Sequence of the 5'-flanking region and promoter activity of the human mucin gene *MUC5B* in different phenotypes of colon cancer cells

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Control of gene expression in intestinal cells is poorly understood. Molecular mechanisms that regulate transcription of cellular genes are the foundation for understanding developmental and differentiation events. Mucin gene expression has been shown to be altered in many intestinal diseases and especially cancers of the gastrointestinal tract. Towards understanding the transcriptional regulation of a member of the 11p15.5 human mucin gene cluster, we have characterized 3.55 kb of the 5'-flanking region of the human mucin gene *MUC5B*, including the promoter, the first two exons and the first intron. We report here the promoter activity of successively 5'-truncated sections of 956 bases of this region by fusing it to the coding region of a luciferase reporter gene. The transcription start site was determined by primer-extension analysis. The region upstream of the transcription start site is characterized by the presence of a TATA box at bases -32/-26, DNA-binding elements for transcription factors c-Myc, N-Myc, Sp1 and nuclear factor κ B as well as putative activator protein (AP)-1-, cAMP-response-element-binding protein (CREB)-, hepatocyte nuclear

factor (HNF)-1-, HNF-3-, TGT3-, gut-enriched Krüppel factor (GKLF)-, thyroid transcription factor (TTF)-1- and glucocorticoid receptor element (GRE)-binding sites. Intron 1 of *MUC5B* was also characterized, it is 2511 nucleotides long and contains a DNA segment of 259 bp in which are clustered eight tandemly repeated GA boxes and a CACCC box that bind Sp1. AP-2 α and GATA-1 nuclear factors were also shown to bind to their respective cognate elements in intron 1. In transfection studies the *MUC5B* promoter showed a cell-specific activity as it is very active in mucus-secreting LS174T cells, whereas it is inactive in Caco-2 enterocytes and HT-29 STD (standard) undifferentiated cells. Within the promoter, maximal transcription activity was found in a segment covering the first 223 bp upstream of the transcription start site. Finally, in co-transfection experiments a transactivating effect of Sp1 on to *MUC5B* promoter was seen in LS174T and Caco-2 cells.

Key words: differentiation, LS174T, Myc, Sp1, transcription.

INTRODUCTION

Human mucins are the main glycoprotein component of epithelial secretions and play important roles in maintaining mucosae integrity. So far, eight genes have been described [1] and among them *MUC6-MUC2-MUC5AC-MUC5B* are clustered on human chromosome 11p15.5 [2]. Previous studies have demonstrated that the expression of these four genes is tissue- and cell-specific, and that their expression is dysregulated during the pathogenesis of several diseases [3]. These results suggest that mucin gene expression is tightly regulated. *MUC5B* is mainly expressed in normal bronchus, submaxillary glands, endocervix, pancreas [4] and gall bladder [5,6]. Interestingly enough, *MUC5B* has been shown to be highly expressed in colon cancer [3] and especially in the mucus-secreting colon cancer cell lines HT-29 MTX (methotrexate) [7] and LS174T [6].

The *MUC5B* gene is 39.09 kb in length, contains 49 exons and encodes a 5701-amino-acid polypeptide with an M_r of approx. 627000. *MUC5B* shows extensive similarity in its 5' and 3' regions to the other genes of the cluster *MUC2*, *MUC5AC* and *MUC6* [8–11]. Regarding *MUC5B* regulation, we showed previously that intron G, now called intron 37 (30 kb downstream of the ATG), contains eight repeats of a perfect Sp1 (GGGCGG)-binding site [9]. It binds Sp1 and a 42-kDa nuclear factor called NF1-MUC5B specific to the mucus-secreting colon cancer cell line HT-29 MTX [12].

Regarding the other 11p15.5 mucin genes, promoter sequences and transcriptional regulation are known for *MUC2* and *MUC5AC*. *MUC2* a prominent mucin gene expressed in the colon, has a TATA and a CCAAT box as well as predicted binding sites for many transcription factors [13,14]. Transfection studies carried out in colon cancer cell lines (HT-29, LS174T) showed that a region in the promoter of *MUC2* that extends 848 bp upstream of the ATG confers maximal transcriptional activity [13]. Gum et al. showed that a short segment including bases -91/-73 relative to the start site was essential for basal promoter activity in several colon cancer cell lines [14]. By the means of transgenesis, the same group showed that *cis*-elements necessary for *MUC2* goblet-cell-specific expression are present within the *MUC2* promoter [15]. More recently, it was shown that Sp1 regulates rat *muc2* gene transcription in lung cells [16]. Li et al. showed that *Pseudomonas aeruginosa* lipopolysaccharide activates a c-Src-Ras-MEK 1/2-pp90rsk signalling pathway that leads to activation of nuclear factor κ B (NF- κ B; p65/p50) [17]. Activated NF- κ B binds to κ B sites in the 5' proximal region of the gene and induces *MUC2* transcription [18,19].

MUC5AC is highly expressed in airway epithelial cells and in the stomach. A TATA box is located at -23/-29 of the transcription start site and putative binding sites for Sp1, activator protein (AP)-2, glucocorticoid receptor element (GRE), NF- κ B as well as a CACCC box were found within the promoter [20]. *MUC5AC* transcription is activated when bronchial ex-

Abbreviations used: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MTX, methotrexate; STD, standard; RT, reverse transcriptase; NF- κ B, nuclear factor κ B; AP, activator protein; UTR, untranslated region; GRE, glucocorticoid receptor element; HNF, hepatocyte nuclear factor; GKLF, gut-enriched Krüppel factor; TTF, thyroid transcription factor.

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plants, airway epithelial cells (NCIH292) or colon cancer cells (HM3) are exposed to *P. aeruginosa* or its exoproducts in cell-free supernatants. Both *MUC2* and *MUC5AC* are up-regulated by other Gram-positive and Gram-negative organisms [20]. These results suggest that the pathology of cystic fibrosis bacterial infection increases *MUC2* and *MUC5AC* mucin mRNA production.

