Transcriptional activation of the murine Muc5ac mucin gene in epithelial cancer cells by TGF-β/Smad4 signalling pathway is potentiated by Sp1

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Changes in the expression of mucin genes in gastrointestinal cancers is thought to contribute to the development of the disease. In our laboratory we have shown previously that MUC5AC is aberrantly expressed in rectosigmoid villous adenomas. However, the regulatory mechanisms underlying that altered profile of expression is unknown. In order to study its regulation at the transcriptional level, we have isolated and characterized 5.5 kb of the 5′-flanking region of the mouse Muc5ac mucin gene. The promoter is flanked by a TATA box and a transcriptional start site located 22 bp downstream of the TATA box. Analysis of the sequence showed a high density of binding sites for Smad4, an essential factor in the signalling cascade activated by TGF-β (transforming growth factor-β), and Sp1, an important factor in the regulation of MUC5AC. This led us to study Muc5ac regulation by TGF-β. We show that exogenous addition of TGF-β to the cells induces Muc5ac endogenous expression, promoter activity and Smad4 binding to the promoter. By co-transfection studies we show that Smad4 is essential for Muc5ac promoter activation and that it does not synergize with Smad2 or Smad3. By gel-retardation and co-transfection assays, we identified Sp1 and Sp3 as important regulators of Muc5ac expression and showed that Smad4 and Sp1 act in a co-operative manner to transactivate Muc5ac promoter activity. Altogether these results bring new insights into the molecular mechanisms of TGF-β-mediated up-regulation of Muc5ac and enhance our understanding as to how Muc5ac is regulated in certain pathologies of the gastrointestinal tract.

Key words: Muc5ac, mucin, Smad4, Sp1, transcription, transforming growth factor-β (TGF-β).

INTRODUCTION

Mucins have been postulated to be important molecules in maintaining epithelial homeostasis in inflammatory diseases and cancer. Mucins are large O-glycoproteins expressed either as transmembrane proteins at the cell surface or as secreted oligomeric molecules to form a protective gel [1–4]. In the gastrointestinal tract, they play a cytoprotective role against acid and pepsin in the gastric juice and against deleterious effects of exogenous agents (pathogens, drugs) and against mechanical damage [5,6]. MUC5AC belongs to the family of secreted mucins that participate in mucus formation and is encoded by a gene located on the p15 arm of chromosome 11 within a cluster of four mucin genes along with MUC2, MUC5B and MUC6 [7,8].

In normal adult, MUC5AC main territories of expression are the surface epithelium of the respiratory tract and stomach [9]. This expression is restricted to mucus-producing lung goblet cells and gastric pit cells. MUC5AC normal pattern of expression is altered in several epithelial diseases of the gastrointestinal tract. It is aberrantly expressed in Barrett’s oesophagus [10,11], in gastric metaplasia in the duodenum [12], in colon adenoma and cancer [6,13]. In rectosigmoid villous adenoma, MUC5AC expression was found very early during the carcinogenetic sequence, in low-grade dysplasia, which makes it a valuable marker for recurrent patients [14]. Despite increasing amounts of data regarding its expression pattern in normal human tissues compared with that in disease [6,15], the precise biological role of MUC5AC as a key gene during sequential steps of carcinogenesis or in inflammatory processes has yet to be proven. Moreover, the molecular mechanisms that govern MUC5AC expression in the gastrointestinal tract are still largely unknown, thus their identification is necessary if one wants to better understand the role of MUC5AC in the pathologies of the gastrointestinal epithelium.

TGF-β (transforming growth factor-β) is a member of the superfamily of cytokines that affect a variety of cell types and elicit a wide array of cell-type-specific biological effects such as differentiation, migration, cell-cycle arrest, adhesion, extracellular matrix production and apoptosis [16]. TGF-β is also an agent involved in gastritis and development of gastric cancer [17]. The two pathologies in which MUC5AC expression is altered. TGF-β-induced signalling occurs when the TGF-β ligand binds to the type II receptor (TGF-β RII), which heterodimerizes with the type I receptor (RI). RI then phosphorylates receptor-activated Smads (Smad2, Smad3). Once activated Smad2 and Smad3 bind to Smad4 and this complex translocates to the nucleus whereupon transcription activation of the target gene occurs [18].

Recent isolation of Muc1 and Muc2 murine mucin genes as well as their regulatory regions has helped a great deal in defining their biological roles in vivo and the molecular mechanisms responsible for their regulation. The studies performed on murine Muc1 mucin gene, which encodes a transmembrane mucin, and on Muc1−/− mice showed that it is overexpressed in most carcinomas, correlates with high metastatic potential and poor survival and participates in tumour progression [19]. More recently, the sequence of the pMS1 clone was submitted to the GenBank Nucleotide Sequence Database under accession number AF288076.

Abbreviations used: EMSA, electrophoretic mobility shift assay; RT-PCR, reverse transcriptase PCR; TBST, Tris-buffered saline/0.2 % Tween 20; TGF-β, transforming growth factor-β; RACE, rapid amplification of cDNA ends; PAS, periodic acid–Schiff; NF-κB, nuclear factor κB; AP-1, activator protein 1; UTR, untranslated region.

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promoter region of murine Muc2, which belongs to the secreted mucins, was characterized [20]. The authors showed, as for its human counterpart and other mucin genes, that Sp1 is an important factor in Muc2 regulation [20]. Interestingly, the knockout mice for Muc2 mucin gene allowed the authors to demonstrate for the first time a direct link between Muc2 expression and tumour formation, as Muc2+/− mice started to develop intestinal adenomas that progressed to invasive adenocarcinoma and colorectal tumours as they were getting older [21]. From this work, the authors concluded that Muc2 is involved in colon cancer and may be considered as a tumour-suppressor gene [21].

Murine Muc5ac mucin gene is partially characterized and part of its tandem repeat region was published by Shekels et al. [22]. Of interest, the authors showed that Muc5ac is located on murine chromosome 7; that is the syntenic chromosomal region corresponding to human chromosome 11. Thus it appears from this work and recent data released from the human and mouse genome databases (NCBI, MGI) that murine Muc2, Muc5ac, Muc5b and Muc6 are clustered on murine chromosome 7, which means that this cluster of mucin genes is conserved throughout evolution [23].

