for HNF4 [30]. Moreover, HNF4α has no functional role in mediating *MUC4* regulation by TDC and TCDC. Taken together, our *in vitro* and *ex vivo* results showing
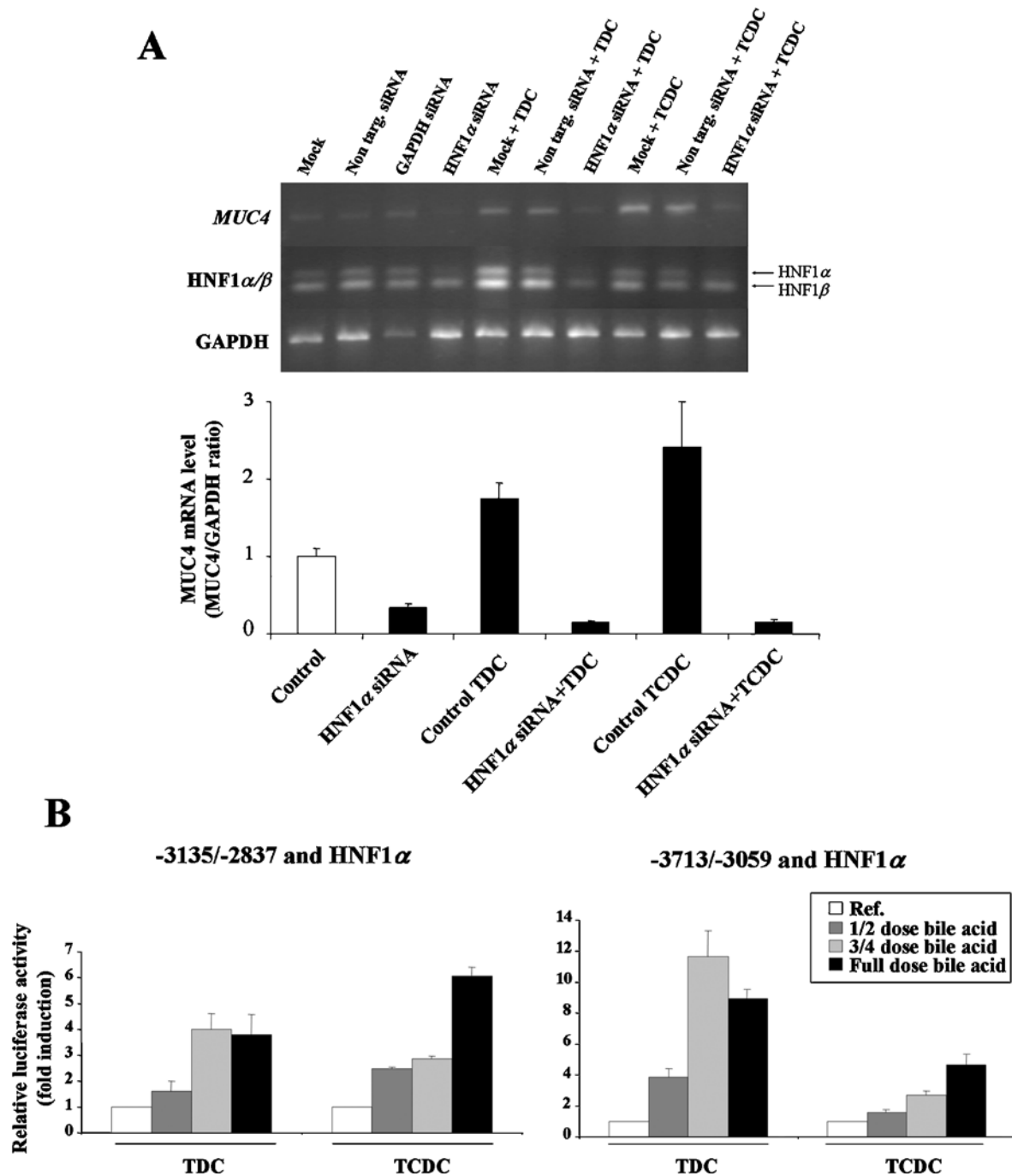
Figure 5   HNF1α-mediated up-regulation of *MUC4* transcription by TDC and TCDC is dose-dependent

(**A**) siRNA experiments were carried out as described in the Materials and methods section. *MUC4*, *HNF1α* and *GAPDH* mRNA levels were assessed by RT–PCR. PCR products (10 μl for *MUC4* and 5 μl for *HNF1α* and *GAPDH*) were analysed on a 1.5 % agarose gel. The lower panel shows results expressed as MUC4/GAPDH ratio. Control corresponds to the mean values 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 μg of pGL3-*MUC4* constructs −3135/−2837 or −3713/−3059 and 0.25 μg of pSG5-HNF1α expression vector. Cells were then incubated for 24 h with one-half, three-quarters or full concentration of TDC or TCDC (1 and 0.5 mM respectively). 'Ref.' refers to the normalized luciferase activity of pGL3-*MUC4* constructs co-transfected with HNF1α expression vector without bile acid treatment; this value was arbitrarily set to 1.

co-expression of HNF1α and MUC4 in Barrett's metaplasia and adenocarcinoma strongly suggest that HNF1α plays a major role in controlling MUC4 expression during oesophageal carcinogenesis.