In this report, we present the genomic sequence of 3.55 kb of the *MUC5B* 5'-flanking region that includes the promoter and the first intron. The promoter and first intron were characterized and several important *cis*-elements that bind nuclear factors (Sp1, N-Myc, NF- κ B, TGT3, AP-2 α and GATA-1) were identified. *MUC5B* transcriptional activity was studied in colon cancer cell lines that are undifferentiated [HT-29 STD (standard)] or that either differentiate in mucus-secreting cells (LS174T) or in enterocytes (Caco-2). It appears that *MUC5B* expression shows cell-specificity and is the highest in mucus-secreting colon cancer cell line LS174T. The roles of the structural and functional properties of the *MUC5B* promoter in *MUC5B* gene transcription are discussed.

MATERIALS AND METHODS

Cloning

Inserts were prepared using the restriction map of two cosmid clones called ELO9 [21] and BEN1 [8], which cover the 5' proximal region of *MUC5B*. Gel-purified fragments (Qiaquick gel extraction kit, Qiagen) were subcloned into the promoterless pGL3 Basic vector (Promega). Internal deletion mutants were generated by PCR using pairs of primers bearing specific restriction sites at their 5' and 3' ends. PCR products were digested, gel-purified and subcloned into the pGL3 vector that has previously been cut with the same restriction enzymes. All clones were sequenced on both strands on an automatic LI-COR sequencer (ScienceTec, France) using infrared-labelled RV3 and GL2 primers (Promega). Plasmids used for transfection studies were prepared using the Endofree plasmid Mega kit (Qiagen).

Cell culture

The LS174T cell line was cultured in a 37 °C incubator with 5% CO₂ in Dulbecco's modified Eagle's minimal essential medium supplemented with non-essential amino acids and 10% foetal calf serum (Boehringer Mannheim). Other human colon adenocarcinoma cell lines were cultured in a 37 °C incubator with 10% CO₂ in Dulbecco's modified Eagle's medium supplemented with either 10% (HT-29 STD, HT-29 MTX) or 20% (Caco-2) foetal calf serum. All cell lines were a kind gift from Dr A. Zweibaum (Unité INSERM N°178, Villejuif, France).

Reverse transcriptase PCR (RT-PCR)

Total RNAs from colon cancer cells were prepared using the RNeasy midi-kit from Qiagen. Cells were harvested at 70% confluence and 1.5 μ g of total RNA was used to prepare cDNA (Advantage[™] RT-for-PCR kit, Clontech). PCR was then performed on 5 μ l of cDNA using specific pairs of primers for *MUC2* and *MUC5B* mucin genes (M.-P. Buisine, N. Moniaux, S. Nollet, E. Destailleur, I. Van Seuningen, A. Laine, N. Porchet and J.-P. Aubert, unpublished work): *MUC2* forward primer, 5'-CTGCACCAAGACCGTCCTCATG-3'; *MUC2* reverse primer, 5'-GCAAGGACTGAACAAAGACTCAGAC-3' (positions nt 15291–15312 and nt 15667–15691, accession number L21998 [22]); *MUC5B* forward primer, 5'-CTGCGAGACCGAGGTCAACATC-3'; *MUC5B* reverse primer, 5'-TGGGCAGCAGGAGCAGGAG-3' (positions nt 9057–9078 and nt 10108–10127,

Table 1 Sequences of the pairs of oligonucleotides used in PCR to produce deletion mutants in the *MUC5B* 5'-flanking region (see Figure 1)

*Kpn*I (GGTACC), *Sac*I (GAGCTC) and *Mlu*I (ACGCGT) sites were added at the ends of the primers to direct subcloning (underlined). Positions of the DNA fragments in *MUC5B* 5'-flanking region relative to the transcription start site are indicated. S, sense; AS, antisense.

pGL3 deletion mutant	Position of the DNA fragment	Orientation	Oligonucleotides used for PCR (5' → 3')
1916	—956/+57	S AS	CGCGGTACCGAGGTATTGCAGCGC CGCACGCGTCTGGGGCTGGCAC
1896	—956/—1	S AS	CGCGGTACCGAGGTATTGCAGCGC CGCACGCGTTGCTTCTCCCTGGGGGC
1597	—223/+57	S AS	CGCGAGCTCTCCCTCCGATTCTCAGA CGCACGCGTCTGGGGCTGGCAC
1596	—223/—1	S AS	CGCGAGCTCTCCCTCCGATTCTCAGA CGCACGCGTTGCTTCTCCCTGGGGGC
1895	—956/—223	S AS	CGCGGTACCGAGGTATTGCAGCGC CGCACGCGTAGAGAAGGGCTACCCCA
1595	—446/—223	S AS	CGCGAGCTCCAGCTCGTGGGCCCTCC CGCACGCGTAGAGAAGGGCTACCCCA
1598	—956/—441	S AS	CGCGGTACCGAGGTATTGCAGCGC CGCGAGCTCAGGCATGCACATGGC

accession number Y09788 [9]). Single-stranded oligonucleotides were synthesized by MWG-Biotech, Ebersberg, Germany. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. PCR reactions were carried out in 50- μ l final solutions [5 μ l of Perkin-Elmer buffer, 5 μ l of 25 mM MgCl₂, 5 μ l of 2.5 mM dNTPs, 10 pmol of each primer, 2 units of *Taq* polymerase (Ampli Taq Gold, Perkin Elmer)]. Cycling conditions were as follows: (i) denaturation, 96 °C, 10 min for one cycle; (ii) denaturation, 96 °C, 30 s; annealing, 60 °C, 30 s; and extension, 72 °C, 1 min for 30 cycles and (iii) final extension, 72 °C, 15 min. PCR products were analysed on 2% E-gels run in 1 × Tris/borate/EDTA buffer (Invitrogen). Mixed ladder was purchased from Invitrogen. Expected sizes of PCR products were 401 bp for *MUC2* and 415 bp for *MUC5B*.

Primer extension

Primer-extension reactions were performed using 25 μ g of total RNA isolated from LS174T and HT-29 MTX cell lines. Primers were end-labelled with [γ -³²P]dATP. Exon 1 reverse primer, 5'-TGCCTGCGGCACCGAGCATG-3' (see Figure 2B below) was annealed to RNA at 62 °C for 20 min followed by a 10 min incubation at room temperature. Extension was performed as described in the Promega primer-extension kit using 200 units of Moloney-murine-leukaemia virus (MMLV) RT (Advantage[™] RT-for-PCR kit, Clontech) for 45 min at 42 °C. Reactions were stopped by adding 20 μ l of loading buffer (98% formamide/10 mM EDTA/0.1% xylene cyanol/0.1% Bromophenol Blue). ϕ X174 DNA/*Hinf*I dephosphorylated markers were radio-labelled with [γ -³²P]dATP just before use (Promega). Manual sequencing of fragment 1429 (DNA fragment of *MUC5B* promoter region) was performed using the T7 Sequenase version 2.0 kit (Amersham). Samples were denatured for 10 min at 90 °C before loading on a 6% sequencing gel (Sequagel-6, National Diagnostic). The gel was then vacuum-dried and autoradiographed for 3–4 days at –80 °C.