Our aim is to better understand the transcriptional regulation of human and murine mucin genes in order to propose new therapeutic targets in epithelial diseases (inflammation and cancer) and better understand their role during embryonic development and differentiation of the gastrointestinal epithelium [8]. In this study, we have isolated, characterized and studied the regulation of the 5′-flanking region of murine Muc5ac mucin gene in order to obtain a base for future studies in animal models [6] and better understand MUC5AC biological role in the pathophysiology of the epithelium. We show for the first time that Muc5ac is regulated at the transcriptional level by TGF-β, an agent involved in gastritis and development of gastric cancer.

**MATERIALS AND METHODS**

**Cloning and characterization of murine Muc5ac 5′-flanking region**

The 5′-flanking region of murine Muc5ac mucin gene was isolated and characterized after screening a murine OLA 129 genomic DNA library made in λGEM12 (kindly provided by Dr A. Berns, The Netherlands Cancer Institute, Amsterdam, The Netherlands) with a probe spanning 750 bp of the human MUC5AC N-terminus (GenBank accession no. AF043909) [24]. Two positive plaques were identified and the corresponding DNA was isolated from the bacteriophages by using the Lambda DNA isolation kit (Qiagen) according to the manufacturer’s protocol. Southern blot analysis and restriction mapping of both inserts identified a 5.5 kb fragment that hybridized with 750 bp of the human MUC5AC. The positive 5.5 kb fragment was subsequently cloned into the SstI site of pBluescript II SK(+-) (Stratagene), which resulted in clone pMS1. That fragment was then digested by Apal and PstI to raise smaller fragments pMS2-pMS7. The clones pMS1–pMS7 were sequenced by Eurogentec (Seraing, Belgium) using T3 and T7 primers, and in the laboratory using the DYEnamic ET Terminator Cycle Sequencing Kit, with fluorescently labelled nucleotides (Amersham Biosciences), according to the manufacturer’s protocol. Sequence reactions were analysed on an ABI-Prism 310 Genetic analyser (Perkin Elmer Applied Biosystems). The obtained sequences were aligned with the BioEdit sequence alignment editor. The derived 5.5 kb nucleotide and amino acid sequences were compared with known sequences using NCBI-Blast database. In addition, the sequence was analysed with PC/Genie software (IntelliGenetics, Mountain View, CA, U.S.A.), Infobiogen database and MatInspector V2.2 and Alibaba2 software based on the Genomatix database to determine the location of putative transcription factor binding sites [25]. The nucleotide sequence of pMS1 clone was submitted to GenBank under the accession no. AF288076.

**Determination of 5′-end of Muc5ac mRNA by RACE (rapid amplification of cDNA ends) PCR**

The transcription start site was determined by 5′-RACE using the 5′/3′ RACE kit (Roche Molecular Biochemicals) according to the manufacturer’s instructions. RACE PCR was conducted on mouse total gastric RNA (2 µg) using 5′-CAAGGAAGTAGAAGACCTGTCC-3′ as first primer and 5′-TGTCATTGCGTGCAGGCACG-3′ as nested primer for reverse transcription and amplification. Resulting amplified PCR products were cloned directly into the TA cloning vector (Invitrogen) and sequenced on an automatic LI-COR sequencer (ScienceTech, France) with T7 and RM13 primers as described below.

**Animals**

Adult specified-pathogen-free Balb/c mice, obtained from Harlan (Zoetermeer, The Netherlands), were killed by cervical dislocation. The stomach was removed and fixed in 4 % paraformaldehyde in PBS and subsequently processed for light microscopy as described previously [26]. The animal experiments were performed with the approval of the Animal Studies Ethics Committee of the Erasmus MC (Rotterdam, The Netherlands).

**Histology**

Sections of mouse stomach tissue (5 μm thick) were routinely stained with haematoxylin and eosin to study the morphology, or stained with Alcian Blue/PAS (periodic acid–Schiff) reagent to stain for acidic and neutral mucins, respectively. Immunolocalization of mouse Muc5ac was carried out as described previously [11] using 45M1 monoclonal antibody (Novocastra).

**Probe preparation for in situ hybridization**

Total RNA was extracted from murine stomach using TRIzol (Life Technologies) following the manufacturer’s protocol. RNA (1 µg) was transcribed at 42 °C into cDNA using Moloney murine leukaemia virus reverse transcriptase (Promega) in a total volume of 20 µl following the manufacturer’s protocol. This was followed by a PCR reaction using 1 µl of cDNA as template in 10 mM Tris/HCl buffer, pH 8.4, containing 50 mM KCl, 2 mM MgCl2, 0.01 % gelatin, 1 unit of Taq polymerase (Eurogentec), 0.2 mM dNTPs and 10 pmol of each primer. The primers were 5′-CCATGGCTAGATGCCAGT-3′ and 5′-AGATCCA-ACCCTCTCTCG-3′, which correspond to nucleotides 374–394 and 552–572 of murine Muc5ac (GenBank accession no. L42292) [22]. The PCR sample (20 µl) was first denatured at 96 °C for 5 min, followed by 30 cycles at 96 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min, with a final extension step at 72 °C for 2 min. The resulting 199 bp PCR product was isolated using the Qiagen gel extraction kit and ligated into the EcoRI site of pBluescript SK vector and subsequently sequenced. The digoxigenin-11-UTP-labelled sense and antisense Muc5ac RNA probes were prepared according to the manufacturer’s protocol (Roche Molecular Biochemicals).