HNF1α is a homoeodomain-containing transcription factor expressed in the liver, kidney, intestine, stomach and pancreas [31].

Inactivation of HNF1α has been associated with the development of human liver adenomas and some hepatocarcinomas, suggesting a potential role for HNF1α as a tumour suppressor gene in these diseases [32]. In the present study, we did not find loss of HNF1α expression during the oesophageal carcinogenetic sequence. On the contrary, expression was induced in Barrett's
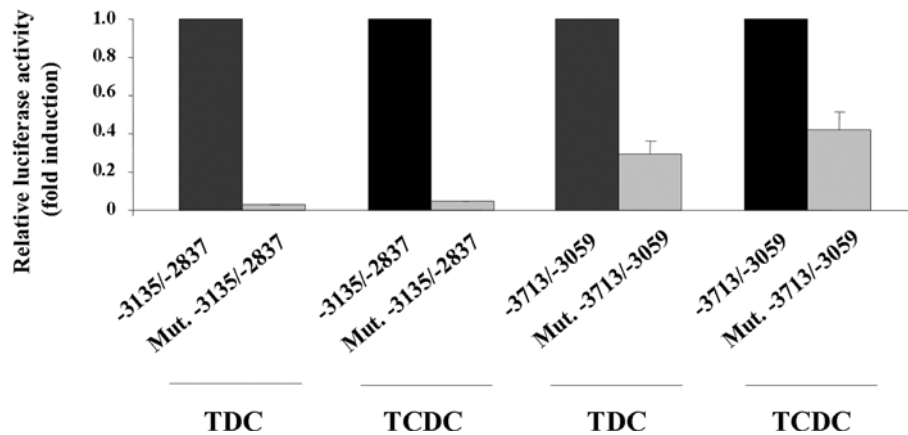
Figure 6   HNF1α *cis*-elements are directly implicated in *MUC4* regulation by TDC and TCDC

Co-transfection experiments in OE33 cells were performed in the presence of 1 μg of pGL3-*MUC4* constructs −3135/−2837, Mut. −3135/−2837, −3713/−3059 or Mut. −3713/−3059 and 0.25 μg of pSG5-HNF1α expression vector. Cells were then treated for 24 h with TDC or TCDC. The values obtained in cells transfected with wild-type pGL3-*MUC4* constructs were arbitrarily set to 1.

metaplasia and adenocarcinoma. This suggests that HNF1α plays a fundamental role in the progression of oesophageal carcinogenesis. Moreover, HNF1α is known to regulate the expression of several intestine-specific genes (lactase and 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 towards development of OA derived from Barrett's metaplasia, it may explain the strong HNF1α expression in Barrett's metaplasia mucosa with concomitant activation of MUC4. A quantitative study of the potential diagnostic value of HNF1α expression during the carcinogenetic sequence leading to oesophagus adenocarcinoma in a higher number of patients would be interesting to investigate.

HNF1α 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 HNF1α, indicating that HNF1α alone is sufficient to convey MUC4 activation via this site. Mutation of the other *cis*-element at −3332/−3327 inhibited HNF1α binding but transactivation was partially affected, suggesting probable involvement of a cofactor. Among transcription factors known to synergize with HNF1α [34] and for which we found putative binding sites in the distal promoter of *MUC4* [18] are Cdx-2 and GATA-4 (GATA-binding protein 4). Moreover Cdx transcription factors are also associated with OA developed on Barrett's metaplasia [38,39]. Preliminary results, however, did not suggest additive or synergistic effects between these three transcription factors in oesophageal OE33 cancer cells (G. Piessen and I. Van Seuningen, 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 justified their use as reliable phenotypic markers [40,41]. During the different steps of the OA carcinogenetic sequence, MUC4, a high-molecular-mass membrane-bound mucin, is overexpressed in the high-grade dysplasia state and in adenocarcinomatous tissues and is presently evaluated by us and other groups as a promising

early diagnostic tumour 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 overexpressed in 11–35 % 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 in the upper aerodigestive tract (good prognosis) [41] or in pancreatic adenocarcinoma (poor prognosis) [45] published in the literature. 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 the present 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 HNF1α 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 to identify potential molecular targets that could have a therapeutic value.

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