Oligonucleotides and DNA fragments

Oligonucleotides used for PCR are shown in Table 1. Those used for gel-shift assays are indicated in Table 2. They were synthesized

Table 2 Sequences of the sense oligonucleotides used for gel-shift-assay experiments

Mutated bases are in bold. Antisense complementary oligonucleotides were also synthesized and annealed to the sense oligonucleotides to produce double-stranded DNA.

Oligonucleotide (sense orientation)	Sequence (5' → 3')
c-Myc/N-Myc/Max site/promoter	GAGAGCAAACACACGTGGCTGGAG
CACCC/Sp1 site/promoter	CTGCTGGGGTGGGGGCGTCTCGG
NF-κB site/promoter	CAGAGGCTGGGATTCCCTCCAGC
CCAAT site/promoter	GGCTGGCATTGGACAAGCGCACGT
TGT3 site/promoter	GAGGACCCCGGGCAGGTCCAGGAC
CACCC box/intron 1	TTAGGGGACACACCCCAAAGT
GA box/intron 1	GCAGGGGAGGGGCTGCCTGGG
Mutated GA box no. 1	GCATTAGAGGGGCTGCCTGGG
Mutated GA box no. 2	GCAGGGATTGGGCTGCCTGGG
Mutated GA box no. 3	GCAGGGGAGCTACTGCCTGGG
Sp1 site/intron 1	CCCTTGGGGCGGGCAACGCCT
AP-2 site/intron 1	ACGTCCTGTGGCCGGGGAAGGCA
GATA-1 site/intron 1	ACGGAGCAGATAACCAAGGGC
NF-κB site/intron 1	GCAGGGGGCTTCCCCAGTGG

by MWG-Biotech. Annealed oligonucleotides were radiolabelled using T4 polynucleotide kinase (Promega) and [γ - 32 P]dATP. Radiolabelled probes were separated from free nucleotides on Sephadex G-25 columns (Boehringer Mannheim). DNA fragment 1472 was produced by PCR (forward primer, 5'-CGCG-AGCTCGATAACCAAGGGCAGTCA-3'; reverse primer, 5'-CGCACGCGTTCCACCCGACCCACAGC-3') and cloned into the pGL3 vector. This fragment contains the eight GA boxes found in intron 1. The fragment was cut out of the vector by *SacI*-*MluI* double digestion, gel-purified and labelled with [α - 32 P]dCTP at the 3' end by a fill-in reaction using the Klenow fragment (Boehringer Mannheim).

Transfections

Cells from the human colon adenocarcinoma cell lines HT-29 STD (parental undifferentiated cell line), Caco-2 (enterocytes) and LS174T (goblet cells, mucus-secreting) were electroporated using Gene Pulser II apparatus (Bio-Rad) on day 7 (70% confluence). Before electroporation, 25 μ g of plasmid were added to the cells. In co-transfection experiments, 20 μ g of pGL3 deletion mutant and 5 μ g of pCMV-Sp1 or pCMV-Sp3 were used. After a 10-min incubation at room temperature, transfected cells were plated and incubated for 48 h at 37 °C. Total cell extracts were prepared using 1 $\times$ Reagent Lysis Buffer (Promega) as described in the manufacturer's instruction manual. Results were corrected for transfection efficiency by co-transfecting 2 μ g of pSV- β Gal vector (Promega). β -Galactosidase activity was measured in 96-well plates as described in the manufacturer's instruction manual using 50 μ l of cell extracts (Promega). Luciferase activity was measured on a Berthold 5901 luminometer on 100 μ l of cell extracts using luciferase assay reagent (Promega). The luciferase activity is expressed as fold induction of the test plasmid activity compared with that of the corresponding control vector (pGL3 control, Promega) after correction for transfection efficiency by dividing by β -galactosidase activity. Each plasmid was assayed in duplicate in at least three separate experiments. pCMV4, pCMV4-Sp1 and pCMV4-Sp3 vectors were a kind gift from Dr J. M. Horowitz (North Carolina State University, Raleigh, NC, U.S.A.).

Nuclear-extract preparation

Nuclear extracts from cell lines of interest were prepared as described in [23], and kept at -80 °C until use. Protein content (5 μ l of cell extracts) was measured using the bicinchoninic acid method in 96-well plates, as described in the manufacturer's instruction manual (Pierce).

Gel-shift assays

Nuclear proteins (5 μ g) were pre-incubated for 20 min on ice in 20 μ l of binding buffer with 2 μ g of poly dI-dC (Sigma) and 1 μ g of sonicated salmon sperm DNA. Radiolabelled DNA probe was added (120000 c.p.m./reaction) and the reaction left for another 20 min on ice. For super-shift analyses, 1 μ l of the antibody of interest (anti-Sp1, anti-AP2 α , anti-GATA-1, anti-c-Myc, anti-N-Myc, anti-Max, anti-c-Fos, anti-NF- κ B p50 and anti-NF- κ B p65, Santa Cruz Laboratories) was added to the proteins and left for 1 h on ice before adding the radiolabelled probe. Negative controls were carried out using 1 μ l of irrelevant antibody in the reaction mixture. Competitions were performed with 50-, 150- and 300-fold excesses of unlabelled oligonucleotide in the reaction mixture before adding the radiolabelled probe. Commercial Sp1 oligonucleotide and HeLa-cell nuclear extract used as positive controls were from Promega. The reactions were stopped by adding 2 μ l of loading buffer, loaded on to a 4% non-denaturing polyacrylamide gel (for oligonucleotides) and electrophoresis conditions were as described in [24]. Gels were pre-run for 30 min at 200 V before loading the samples and running the electrophoresis at 200 V for 2–3 h in 0.5 $\times$ Tris/borate/EDTA buffer at 4 °C. For DNA probes longer than 250 bp (1472), 6% gels were run as described in [25], with slight modifications: the pre-run was 1 h and 15 min long and electrophoresis was run for 4 h and 15 min at 4 °C. Gels were then vacuum-dried and autoradiographed overnight at -80 °C.