**In situ hybridization**

Tissue sections were deparaffinized with xylene and rehydrated through RNase-free ethanol/water solutions. The non-radioactive
in situ hybridization was essentially carried out as described previously [27]. Briefly, the riboprobes were diluted in hybridization solution [50% deionized formamide (v/v), 10% dextran sulphate (w/v), 2 × SSC (where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate), 1 × Denhardt’s solution, 1 µg/ml tRNA and 250 µg/ml herring sperm DNA] to a concentration of 100 ng/ml, hybridized overnight at 55 °C in a humid chamber. Post-hybridization washes were performed at 45 °C using the following steps: 50% formamide (v/v) in 2 × SSC, 50% formamide (v/v) in 1 × SSC and 0.1 × SSC. A 15 min incubation with RNase T1 (2 units/ml in 5 × SSC) followed by washes of 0.1 × SSC at 45 °C and 2 × SSC at room temperature. The digoxigenin-labelled hybrids were detected by incubation with anti-digoxigenin (Fab; 1:2000) conjugated to alkaline phosphatase for 2.5 h at room temperature. Thereafter, sections were washed in 0.025% (v/v) Tween in Tris-buffered saline, pH 7.5. For staining, sections were layered with detection buffer (0.1 M Tris/HCl, pH 9.5, containing 0.1 M NaCl and 0.05 M MgCl₂) containing 0.33 mg/ml 4-Nitro Blue Tetrazolium chloride, 0.16 mg/ml 5-bromo-4-chloro-3-indolyl phosphate, 8% (v/v) polyvinyl alcohol (31 000–50 000 Da; Aldrich) and 1 mM levamisole (Sigma). Development of the reaction was performed overnight in the dark and was stopped when the desired intensity of the resulting blue precipitate was reached. Finally, sections were washed in distilled water and mounted with Aquamount improved (Gurr, Brunschwig, Germany).

Muc5ac-pGL3 deletion mutant constructions

The Muc5ac-pGL3 deletion mutants that cover 1.2 kb of the promoter were constructed into pGL3 Basic vector (Promega) using a PCR-based method as described previously [28,29]. PCR reactions were carried out on pMS1 clone in order to subclone the promoter region of Muc5ac. PCR products were then subcloned into pCR2.1 vector (Invitrogen) before subcloning into SacI–MluI sites of the promoterless pGL3 Basic vector. Internal deletion mutants were generated by PCR using pairs of primers bearing specific restriction sites at their 5′ and 3′ ends (Table 1). PCR products were digested, gel-purified (Qiagel quick gel extraction kit; Qiagen) and subcloned into the pGL3 Basic vector that had been previously cut with the same restriction enzymes. All clones were sequenced on both strands on an automatic LI-COR sequencer using infrared-labelled RV3 and GL2 primers (Promega). Plasmids used for transfection studies were prepared using the Endofree plasmid Mega kit (Qiagen).

Cell culture

Murine rectal cancer cell line CMT-93 was a kind gift of Dr D. Podolsky (Massachusetts General Hospital, Boston, MA, U.S.A.). CMT-93 cells were cultured in Dulbecco’s modified essential medium containing 10% fetal bovine serum, 4 mM L-glutamine, penicillin (100 units/ml) and streptomycin (100 µg/ml). IEC-6 cells were purchased from the ECACC (European Collection of Animal Cell Cultures). This cell line was established from rat small-intestine crypt cells and was cultured in Dulbecco’s modified essential medium containing 5% fetal bovine serum, 2 mM L-glutamine, 10 µg/ml insulin, 50 units/ml penicillin and 50 µg/ml streptomycin. HCT116-Smad4⁺/⁻ and HCT116-Smad4⁻/⁻ were a kind gift of Dr A. Atfi (INSEMM U482, Paris, France). Cells were cultured in Dulbecco’s modified essential medium supplemented with 2 mM glutamine and 10% fetal calf serum. Human gastric cancer cell line KATO-III was cultured as described previously [30]. All cells were cultured at 37 °C in a humidified 5% CO₂ water-jacketed incubator. To study TGF-β effect, cells were incubated for 24 h with TGF-β (recombinant human TGF-β; 10 ng/ml). All reagents were from Sigma unless otherwise indicated.

RT-PCR (reverse transcriptase PCR)

Total RNAs from cultured cells and mouse tissues were prepared using the QiAamp RNA blood mini-kit and midi-kit (Qiagen), respectively. Total RNA (1.5 µg) was used to prepare first-strand cDNA (Advantage™ RT-for-PCR kit; Clontech). PCR was performed on 2 µl of cDNA using specific pairs of primers as follows: Muc5ac forward primer, 5′-GAGGGCCCATGGA-GCATCCTCC-3′; Muc5ac reverse primer, 5′-TGGGACAGCAGCAGTATTACGT-3′ (accession number AJ010792). β-Actin was used as an internal control: mouse β-actin forward primer, 5′-TGGGCGCGCTCTAGGCCACCA-3′; mouse β-actin reverse primer, 5′-TGCCTTAAGGGTGAGGCGG-3′ (accession number M12481). Muc5ac and β-actin PCR product sizes are 361 and 241 bp, respectively. Mouse TGF-βRII forward primer, 5′-CTGGTGAGAGAGAAACACACAA-3′; reverse primer, 5′-TCCTCAAACTGCCCTTGGAGTGG-3′ (accession number S69114). The PCR product was 560 bp long. Rat β-actin forward primer, 5′-ATATCGCTGCGCTGCTGCTGAGAA-3′; rat β-actin reverse primer, 5′-AACACAGCTGGATGCGTACAT-3′ (accession number V01217). PCR reactions were carried out in 50 µl final solutions as described in [31]. Annealing temperature was 58 °C. PCR products were analysed on 1.5% ethidium bromide-stained agarose gels run in 1 × Tris/borate/EDTA buffer. A 100 bp DNA ladder was purchased from Amersham Biosciences.

Transfections

Transfections and co-transfections experiments were performed using Effectene® reagent (Qiagen) as described previously using 1 µg of Muc5ac-pGL3 deletion mutants [28]. Total cell extracts were prepared after a 48 h incubation at 37 °C using 1 × Reagent Lysis Buffer (Promega) as described in the manufacturer’s instruction manual. Luciferase activity (20 µl) was measured on
a TD 20/20 luminometer (Turner Design). Total protein content in the extract (4 µl) was measured using the bicinchoninic acid method in 96-well plates as described in the manufacturer’s instruction manual (Pierce, Bezons, France). In co-transfection experiments, 1 µg of the deletion mutant of interest was transfected with 0.25 µg of the expression plasmid encoding the transcription factor of interest. Results were expressed as fold activation of luciferase activity in samples co-transfected with the control co-transfected with the corresponding empty vector.