Southwestern- and immuno-blotting

Nuclear proteins (25 μ g) were resolved by SDS/PAGE (10% gel) and then electrotransferred on to a nitrocellulose membrane (pore size 0.22 μ m, Schleicher and Schuell). Proteins were submitted to a denaturation/renaturation cycle as described by Jackson [26] before radiolabelled probe (1 $\times$ 10⁶ c.p.m./ml of buffer) was added to the mixture and left for 30 min at room temperature. When necessary, unlabelled competitor (150-fold excess) was pre-incubated with the membrane before adding the radiolabelled probe. The membrane was then washed and autoradiographed overnight at -80 °C. The radiolabelled probe was washed off by incubating the membrane twice for 5 min in boiling 0.1 $\times$ SSC/0.1% SDS solution (where 1 $\times$ SSC is 0.15 M NaCl/0.015 M sodium citrate) and rinsed once with 3 $\times$ SSC. Immunostaining was then performed using the anti-Sp1 antibody PEP2 (Santa Cruz Laboratories) at a 1:1000 dilution. Alkaline phosphatase-conjugated goat anti-rabbit antibody (Promega) was used at a 1:4725 dilution. Protein bands were developed using Nitro Blue Tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate *p*-toluidine salt (Life Technologies) as chromogenic substrates for alkaline phosphatase. Prestained protein molecular-mass standards were from Life Technologies.

DNA sequence and transcription-factor-binding site analyses

DNA sequences were analysed using PC-GENE software and the TRANSFAC database [27] was used to define potential transcription-factor-binding sites within the clones of interest. The search was conducted using MatInspector V2.2 software [27].

Figure 1 For legend see opposite.

1821 GGGTTGGAGCTGCCTCCTCCAGCCTCCATGGGGTTGGTGGGTGAGGCCTTGCC*CGGAGG
 1881 CGGTGGTCAGCCTGGGGGACCTTGGGGGCGCCATCCCAGTATCAACGGCCACACAGCTTG C
 1941 GCGGCCACAGAGTCCTGCCCCAGCCTGCCCACTCGCCCTGACTTAGGATCTAGTTCGAA
 2001 ACTGGTTCTGTGTTTAGGTTTCTGCTAAGTCACGCCTGGAAGGCTCCAAGTGTGTCTCTC
 2061 TAAACAAAGCTGGTCTTTGTCTTCTCCAAGGGATGTGTGGGATGGGGCGAAATCCCTCCCT
 2121 TGGGGGCGCCCAACGCCCTTTCTCTGATTCCATTTTCTCCCCCATCCCTTGAGAAGGAGGCA
 2181 CCATCCCCGCTGTCAAGTCGGGGACAGGGCAGGCCGTGCTGGGGGACAGCTCAGGGCTCCC
 2241 TGCTGGAAGCTTCCATCCCGCAGGCTTTCCATAGCATTGAGCAGGAGC*CGGAGGCATCTG
 2301 CGGCTGACGGTTGGGGTGGCCTGAGCGGCTGGGGAGGAGTCC*CGGCCTTGGCCACAGTGT
 2361 GTCGTGAGGGTGAACCTGCAGGGCATGGAGACCGCCACCAAGGAACCCACATGCGCGTGC
 2421 CGCACCAGGGATGTGGCCAGGTCCGTGGTGGGTTTCGGCTGGCAGCCACATCTAGTTCC
 2481 TCACTGGAAGCTCCCATTTCTCTTCCACAGAGCCAGGGCCCTGTGGAGGCCGAGCTGG
 GGGAAATGCAGGGCACACCATGGATGGCGGT
 G N A G H T M D G G

Figure 1 DNA sequence of the human *MUC5B* promoter (A) and intron 1 (B)

(A) The TATA-like box (TACATAA) at $-32/-26$ is double-underlined and potential binding sites for known transcription factors are in grey. The transcription start site is designated as $+1$ and first ATG is bold and italicized. *HpaII* (C*Gg) potential methylation sites are also indicated. (B) DNA and peptide sequences of exon 1 and exon 2 surrounding intron 1 are bold and italicized. Square brackets indicate the beginning and end of intron 1 sequence. DNA sequences used for binding assays are boxed.

Figure 2 Transcription start site of human *MUC5B* mucin gene

Schematic representation of the 5'-flanking region of *MUC5B*. The bent arrow indicates the transcription start-site position (upper panel). (A) Primer-extension reactions were performed on 25 μ g of total RNA from LS174T and HT-29 MTX cell lines. A product of 126 bp was produced when using the exon 1 reverse primer. ϕ X174 DNA/*HinfI* dephosphorylated markers previously radiolabelled and denatured are shown on the left. (B) DNA sequence surrounding the transcription start site. The reverse exon 1 oligonucleotide used for primer-extension experiments was designed from the underlined sequence.

RESULTS

DNA sequence and characterization of *MUC5B* 5'-flanking region

Two genomic clones, ELO9 and BEN1, which we previously isolated to study *MUC5B* and *MUC5AC* gene organization [9,21], were used in this work to characterize the 5'-flanking region of *MUC5B*. Here, we report the genomic sequence obtained and an analysis of transcriptional activity of the 5'-

flanking region including 956 bases of DNA sequence located upstream of the transcriptional start site as well as 2637 bases downstream that include the first two exons and the first intron. Between the transcription start site and the ATG codon coding for the first methionine lies the 5' untranslated region (UTR) of 56 nucleotides (Figure 1A). The first exon encodes the first 23 amino acids (MGAPSACRTLVLALAAMLVVPQA) and is separated from exon 2, which is composed of 19 amino acids

Figure 3 Mucin gene-expression profiles in colon cancer cells by RT-PCR

RT-PCR was performed on total RNA extracted from cells at day 7. Specific oligonucleotides for *MUC2* and *MUC5B* generate 401- and 415-bp PCR products, respectively. The *GAPDH* PCR product is 980 bp long. PCR products were separated on a 2% agarose gel. Molecular-mass markers are shown on the right.