**Nuclear extract preparation**

Nuclear extracts from the different cells were prepared as described by Van Seuningen et al. [32], and kept at −80 °C until use. Protein content (2 µl of cell extracts) was measured using the bicinchoninic acid as described above.

**Western blotting**

Nuclear proteins (20 µg) and prestained molecular-mass markers (8 µl; Life Technologies) were loaded on a SDS/PAGE gel (10%) and electrophoresed for 1 h 15 min at 35 mA until Bromophenol Blue reached the bottom of the gel. The polyacrylamide gel was then electrotransferred on a 0.45 µm PVDF membrane (Millipore) for 30 min at room temperature with TBST and for 5 min with substrate buffer (0.1 M phosphate-citrate buffer, 0.05 M MgCl2, pH 9.5, containing 0.1 M NaCl and 0.05 M MgCl2).

**Oligonucleotides and DNA probes**

The sequences of the oligonucleotides used for gel-shift assays are indicated in Table 2. They were synthesized by MWG-Biotech (Ebersberg, Germany). Equimolar amounts of single-stranded oligonucleotides were annealed and radiolabelled using T4 polynucleotide kinase (Promega) and [γ-32P]dATP. Radiolabelled probes were purified by chromatography on a Bio-Gel P-6 column (Bio-Rad).

**EMSA (electrophoretic mobility shift assay)**

Incubation of nuclear proteins (8 µg) with radiolabelled probes, supershift analyses and unlabelled competition (‘cold competition’) experiments were carried out as in [31]. Anti-Sp1, anti-Sp3, anti-Smad2, anti-Smad4 and anti-NF-xB (nuclear factor xB) p65 antibodies and consensus Smad4 oligonucleotide were purchased from Santa Cruz Biotechnology (Tebu, France). Reactions were stopped by adding 2 µl of loading buffer. Samples were then electrophoresed on a 4% non-denaturing polyacrylamide gel and electrophoresis conditions were as described in [29]. Gels were vacuum-dried and autoradiographed overnight at −80 °C.

Table 2 Sequences and positions within the promoter of Muc5ac of the sense oligonucleotides used for EMSAs

<table>
<thead>
<tr>
<th>Probe Position of putative binding site</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>−57/−34 Spl (−51/−42)</td>
<td>5′-GTGCTGGGGCTGAGCCAGCTCT-3′</td>
</tr>
<tr>
<td>−83/−56 Spl/CACCC (−76/−65)</td>
<td>5′-AGAACCTGCTCCACCCACCCACAGCTGAAG-3′</td>
</tr>
<tr>
<td>−113/−81 Spl (−100/−90)</td>
<td>5′-GCAACCTTATCCGAGGGAGAACCAAGAGA-3′</td>
</tr>
<tr>
<td>−92/−71 Smad (−84/−80)</td>
<td>5′-GGGACCCAGAACCCCTGCAC-3′</td>
</tr>
<tr>
<td>−467/−444 Smad (−458/−454)</td>
<td>5′-CTGCTGCCATCCAGACGACGCGAA-3′</td>
</tr>
<tr>
<td>−552/−529 Smad (−539/−534)</td>
<td>5′-CCTGCTGCTGCAACAGGGTACGC-3′</td>
</tr>
<tr>
<td>−645/−622 Smad (−641/−637)</td>
<td>5′-CCCTGCTGCCACAGGGTACGC-3′</td>
</tr>
<tr>
<td>−1146/−1126 Smad (−1131/−1128)</td>
<td>5′-GCCACTGCTGCCACAGGGTACGC-3′</td>
</tr>
</tbody>
</table>

Probe Position of putative binding site Sequence

**RESULTS**

**Cloning and characterization of murine Muc5ac mucin gene 5′-flanking region**

In order to obtain the mouse Muc5ac 5′-flanking region, a mouse genomic library in λGEM12 was screened with a 750 bp DNA fragment encoding the N-terminal region of human MUC5AC [24]. The two positive plaques contained inserts of approx. 18 kb. After restriction mapping and Southern blot analysis, seven clones (pMS1-pMS7) were isolated and sequenced (see the Materials and methods section). Sequencing of the 5.5 kb pMS1 clone indicated that it contains the promoter region, the first three exons and the first three introns of the mouse MUC5AC gene (Figure 1B).

**Determination of the transcription start site of Muc5ac**

To determine the transcription start site, 5′-RACE assays were performed on mouse gastric RNA using two nested antisense primers selected at about 300 and 70 bp downstream of the presumed start codon, respectively, based on comparison with the human MUC5AC gene [33]. The sequences showed 100% homology when aligned with the 5′-flanking sequence of mouse Muc5ac. The 5′-end of the longest product (117 bp) corresponded to a thymidine residue located 33 nucleotides upstream of the first ATG and 22 nucleotides downstream of the TATA box (Figure 2).
Figure 1  Isolation and characterization of a 5.5 kb genomic DNA fragment containing the 5′-flanking region of murine Muc5ac mucin gene
(A) Schematic representation of the organization of the 5′-flanking region of Muc5ac gene showing the promoter, the first three exons, ATG and transcription initiation site locations. (B) Alignment of deduced N-terminal amino acid sequences of mouse (mMuc5ac), rat (rMuc5ac) and human (hMuc5ac) MUC5AC peptides. Black boxes indicate conserved amino acid residues in the three species and grey boxes amino acid residues identical in two different species. The deduced consensus sequence is indicated at the bottom.

Figure 2  Sequence of the 5.5 kb genomic fragment containing the Muc5ac promoter
The transcription start site +1 (bold and underlined) is located 22 nucleotides downstream of the TATA box (underlined). The first ATG (+34) is bold, italicized and underlined. Nucleotides representing the first exon are italicized and underlined. Grey boxes indicate putative binding sites for transcription factors and boxed sequences indicate the sequences of oligonucleotides used in gel-shift assays. Arrows delineate the sequence of the deletion mutants used in this study.