(ETQGPVEPSWGNAGHTMDG), by an intron of 2511 nucleotides (Figure 1B).

Analysis of the DNA sequence using PC-GENE software and the TRANSFAC database [27] showed that the 5'-flanking region upstream of the transcription start site contained a TATA-box-like sequence TACATAA located at bases -32/-26 upstream of the transcription start site (Figure 1A). In the close vicinity to the TATA-box-like sequence were found potential binding sites for c-Myc/N-Myc/Max transcription factors (consensus CACGTG), Sp1 and numerous GC and CACCC boxes. It is of interest to note that GC and CACCC boxes are always clustered. Further upstream, putative binding sites were found for AP-1, NF- κ B, CCAAT, cAMP-response-element-binding protein (CREB) and GRE transcription factors as well as for a silencer called CIIS1. A striking feature was the presence, throughout the 5'-flanking region upstream of the TATA box, of numerous putative binding sites for transcription factors known to be involved in intestinal [hepatocyte nuclear factor (HNF)-1, HNF-3, TGT3, gut-enriched Krüppel factor (GKLF)] or respiratory [HNF-3, thyroid transcription factor (TTF)-1] cell differentiation. Numerous *Hpa*II sites that could be potential methylation sites were found, two of them being located in the 5'-UTR region.

In the first intron (Figure 1B), a high amount of GC boxes, Sp1 binding sites and CACCC boxes were present. More

Figure 4 Position and transcriptional activity of the pGL3 deletion mutants in *MUC5B* promoter

(A) Schematic representation of the localization of the different pGL3 deletion mutants covering 1013 nucleotides upstream of exon 1. Numbering refers to the transcription start site designated +1. The TATA-box location in fragments 1916, 1896, 1597 and 1596 is indicated. (B) Transcriptional activity of the deletion mutants was studied in enterocytic Caco-2 (white bars), in mucus-secreting LS174T (black bars) and in undifferentiated HT-29 STD (grey bars) colon cancer cell lines. The background activity of pGL3 Basic promoterless vector used to subclone *MUC5B* fragments is shown. The results are means $\pm$ S.D. and represent more than three different experiments performed in duplicate for each fragment.

Figure 5 Binding of nuclear factors to the promoter of *MUC5B*

Autoradiograms of the gel-shift assays in which 5 μ g of LS174T or Caco-2 nuclear extracts were incubated with double-stranded radiolabelled oligonucleotides representative of the c-Myc/N-Myc/Max (–108/–98, **A**, lanes 2–5 and 7–10), CACCC/Sp1 (–196/–185, **A**, lanes 11–14), NF-κB (–372/–364, **B**, lanes 2–7), TGT3 (–791/–785, **B**, lanes 9 and 10) and CCAAT (–662/–658, **C**, lanes 2 and 3) binding sites found in the *MUC5B* promoter. (**A**) No proteins added (lanes 1, 6 and 15). Nuclear extracts were either incubated in the absence (–; lanes 2, 7, 11 and 13) or the presence (lanes 3–5, 8–10, 12 and 14) of 1 μ l of the antibody of interest. (**B**) No proteins added (lanes 1 and 8). Nuclear extracts were either incubated in the absence (–; lanes 2, 5, 9 and 10) or the presence (lanes 3, 4, 6 and 7) of 1 μ l of the antibody of interest. There was no antibody added in lanes 9 and 10. (**C**) No proteins added (lane 1). The CCAAT radiolabelled probe was incubated with Caco-2 (lane 2) or LS174T (lane 3) nuclear extracts. DNA–protein complexes are indicated by the numbers on each side of the autoradiograms.

importantly, in the central part of the intron were found clustered a CACCC box followed by an array of eight GA boxes repeated in tandem containing the consensus sequence GGGGAGG-GGCT, each separated by 8–10 bp. Other putative sites for known transcription factors are shown in Figure 1(B) and include three canonical Sp1 binding sites, two AP-2 sites, one NF-κB site, one GATA-1 site and one Adh-1 site.

Characterization of the *MUC5B* transcription start site

Primer-extension reactions were performed using RNAs from the two mucus-secreting colon cancer cell lines LS174T and HT-29 MTX to identify the transcription start site in the *MUC5B* 5'-flanking region. These two cell lines were chosen as they both express *MUC5B*. The result, shown in Figure 2(A), indicates that the reactions carried out with reverse primer E1 localized in exon 1 (sequence underlined in Figure 2B) produced one extension product of the same size (126 bp) in both cell lines. In the sequence, the start site matches a cytosine residue that we numbered +1. The 5'-UTR region is 56 nucleotides long (Figure 2B).

MUC5B gene expression in LS174T, Caco-2 and HT-29 STD colon cancer cells

To investigate the cell-specific expression of *MUC5B*, three colon cancer cell lines showing different phenotypes were chosen: the mucus-secreting LS174T, the Caco-2 enterocyte and the un-

differentiated HT-29 STD cell lines. *MUC2* expression was studied in parallel as a positive control of RT-PCR as it is the main mucin expressed in colon. *MUC5B* (415-bp PCR product) and *MUC2* (401-bp PCR product) mRNA expression was studied by RT-PCR from 70 % confluent cells in order to correlate RT-PCR results with transfection experiments. As shown in Figure 3, undifferentiated cell line HT-29 STD slightly expresses *MUC2* and *MUC5B* mRNAs. Caco-2 cells that spontaneously differentiate in enterocytes also show a very low expression of the two genes. Overall, in these two cell lines, mucin gene expression remains very discrete when compared with the GAPDH internal control. On the other hand, *MUC2* and *MUC5B* are highly expressed in the mucus-secreting cell line LS174T. The level of expression of both genes is maximal after cells have differentiated into mucus-secreting cells. Moreover, one can notice that *MUC2* and *MUC5B* follow the same pattern of expression in the three colon cancer cell lines studied.

Promoter activity of the *MUC5B* 5'-flanking region

In order to identify DNA sequences involved in *MUC5B* transcriptional regulation, constructs were generated in the promoterless pGL3 Basic vector and analysed for transcriptional activity after cell electroporation. Insert sequences were confirmed by infrared sequencing of both strands and aligned with ELO9 and BEN1 sequences.

Figure 6 Sp1 binds to the eight clustered GA boxes and the CACCC box found in intron 1

(A) Autoradiogram of the gel-shift assay in which 5 µg of LS174T nuclear extracts were incubated with double-stranded oligonucleotides representative of the CACCC (lane 2), GA (lane 3), Sp1 (lane 4) boxes found in *MUC5B* intron 1 and with the GC box located in intron 37 [12] (lane 5). Super-shift analyses were performed by adding an antibody directed against Sp1 (lanes 6–10 and 12). A positive control was included by performing shift analysis with a commercial Sp1 oligonucleotide mixed with LS174T extracts (lane 10) and HeLa cell extracts (lane 11). Sp1 super-shift is shown with HeLa cells in lane 12. Arrow 1 indicates the position of the DNA–protein complex and the arrow 2 the position of the super-shifted complex when the antibody of interest was added. (B) Autoradiogram of the Southwestern blot of 25 µg of nuclear proteins from Caco-2 (lanes 2, 5, 8, 11 and 14), LS174T (lanes 3, 6, 9, 12 and 15) and HT-29 STD (lanes 1, 4, 7, 10 and 13) cells incubated with the GC- (lanes 1–6) and GA- (lanes 7–12) box radiolabelled probes. 'Cold' competition with a 150-fold excess of GC (lanes 4–6) and GA (lanes 10–12) boxes. Sp1 immunostaining of the same blot with a commercial antibody directed against Sp1 (PEP2; lane 13–15). (C) Autoradiogram of the gel-shift assay with 1472 as the probe with 5 µg of LS174T nuclear proteins. No extracts (lane 1), proteins and 1472 probe (lane 2), super-shift with anti-Sp1 antibody (lane 3). Competition with unlabelled 1472 DNA, molar ratios of $\times 50$ (lane 4), $\times 150$ (lane 5) and $\times 300$ (lane 6). Competition with unlabelled GA-box oligonucleotide, molar ratios of $\times 50$ (lane 7), $\times 150$ (lane 8) and $\times 300$ (lane 9). Competition with unlabelled mutated GA-box oligonucleotides with molar ratio $\times 150$, *GA box no. 1 (lane 10), *GA box no. 2 (lane 11) and *GA box no. 3 (lane 12). Arrow 1 indicates the position of the DNA–protein complex and arrow 2 the position of the super-shifted complex when the anti-Sp1 antibody was added.

Transfection experiments on deletion fragments of the *MUC5B* promoter were carried out in Caco-2, LS174T and HT-29 STD cell lines. Seven fragments were generated that cover 956 nucleotides upstream of the transcription start site (Figure 4A). The fragments 1916, 1896, 1597 and 1596 contain the TATA-box-like sequence (TACATAA), the three Sp1-binding sites and the CACCC box. In fragments 1916 and 1597, the 5'-UTR region was included. In LS174T cells, the *MUC5B* 5'-flanking region shows a highly active promoter activity. In these cells, the fragment 1896 that covers the whole region shows a lower luciferase activity when compared with the region restricted to the first 223 nucleotides upstream of the transcription start site (1596; Figure 4B). Interestingly enough, when the 5'-UTR region was included in the fragments corresponding to the whole region (1916) or to the 223 first bases (1597), luciferase activity dropped by approx. 2-fold. This result indicates that the 5'-UTR region of *MUC5B* plays a role in regulating promoter activity. In conclusion, the first 223 bp (fragment 1596) adjoining the tran-

scription start site suffice to drive basal promoter activity of the luciferase reporter gene. Three other deletion mutants were then constructed to identify a potential inhibitory domain in the promoter that would explain the low luciferase activity of the fragment 1896. Fragment 1895 covers the upstream 734 bases (–956/–223) and fragments 1595 and 1598 include bases –446/–223 and –956/–441, respectively. Experiments show that in all cell lines 1595 has no activity whereas fragments 1895 and 1598 act as inhibitory domains. Thus this result confirms the fact that inhibitory factors bind to *cis*-elements present in the –956/–441 region of the promoter. Finally, *MUC5B* promoter activity is minimal in Caco-2 and HT-29 STD cell lines when compared with that in LS174T cells.

Binding studies of *MUC5B* promoter with nuclear proteins

In order to characterize *cis*-elements and *trans*-nuclear factors that could account for *MUC5B* transcription regulation in colon

Figure 7 AP-2 α and GATA-1 transcription factors bind to the first intron of *MUC5B*

Autoradiogram of the gel-shift assay in which radiolabelled oligonucleotides including the AP-2- (lanes 1–4) and GATA-1- (lanes 5–8) binding sites found in intron 1 were incubated with 5 μ g of Caco-2 nuclear extracts in the absence (lanes 2 and 6) or presence (lanes 3–5 and 8) of 1 μ l of specific antibody as indicated. Radiolabelled probe alone (lanes 1 and 7).

cancer cells, electrophoretic mobility-shift assays were carried out with double-stranded DNA probes containing binding sites for specific nuclear factors (Figure 5). In Figure 5(A), two retarded bands (lanes 1 and 2) were obtained when Caco-2 nuclear extracts were incubated with the double-stranded oligonucleotide comprising the c-Myc/N-Myc/Max putative binding site (Figure 5A, lane 2). Addition of c-Myc or N-Myc antibodies prior to the radiolabelled probe dramatically reduced the intensity of complex no. 1 but had no effect on complex no. 2 (Figure 5A, lanes 3 and 4). On the other hand, addition of the Max antibody had no effect (Figure 5A, lane 5). Complex no. 1 was not visualized in LS174T cells (Figure 5A, lanes 7–10). Sp1 binds to the CACCC box in both cell lines (complex no. 3) as addition of Sp1 antibody resulted in a totally super-shifted complex (band no. 4, Figure 5A, lanes 11–14).

In Figure 5(B) is shown the binding of NF- κ B (lanes 1–7). When Caco-2 nuclear proteins were incubated with the double-stranded oligonucleotide containing the putative NF- κ B binding site (Figure 5B, lane 2), three retarded bands were visualized on the autoradiogram (bands 1–3). When incubated with LS174T nuclear proteins (Figure 5B, lane 5) only bands 1 and 3 could be seen. Upon addition of the specific NF- κ B p50 antibody (Figure

5B, lane 3), complexes 2 and 3 were abolished. With the antibody directed against the NF- κ B p65 subunit, only complex 2 disappeared. Complex 1 is therefore due to an interaction with another nuclear protein since the addition of these two antibodies did not have any effect on the shifted band. Binding studies with the TGT3 putative binding site (Figure 5B, lanes 8–10) resulted in two high-mobility retarded bands with Caco-2 nuclear proteins (Figure 5B, lane 9, complexes 5 and 6) but only one low-mobility-shifted band with LS174T nuclear extracts (Figure 5B, lane 10, complex 4).