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In conclusion, the Muc5ac transcription start site is a T residue located 22 nucleotides downstream of the TATA box.

Characterization of the promoter sequence of Muc5ac

The sequence found upstream of the transcription initiation site is characterized by the presence of a TATA box (TACAAAA) at −28/−22 (Figure 2). The first 150 nucleotides upstream of the TATA box are rich in GC and CACCC boxes and Sp1 binding sites. Putative binding sites for Smad factors are present throughout the promoter sequence. It is interesting to note that the Smad sites are always found in close vicinity or embedded within Sp1-binding sites and/or GC-rich sequences. More upstream (−944/−938) is found an AT-rich sequence that is a putative site representative of a TATA box. Note that this sequence may also bind the CdX transcription factor. Two putative binding sites for GATA factors were found at −1001/−998 and −774/−771. Consensus binding sites for retinoid [ATF (activating transcription factor)/RXRa (retinoid X receptor), −837/−832] or thyroid hormone [T3R-α (thyroid hormone receptor α), −634/−626] receptors were also found. In the 5′-UTR region, which is 33 nucleotides long, putative binding sites for AP-1 (activator protein 1) and Sp1 were found at +2/+10 and +14/+23, respectively. In the first exon, a putative binding site for the YY1 transcription factor is found at +47/+56 adjoining an Sp1 binding site at +54/+63.

Expression of Muc5ac in mouse tissues

Expression of Muc5ac mRNA in mouse tissues was studied by RT-PCR and in situ hybridization. By RT-PCR, Muc5ac expression is only seen in stomach (Figure 3A). No expression was found in submaxillary glands, parotid glands, trachea, thymus, gallbladder, liver, small intestine, colon or kidney. In situ hybridization was performed to localize Muc5ac expression in the stomach. Alcian Blue/PAS staining of acidic mucus was found in mucus granules of surface epithelial cells (Figure 3C). Labelling with Muc5ac antisense probe showed that Muc5ac mRNA is only expressed in the surface epithelium of the stomach (Figure 3D). The specificity of the labelling was confirmed by the absence of signal when using a sense probe (Figure 3E) and by absence of signal in colon when using the antisense probe (Figure 3F). Immunohistochemical staining of a mouse gastric mucosa with 45M1 monoclonal anti-MUC5AC antibody confirmed the expression of Muc5ac mucin in surface gastric epithelial cells (Figure 3G). In conclusion, Muc5ac expression in normal mice is restricted to the surface epithelium of the stomach.

Characterization of Muc5ac promoter activity

To study Muc5ac promoter activity we used two Muc5ac-expressing (CMT-93, IEC-6) and one Muc5ac-non-expressing (KATO-III) cell lines. Expression of Muc5ac in the murine rectal cancer cells (CMT-93, lane 3) and in the rat intestinal cell line (IEC-6, lane 6) is shown in Figure 4(A). Absence of Muc5AC expression in KATO-III cells was reported previously [8]. To define essential regions that drive transcription of Muc5ac promoter, 10 deletion mutants that cover 1.2 kb of the promoter were constructed in the promoterless pGL3 Basic vector (Figure 4B). Numbering refers to the transcription start site designated as +1. Deletion mutants −1021/−828 and −1171/−828 were made in order to check the functional activity of the distal TATA box found at −944/−939.

The luciferase results obtained in the three cell lines indicate that Muc5ac promoter activity is the strongest in Muc5ac-expressing cell lines (Figure 4C). In CMT-93 and IEC-6 cells, the highest luciferase activity was obtained with fragment −199/+3 (17−20-fold activation), which indicates that this region possesses essential positive regulatory elements that confer maximal activity to the promoter. In CMT-93 cells, the luciferase activity gradually decreases as the constructs include longer portions of the promoter's distal region. In IEC-6 cells, a strong decrease in luciferase activity is seen with fragment −376/+3 (6-fold activation) and this decrease is maintained in fragments −1021/+3 and −1171/+3. This indicates that inhibitory elements, active in IEC-6 cells, are present within the −376/−200 region of the promoter. Interestingly, one can note that the fragments, in which the +3/+132 region was added, have a decreased luciferase activity of about 50 to 100%. The inhibitory activity was found in the three cell lines. That region includes the 5′-UTR (untranslated region) and first exon in which a binding site for YY1 repressor (+47/+56) is clustered between two Sp1-binding sites at +29/+38 and +54/+63, respectively. The −1021/+3 and −1171/+3 mutants that contain the distal TATA box located at −944/−939 are not active in the cell lines tested. In conclusion, essential regulatory elements for the basal activity of the promoter are found within the −199/+3 proximal region of Muc5ac promoter and negative elements are present within the 5′-UTR.

Figure 3 Expression of Muc5ac in mouse tissues by RT-PCR, in situ hybridization and immunohistochemistry

(A) Muc5ac (10 µl) and β-actin (2 µl) PCR products were separated on a 1.5 % agarose gel. In situ hybridization (B–F) and immunohistochemistry (G) studies. (B) Haematoxylin and eosin staining of gastric mucosa. (C) Alcian Blue and PAS staining of gastric mucosa. (D) Staining of gastric mucosa with Muc5ac digoxigenin-labelled antisense RNA probe. (E) Staining of gastric mucosa with Muc5ac digoxigenin-labelled sense probe. (F) Staining of colon mucosa with Muc5ac digoxigenin-labelled antisense probe and (G) Immunostaining of Muc5ac in gastric mucosa with monoclonal 45M1 antibody. Magnification, ×200.
Regulation of Muc5ac promoter by TGF-β and Smads transcription factors

The high number of putative binding sites for Smad transcription factors within the Muc5ac promoter (see Figure 2) supports a regulatory role for TGF-β on Muc5ac promoter activity. Smads are the main factors activated by TGF-β [18]. For this reason, and because it was previously shown that MUC5AC mucin gene expression is altered in inflammatory pathologies of the epithelium in which TGF-β is implicated [34], we undertook to study the regulation of Muc5ac promoter by TGF-β and Smad factors. TGF-β sends intracellular signals after binding to TGF-β RIIs on the cell membrane. Thus before studying TGF-β signalling pathway on Muc5ac expression in CMT-93 cells, we checked whether the cells expressed TGF-β RII. As shown in Figure 5(A), TGF-β RII mRNAs are highly expressed in CMT-93 cells. Treatment of CMT-93 cells with TGF-β substantially induced the amount of Muc5ac mRNA in the cells (Figure 5B). Identification of TGF-β-responsive elements within the Muc5ac promoter was then tested in transfection studies in which transfected cells were treated with TGF-β under the same conditions (Figure 5C). The luciferase result indicates that exogenous TGF-β induces the activity of the fragments covering the −1021/+3 region (2.0-, 2.7- and 1.8-fold, respectively). Induction was lost when the longest fragment (−1171/+3) was used. These results indicate that TGF-β-responsive elements are present within the −1021/+3 region of the promoter of Muc5ac, which contains seven putative Smad-binding sites (CAGAC).

In order to identify Smad-binding sites, EMSAs were performed with double-stranded oligonucleotides representative of the Smad-binding sites found at −538/−534, −641/−637, −1131/−1128, −458/−454 and −84/−80, respectively (Table 2). Incubation of the radiolabelled probes with CMT-93 nuclear extracts resulted in one shifted band characteristic of Smad–DNA complexes (Figure 6A, lanes 2, 7 and 12) when compared with the mobility of the Smad4 consensus radiolabelled probe (Figure 6A, lane 17). The specificity of the complexes were confirmed by total disappearance of the shifted bands when unlabelled competition was performed (Figure 6A, lanes 3, 8, 13 and 18). Involvement of Smad4 in the DNA–protein shifted complex was then proven by inhibition of complex formation upon addition of Smad4 antibody (Figure 6A, lanes 5, 10 and 14), whereas no effect was observed upon addition of Smad2 antibody (Figure 6A, lanes 4, 9, and 15). The same result was obtained with −552/−529 and −92/−71 probes (results not shown). Induction of Smad4 binding by TGF-β was then tested on nuclear extracts from TGF-β-treated cells. Increase of Smad4 binding to the Smad cis-elements was indeed observed with nuclear extracts from TGF-β-treated cells (Figure 6A, lane 21) when compared with untreated cells (Figure 6A, lane 20). These
studies indicate that Smad4 binds to five cognate cis-elements at −84/−80, −458/−454, −538/−534, −641/−635 and −1131/−1128 within the TGF-β-inducible region of Muc5ac promoter.

In order to show whether the TGF-β-responsive region that binds Smad4 is indeed regulated by Smad factors, we then performed co-transfection experiments in which Muc5ac-pGL3 deletion mutants were co-transfected with Smad4 alone or in combination with expression vectors encoding Smad2 or Smad3, which are co-factors of Smad4. As shown in Figure 6(B), Smad2 induces Muc5ac promoter activity with the strongest effect on fragment −376/+3 (10-fold). Smad3 transactivation of Muc5ac promoter is confined to the −376/+3 region of the promoter and is not as strong (4–5-fold activation, fragments −199/+3 and −376/+3). The transactivating effect of Smad3 is lost when co-transfected with longer fragments of the promoter (−1021/+3, −1171/+3). Smad4, as expected, strongly transactivates Muc5ac promoter activity throughout the sequence (12–22-fold activation). Co-transfection experiments in the presence of Smad2 and Smad4 or Smad3 and Smad4 did not lead to synergistic activation of the promoter (results not shown). The involvement of Smad4 in up-regulating Muc5ac transcription was then confirmed by comparing Muc5ac promoter activity in a cell line that either constitutively expresses active Smad4 (HCT116-Smad4+/+) or is mutated for Smad4 (HCT116-Smad4−/−; Figure 6C). The luciferase diagram shows that Muc5ac promoter activity (fragment −1021/+3) is three times more active in cells expressing Smad4 (Figure 6C, black bars) compared with cells mutated for Smad4 (Figure 6C, white bars). The same result was obtained with fragment −376/+3 (results not shown).

From these studies, it can be concluded that Smad4 is an activator of Muc5ac transcription, that TGF-β-responsive Smad4-binding sites are present throughout the promoter and that Smad2 and Smad3 factors do not act in synergy with Smad4 to induce Muc5ac transcription.

Role of Sp1 and Sp3 in the regulation of Muc5ac promoter

Since we showed that Smad2 and Smad3 are not the partners of Smad4 to activate Muc5ac transcription, we looked for other partners. Interestingly, when we looked at the location of the Smad4-binding sites in the promoter, we noticed that they were often either embedded in or neighbouring Sp1 cis-elements, and those transcription factors are known to synergize to activate transcription of many genes. Before testing the synergistic effect between Smad4 and Sp1, we studied the regulation of Muc5ac promoter by Sp1 and Sp3, since the proximal region is GC-rich and Sp1/Sp3 are important regulators of mucus gene expression [8].

EMSAs studies were performed with nuclear extracts from CMT-93 cells in which we checked the presence of Sp1 (96 kDa) and Sp3 (two isoforms, 100 and 60 kDa) proteins by Western blotting (Figure 7A). Three double-stranded radiolabelled probes (Table 2), each containing a putative Sp1 binding site (−113/−81, −57/−34) and CACCC boxes (−83/−56), were tested. Incubation of the −113/−81 probe with CMT-93 nuclear proteins did not produce any shift. Incubation with −57/−34 probe produced a strong retarded band that could not be supershifted in the presence of anti-Sp1 or anti-Sp3 antibodies (results not shown). On the other hand, incubation of the −83/−56 probe (Figure 7B), which contains CACCC box-binding sites, with CMT-93 nuclear proteins produced three shifted complexes (Figure 7B, lane 2, arrows). Specificity of these complexes was confirmed by complete inhibition of complex formation when unlabelled competition was performed with a 50× excess of the unlabelled probe (Figure 7B, lane 3). Complex 1 was completely supershifted upon addition of specific anti-Sp1 antibody in the reaction mixture (Figure 7B, lane 4, SS Sp1), whereas complex 2 was specifically supershifted upon addition of anti-Sp3 antibody (Figure 7B, lane 5, SS Sp3). No supershift was observed upon addition of irrelevant NF-κB p65 antibody (Figure 7B, lane 6). In conclusion, Sp1 and Sp3 bind to the same cis-element located at −76/−65 within the Muc5ac proximal promoter.