In Figure 5(C), studies with the CCAAT DNA probe showed one retarded band with Caco-2 nuclear extracts (lane 2, complex 1). Two more retarded bands with a higher mobility (complexes 2 and 3) were visualized when incubated with LS174T nuclear extracts (lane 3).

Binding studies of *MUC5B* first intron with nuclear proteins

Given that first introns have been shown to play roles in the regulation of tissue-specific intestinal genes, we investigated whether such potential DNA-binding *cis*-elements could be found in *MUC5B*'s rather large (2.5 kb) first intron. A highly striking feature was the presence of eight clustered GA boxes repeated in tandem located near to a CACCC box. All these *cis*-elements represent potential binding sites for the ubiquitous nuclear factor Sp1. Gel-shift-assay experiments were performed with nuclear extracts from the three cell lines of interest. As they gave the same results only those with LS174T nuclear proteins are shown. When we used double-stranded DNA representative of the CACCC box one low-mobility retarded band was visualized (complex 1, Figure 6A, lane 2) that was totally super-shifted when an anti-Sp1 antibody was added in the reaction mixture (Figure 6A, lane 6). The same result was obtained with the double-stranded oligonucleotide containing the GA-box consensus sequence GGGGAGGGGCT (Figure 6A, lanes 3 and 7). A similar experiment conducted with an oligonucleotide containing an Sp1 found in the 3' region of the intron did not produce any retarded bands (Figure 6A, lanes 4 and 8).

In order to confirm the binding of Sp1 to the GA boxes, Southwestern- and immuno-blotting studies were performed (Figure 6B). The nuclear protein recognized by the radioactive GA-box DNA probe shows an M_r of around 95000 in the three cell lines (Figure 6B, lanes 7–9). When 'cold' competition was performed with a 150-fold excess of the unlabelled GA-box DNA probe the signal was almost totally abolished, demonstrating the specificity of the interaction (Figure 6B, lanes 10–12). As a positive control for Sp1 binding, the blot was then probed with a DNA probe representative of one of the Sp1-binding sites present in *MUC5B* promoter (see Figure 5A, lanes 11–14). The same band was visualized (Figure 6B, lanes 1–3) and 'cold' competition also resulted in the loss of the signal (Figure 6B, lanes 4–6). The same blot was then probed with the anti-Sp1 antibody (Figure 6B, lanes 13–15). The band visualized after immunostaining could be superimposed on the radioactive band obtained from the Southwestern-blotting study, confirming the fact that Sp1 binds to the GA box. We can conclude from these results that the Sp1 nuclear factor engages with the CACCC and GA boxes found in *MUC5B* intron 1. It is likely that the other seven GA boxes that contain the same consensus sequence GGGGAGGGGCT also interact with Sp1.

To verify this hypothesis, gel-shift experiments were performed using the fragment 1472 that contains the eight GA boxes as the DNA probe. Figure 6(C) shows that the shifted band obtained when 1472 was incubated with nuclear proteins from LS174T

Figure 8 Sp1 and Sp3 effects on *MUC5B* promoter activity

Co-transfections were performed in enterocyte Caco-2, mucus-secreting LS174T and undifferentiated HT-29 STD colon cancer cell lines. pGL3 deletion mutants (20 µg) 1596 or 1896 were co-transfected with 5 µg of pCMV4 expression vector either empty (pCMV4) or carrying the coding sequence of Sp1 (pCMV4-Sp1) or Sp3 (pCMV4-Sp3) transcription factors. The results are means $\pm$ S.D. and represent more than three different experiments performed in duplicate for each fragment.

cells (Figure 6C, lane 2, complex 1) was completely super-shifted when the anti-Sp1 antibody was added to the reaction mixture (Figure 6C, lane 3, complex 2). The shift was shown to be specific by performing competition experiments with unlabelled DNA fragment 1472 (Figure 6C, lanes 4–6) or unlabelled GA box oligonucleotide (Figure 6C, lanes 7–9) as competitors. The shift is almost totally inhibited when competitors are added at a molar ratio of 150 (Figure 6C, lanes 5 and 8) and totally disappears at a ratio of 300 (Figure 6C, lanes 6 and 9). Using mutated oligonucleotides with a modified GA-box sequence (see Table 2) only produced a partial inhibition of the retarded band (Figure 6C, lanes 10–12).

Among the other predicted binding sites present in intron 1, we studied NF- κ B, AP-2 and GATA-1 binding (Figure 7). Two shifted bands were obtained when Caco-2 nuclear proteins were incubated with the AP-2 oligonucleotide (Figure 7, lane 2). Of these two bands, the upper one disappeared upon addition of the anti-AP-2 α antibody (Figure 7, lane 3). The specificity of the binding was confirmed by the absence of any effect when an irrelevant anti-c-Fos antibody was used (Figure 7, lane 4). Complex 1 was not seen in LS174T cells (results not shown). When we used the double-stranded GATA oligonucleotide, one shifted band (complex 3) was visualized with Caco-2 nuclear proteins (Figure 7, lane 6). The intensity of the shifted band decreased substantially when an anti-GATA-1 antibody was added to the reaction mixture (Figure 7, lane 5), whereas there was no effect with the anti-Sp1 antibody (Figure 7, lane 8). No shift was visualized with the NF- κ B oligonucleotide in either cell line (results not shown).

Sp1 transactivation of the *MUC5B* promoter

In order to demonstrate the active participation of Sp1 in *MUC5B* promoter activity, co-transfection experiments were performed in the three cell lines of interest. Sp3, another member of the Sp family known to compete with Sp1 for the same binding sites, was also studied (Figure 8). Co-transfection of Sp1

with fragment 1896 (–956/–1) led to an increase in luciferase activity in LS174T and Caco-2 cells (2- and 3.5-fold, respectively), whereas co-transfection with Sp3 led to a slight decrease of activity in LS174T cells and had no effect in Caco-2 cells. When the same experiments were conducted with the fragment 1596 (–223/–1), no significant effect was observed in either cell line. In HT-29 STD cells, co-transfection with Sp1 had no effect, whereas co-transfection with Sp3 dramatically decreased the luciferase activity of both fragments by about 5-fold.