To study the role of Sp1 and Sp3 in regulating Muc5ac transcription, co-transfection experiments were carried out in CMT-93 cells in the presence of an expression vector encoding either Sp1 (Figure 7C, black bars, pCMV4-Sp1) or Sp3 (Figure 7C, white bars, pCMV4-Sp3). Sp1 strongly transactivates both the proximal (fragments −199/+3 and −376/+3, 8–9-fold activation) and distal (fragments −1021/+3 and −1171/+3, 10–15-fold activation) regions of the promoter. Addition of the 5′-UTR to the promoter construct −1171/+3 (construct −1171/+3) inhibited the transactivating effect of Sp1. Sp3 only has a mild transactivating effect on the distal part of the promoter (2–3-fold activation). Again, addition of the 5′-UTR inhibited that effect. Altogether, these studies indicate that Sp1 is a strong transactivator of Muc5ac promoter activity in CMT-93 cells.

Co-operation between Sp1 and Smad4 to activate Muc5ac promoter

Having shown that both Smad4 and Sp1 were strong activators of Muc5ac transcription in CMT-93 cells, we then undertook to look at their co-operative effect on the promoter. Co-transfections in the presence of Smad4 and Sp1 were performed on fragments −199/+3, −376/+3 and −1171/+3 (Figure 8). No synergistic effect was seen on the shortest fragment −199/+3

Figure 5 Regulation of Muc5ac mRNA expression and promoter activity by exogenous TGF-β in CMT-93 cells

(A) Expression of TGF-βR II in CMT-93 cells by RT-PCR. 10 μl of the PCR product was loaded. (B) Expression of Muc5ac mRNA by RT-PCR in untreated cells (control) and TGF-β-treated (TGF-β) cells. (C) Luciferase diagram showing effect of TGF-β on Muc5ac promoter activity. Results are expressed as fold activation of luciferase activity in samples treated with TGF-β. transcription. 

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Transcriptional regulation of murine Muc5ac mucin gene

Figure 6 Identification of Smad4-binding sites within the promoter of Muc5ac by EMSA and regulation of the promoter by the Smads by transient transfection

(A) Nuclear extracts from CMT-93 cells were incubated with radiolabelled DNA probes, −645/−622 (lanes 1–5), −1146/−1126 (lanes 6–10), −467/−444 (lanes 11–15) and consensus Smad4 (lanes 16–19) probes. Radiolabelled probes alone (lanes 1, 6, 11 and 16), nuclear extract incubated with radiolabelled probe (lanes 2, 7, 12 and 17), unlabelled competition with a 50× excess of unlabelled probe (lanes 3, 8, 13 and 18), supershift analysis by preincubating nuclear extract with 1 µl of anti-Smad2 (lanes 4, 9 and 15) or anti-Smad4 (lanes 5, 10, 14 and 19) antibodies before adding the radiolabelled probe. The arrow on the left indicates the position of the DNA–protein complex engaging Smad4. Lanes 20 and 21, Smad4–DNA complex formation with nuclear extracts from untreated (lane 20) and TGF-β-treated (lane 21) cells.

(B) Regulation of Muc5ac promoter by Smad2, Smad3 and Smad4 transcription factors in CMT-93 cells. Co-transfection experiments in the presence of pCMV-Smad2 (grey bars), pCMV-Smad3 (white bars) or pCMV-Smad4 (black bars) expression vectors.

(C) Luciferase activity of Muc5ac promoter in HCT116-Smad4−/− (white bars) and in HCT116-Smad4+/+ (black bars) cells.

In this paper, we have isolated and characterized the 5′-flanking region of the murine mucin gene Muc5ac, including the promoter and first three exons/introns. Analysis and alignment of the first three exons with its human and rat counterparts show a high degree of homology with rat Muc5ac (79%) whereas it shares less with human MUC5AC (52%). The first ATG is identical with rat Muc5ac (GenBank accession no. AB042530), which adds four amino acid residues (MLHS) when compared with the human MUC5AC N-terminal amino acid sequence [33]. The TATA box sequence (TACAAAA) is conserved throughout evolution, since both mouse (this study) and human TATA boxes are identical [33]. The sequence of the TATA box in rat Muc5ac gene is not known.

Overall, it can be stressed that the 5′ region of Muc5ac mucin gene is relatively well conserved between human, mouse and rat. The sequence of the TATA box in rat Muc5ac gene is not known. In this report we found that expression of murine Muc5ac gene and protein was very specific and restricted to the surface epithelial cells of the gastric mucosa, like that described previously for rat Muc5ac [37]. No expression was found in trachea. This latter result is in contrast with expression territories in humans in which MUC5AC is expressed in the surface epithelium of stomach but also in goblet cells of the tracheobronchial tract [9].

DISCUSSION

The organization, evolution and regulation of expression of the four 11p15 mucin genes, MUC2, MUC5AC, MUC5B and MUC6, have been studied extensively over the past few years [2,7,35]. In situ hybridization and promoter functional studies have led the investigators to the conclusion that these mucin genes are tightly regulated at the transcriptional level [8]. They often harbour an altered profile of expression in human tumours of the respiratory, gastrointestinal and urogenital tracts [2,5,6,36] and such alterations (overexpression, repression, neo-expression) are usually characteristic of genes playing important roles in carcinogenesis. In order to study mucin gene regulation in animal models, isolation of the 5′-flanking regions including promoter of the murine counterparts are now mandatory. The 5′-flanking region of the murine mucin gene Muc5ac, including the promoter and first three exons/introns, is conserved throughout evolution, since both mouse (this study) and human TATA boxes are identical [33]. The sequence of the TATA box in rat Muc5ac gene is not known. Overall, it can be stressed that the 5′ region of Muc5ac mucin gene is relatively well conserved between human, mouse and rat and shows typical features of genes with a cell-specific profile of expression. Indeed, in this report we found that expression of murine Muc5ac gene and protein was very specific and restricted to the surface epithelial cells of the gastric mucosa, like that described previously for rat Muc5ac [37]. No expression was found in trachea. This latter result is in contrast with expression territories in humans in which MUC5AC is expressed in the surface epithelium of stomach but also in goblet cells of the tracheobronchial tract [9].

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Figure 7 Regulation of Muc5ac promoter by Sp1 and Sp3 transcription factors in CMT-93 cells: identification of an Sp1 cis-element in the promoter by EMSA

(A) Western blotting of nuclear proteins prepared from CMT-93 cells. Prestained molecular-mass markers. Immunostaining of the membrane with polyclonal PEP-2 anti-Sp1 (Sp1) and with polyclonal anti-Sp3 (Sp3) antibodies. (B) Identification of an Sp1 cis-element by EMSA. Nuclear extracts from CMT-93 cells were incubated with the −83/−56 radiolabelled DNA probe. Radiolabelled probe alone (lane 1), incubation of −83/−56 probe with CMT-93 nuclear proteins (lane 2), unlabelled competition with 50× excess of unlabelled probe (lane 3), supershift analysis upon addition of anti-Sp1 (lane 4) or anti-Sp3 (lane 5) antibodies. Lane 6, addition of irrelevant NF-κB p65 antibody. DNA–protein complexes are indicated by arrows on the left. Asterisks indicate the positions of Sp1 (SS Sp1) and Sp3 (SS Sp3) supershifted complexes. A longer exposure of the upper part of the same autoradiogram is shown at the bottom. (C) Co-transfection experiments in the presence of pCMV-Sp1 (black bars) or pCMV-Sp3 (white bars) expression vectors. Ref. refers to the normalized luciferase activity of the pGL3 deletion mutants of interest co-transfected with the empty expression vector pCMV4. Results are the means ± S.D. obtained in triplicate from three separate experiments.

epithelium of their conducting airways mostly consists of ciliated cells (upper tract) and Clara cells (lower tract), whereas mucus cells remain rare [38].

Analysis of the promoter nucleotide sequence immediately upstream of the TATA box (over the first 199 nucleotides) shows that it is GC-rich and bears numerous putative consensus Sp1-binding sites or CACCC boxes also known to bind transcription factors of the Sp family [8]. Consistent with a role in cancer for factors of the Sp family is their ability to be oncogenic themselves or to interact with oncogenes or tumour suppressors [39]. The high GC content of proximal region of promoters is a common feature in mucin genes, as it was also found in human MUC2, MUC5AC, MUC5B and murine Muc2 promoters [8,20,30,40], and functional studies pointed to an important role for the transcription factors of the Sp family as mucin gene regulators. The fact that these cis-elements are conserved between mouse and human MUC5AC genes is also in favour of an important role for Sp1 in MUC5AC transcriptional regulation. Here, we confirmed that hypothesis by means of co-transfection experiments in which we showed that Sp1 strongly transactivates the promoter of Muc5ac. Having previously shown that Sp3 interferes with Sp1-mediated regulation of human MUC5AC promoter [28], we studied its effect on murine Muc5ac promoter as well. Our results indicate that Sp3 is also a regulator of Muc5ac transcription but with a weaker effect. Since Sp1 and Sp3 are ubiquitously expressed in the cells and recognize the same DNA motif, it also implies that Sp1 and Sp3 will compete when they are both present in equimolar amounts in the cell.

Interestingly, Muc5ac promoter activity was repressed by 50% when pGL3 constructs contained the 5′-UTR (33 nucleotides long) and part of the first exon. Analysis of the sequence indicated that AP-1 and Sp1 binding sites are present within the 5′-UTR. Those factors are generally considered as activators of the transcription and could not be responsible for the repression observed. In close vicinity of the Sp1-binding site was found a second Sp1 cis-element overlapping a YY1-binding site. YY1
possesses dual activity (repressor or activator), depending on the molecular context and is known to interact with Sp1 [41]. As it was recently shown for hamster Muc1 mucin gene [42], YY1 may repress Muc5ac transcription via interactions with Sp1.

The Muc5ac promoter contains binding sites for the Smad4 transcription factor throughout its sequence. Alignment of human [33] and mouse (this study) sequences of the promoters indicates that Smad putative sites are present in both genes but not at the same location. The response of the two genes to TGF-β is different and seems to depend on the cellular situation as it was recently shown that human MUC5AC is negatively regulated when non-typeable *Haemophilus influenzae*-infected cells are treated with TGF-β [43]. Smad transcription factors are activated by growth factors of the TGF-β family in a sequential manner [18] and form either Smad2–Smad4 or Smad3–Smad4 complexes that are translocated into the nucleus where they bind to the promoter of the target gene to activate transcription. However, once bound to the promoter, Smad4 may interact with other factors to activate transcription. Our results indicate that Muc5ac is a target gene of TGF-β. However, they also suggest that the pathway induced by TGF-β does not imply complex formation between Smad4 and Smad2 or Smad4 and Smad3. Apart from activating Smad factors, TGF-β is also known to activate Sp1 site-dependent transcription of its target genes, Smad factors are known to activate transcription through Sp1 sites and Sp1 interacts with Smad4 to induce transcription and this mechanism is TGF-β-dependent [39,44,45]. The data presented in this article are in favour of such a positive regulatory mechanism between Smad4 and Sp1 to explain the TGF-β-mediated up-regulation of Muc5ac expression in cancer cells. Since TGF-β is a pleiotropic cytokine with diverse functions during development and in adult tissue homeostasis, carcinogenesis and inflammation [46,47], it will be interesting in the future to study the correlation between TGF-β-mediated processes with that of Muc5ac expression and their consequences on epithelium homeostasis.

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