DISCUSSION

Molecular mechanisms involved in the control of gene expression in intestinal cells are still poorly understood. An understanding of the regulatory network of nuclear proteins that direct transcriptional initiation of genes will provide insight into the mechanisms of normal intestinal development and differentiation as well as disease processes such as neoplasia [28]. Since mucins are the main components of human epithelial secretions, responsible for the mucus rheology and physical properties, and since mucin genes display altered expression in neoplastic and pre-neoplastic processes affecting the colon [3,29], it is essential to understand the molecular mechanisms that prevail to their expression. Thus characterization of the promoter regions as well as the nuclear factors involved in the regulation of mucin gene expression is necessary.

In order to identify regulatory regions involved in *MUC5B* transcription, the *MUC5B* promoter region as well as the first intron have been sequenced and analysed. Transcriptional activity was studied in three colon cancer cell lines and transcription-factor binding to *cis*-elements present in either the promoter or the first intron were identified. The 5'-flanking region upstream of the transcriptional start site revealed the presence of a TATA-like sequence TACATAA at –26/–32, which is a characteristic of tissue-specific genes. The *MUC5B* promoter contains a high number of GC and CACCC boxes that are known to bind Sp1. In this study, Sp1 binding was

demonstrated to occur in this region in the three cell lines. Involvement of Sp1 in regulation of mucin genes has already been shown for *MUC1* [30] and *MUC2* [13,14]. Also in this study, the transactivating effect of Sp1 on the *MUC5B* promoter was shown to be effective on the whole promoter region but not on the fragment containing the first 223 nucleotides adjacent to the TATA box. This result may be explained by the fact that other transcription factors or epigenetic mechanisms like methylation are required to mediate Sp1 activity. In the *MUC5B* promoter, Sp1 binding sites were often found to be nearby binding sites for other specific factors. This phenomenon usually indicates that the clustered factors act in conjunction with each other to modulate transcription [31]. Among the other factors, NF- κ B may be a good candidate as it has already been shown to increase *MUC2*-promoter activity in epithelial cells via an Src-dependent pathway in response to bacterial exoproducts [18]. The *MUC5B* promoter contains a κ B element that binds NF- κ B in Caco-2 cells. In these cells, N-Myc, c-Myc as well as AP-2 and GATA factors may also control regulation of *MUC5B* since these factors were shown in this report to bind to the *MUC5B* promoter and first intron, respectively.

The first intron of *MUC5B*, whose sequence is described for the first time in this report, is very large (2.5 kb) and contains a striking feature, a central domain of 259 bp, which contains eight GA boxes repeated in tandem. Sp1 was shown to bind throughout this domain suggesting that this domain may serve as a docking platform for Sp1. Such repetitive sequences located in introns of 5'-flanking regions have already been described for human immunoglobulin heavy chain, insulin and serotonin transporter genes. However, this type of sequence remains relatively rare in the human genome [32]. They are usually rich in GC and GA residues and induce looping of DNA, and therefore their presence is often associated with a role in regulating gene transcription [32]. Such a role may thus be played by the first intron of *MUC5B*.

MUC5B-promoter activity was studied in three intestinal cancer cell lines showing different phenotypes in order to address cell-specific activity. The cells were either undifferentiated (HT-29 STD), differentiated in enterocytes (Caco-2) or mucus-secreting (LS174T). Clearly, the *MUC5B* promoter is not active in undifferentiated and enterocyte colon cancer cells. It is however very active in LS174T mucus-secreting cells. This result confirmed our RT-PCR results in which *MUC5B* mRNA expression was important in LS174T cells and almost absent from HT-29 STD and Caco-2 cells. Our results regarding *MUC2* and *MUC5B* expression in LS174T cells are in good agreement with those published by Van Klinken et al. [33]. A recent study by these authors also showed that *MUC2* and *MUC5B* were expressed in the same subset of colonic cells, that is goblet cells in the crypt base; co-localized expression was also shown in the small granules within goblet cells [6]. Why the cell needs to express several mucin genes simultaneously is another issue that will have to be addressed. Furthermore, knowledge of the mechanisms that control transcription of the four human mucin genes, *MUC6*, *MUC2*, *MUC5AC* and *MUC5B*, clustered on the chromosome 11 will certainly help in understanding the role of some regulatory factors in intestinal diseases in which altered expression of mucin genes has already been demonstrated [34].

Numerous putative binding sites for transcription factors involved in intestinal (HNF-1, HNF-3, three consensus binding sites for TGT3, GKLf) or respiratory (10 consensus binding sites for TTF-1) cell differentiation were found throughout the *MUC5B* promoter. *In situ* hybridization studies performed in our laboratory showing early expression of human mucin genes during embryonic and foetal development in intestine and

respiratory tracts suggest the involvement of these genes during ontogenesis [35,36]. Identification of silencers is also important to elucidate *MUC5B* down-regulation in certain cells. A putative binding site for such a factor, called CHIS1 [37], was found in the *MUC5B* promoter in fragments that showed inhibitory activity (1895 and 1598).

In conclusion, we have characterized the 5'-flanking region of *MUC5B* and shown that promoter activity was the highest in mucus-secreting cells. Important features and *cis*-elements that bind specific transcription factors were identified in the promoter (TATA box, Sp1, NF- κ B, c-Myc and N-Myc) and the first intron (Sp1, GATA-1 and AP-2 α) of *MUC5B* that might account for the cell-specific expression pattern of that gene. We also showed that Sp1 is involved in the regulation of *MUC5B* promoter activity. Experiments are now in progress to identify transcription factors and cell-signalling pathways responsible for the altered expression of *MUC5B* in intestinal diseases. Of particular interest will be the deciphering of the molecular mechanisms implicating the *MUC5B* gene in gastrointestinal cell differentiation. Moreover, experiments are now in progress to determine the influence of intron 1 and of intron 37, which has already been shown to bind Sp1 and NF1-MUC5B nuclear factors [12], on *MUC5B* promoter activity.

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