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

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Abbreviations

aa : amino acid

bp : basepair

DMEM : Dulbecco's Modified Essential Medium

EMSA : Electrophoretic Mobility Shift Assay

kb : kilobase

PCR : Polymerase Chain Reaction

RT : Reverse Transcriptase

TBE : Tris-borate-EDTA

TBST : Tris-Buffered Saline-Tween 20

TGF-β : Transforming Growth Factor-β

SSC : Saline-Sodium Citrate

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Abstract

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 previously shown 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 is 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 signaling cascade activated by TGF-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 cooperative 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.

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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 at the cell surface as transmembrane proteins 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 esophagus [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.

Transforming growth factor-beta (TGF-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], two pathologies in which *MUC5AC* expression is altered. TGF-β-induced signaling 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 to tumor progression [19]. More recently, the 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 knock-out mice for *Muc2* mucin gene allowed the authors to demonstrate for the first time a direct link between *Muc2* expression and tumor formation as $Muc2^{-/-}$ mice started to develop intestinal adenomas that progressed to invasive adenocarcinoma and colorectal tumors 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 tumor 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.

Material 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, Amsterdam, the Netherlands) with a probe spanning 750 basepairs (bp) of the human *MUC5AC* N-terminus (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 kilobases (kb) fragment that hybridized with 750 bp of the human *MUC5AC*. The positive 5.5 kb fragment was subsequently cloned into the *Sst*I site of pBluescript II SK (+/-) (Stratagene), which resulted in clone pMS1. That fragment was then digested by *Apa*I and *Pst*I 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 fluorescent-labeled nucleotides (Amersham), according to the manufacturer's protocol. Sequence reactions were analyzed on an ABI-PRISM 310 Genetic analyzer (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 to known sequences using NCBI-blast database. In addition, the sequence was analyzed with PC/Gene software (Intelligenetics, Inc., Mountain View, CA, USA), INFOBIOGEN database and MatInspector V2.2 and Alibaba2 softwares 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 number AF288076.

Determination of 5'-end of Muc5ac mRNA by RACE-PCR

The transcription start site was determined by 5'-RACE (rapid amplification of cDNA ends) 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 oligonucleotides 5'-CAGGGAAGTAGAAGACCTGTCC-3' as first primer and 5'-TGTGCATTGGCTGCAGGCCAG-3' as nested primer for reverse transcription and amplification. Resulting amplified PCR products were cloned directly into TA cloning vector (Invitrogen) and sequenced on an automatic LI-COR sequencer (ScienceTech, France) with T7 and RM13 primers as described thereafter.

Animals

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

Histology

Five μm-thick sections of mouse stomach tissue were routinely stained with hematoxylin and eosin to study the morphology, or stained with Alcian Blue/Periodic Acid Schiff (PAS) reagent to stain for acidic and neutral mucins, respectively. Immunolocalization of mouse *Muc5ac* was carried out as previously described [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 M-MLV 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 cDNA as template in 10 mM Tris-HCl buffer pH 8.4, containing 50 mM KCl, 2 mM $MgCl_2$, 0.01% gelatin, 1 U Taq polymerase (Eurogentec), 0.2 mM dNTPs, and 10 pmoles of each primer. The primers were (5'-CCAATTGGCTAGATGGCAGT-3') and (5'-AGATCAAACCCTCCTCTCG-3'), which corresponds to nucleotides 374-394 and 552-572 of murine *Muc5ac*, Genbank 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 and 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 *Eco*RI site of pBluescript SK vector and subsequently sequenced. The digoxigenin-11-UTP-labeled sense and antisense *Muc5ac* RNA probes were prepared according to the manufacturer's protocol (Roche Molecular Biochemicals).

In situ hybridization

Tissue sections were deparafinized 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 sulfate (w/v), 2X SSC, 1X Denhardt's solution, 1 μg/ml tRNA, 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 2X SSC, 50% formamide (v/v) in 1X SSC and 0.1X SSC. A 15 min incubation with RNase T1 (2 U/ml in 1 mM EDTA in 2X

SSC) at 37 °C was followed by washes of 0.1X SSC at 45°C and 2X SSC at room temperature (RT). The digoxigenin-labeled hybrids were detected by incubation with anti-digoxigenin (Fab, 1:2000) conjugated to alkaline phosphatase for 2.5 h at RT. Thereafter, sections were washed in 0.025% Tween (v/v) in Tris-buffered saline pH 7.5. For staining, sections were layered with detection buffer (0.1M Tris-HCl, pH 9.5 containing 0.1M NaCl and 0.05M $MgCl_2$) containing 0.33 mg/ml 4-nitroblue tetrazolium chloride, 0.16 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate, 8% (v/v) polyvinylalcohol (Mw 31000-50000, Aldrich) and 1 mM levamisol (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).

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 previously described [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 *Sac*I-*Mlu*I 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 I). PCR products were digested, gel-purified (Qiaquick 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 infra-red labeled 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 (Boston, USA). CMT-93 were cultured in DMEM medium containing 10% fetal bovine serum, 4 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml). IEC-6 cells were purchased from ECACC (European collection of animal cell cultures). This cell line was established from rat small intestine crypt cells and was cultured in DMEM medium containing 5% fetal bovine serum, 2 mM L-glutamine, 10 μg/ml insulin, 50 U/ml penicillin and 50 μg/ml streptomycin. HCT116-Smad4$^{+/+}$ and HCT116-Smad4$^{-/-}$ cells were a kind gift of Dr. A. Atfi (INSERM U482, Paris). Cells were cultured in DMEM supplemented with 2 mM glutamine and 10 % fetal calf serum. Human gastric cancer cell line KATO-III was cultured as previously described [30]. All cells were cultured at 37°C in a humidified 5% CO_2 water-jacketed incubator. To study TGF-β effect, cells were incubated for 24h with TGF-β (recombinant human TGF-β, 10 ng/ml). All reagents were from Sigma unless otherwise indicated.

RT-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^{TM} RT-for-PCR kit, Clontech). PCR was performed on 2 μl of cDNA using specific pairs of primers as follows : *Muc5ac* forward primer: 5'-GAGGGCCCAGTGAGCATCTCC-3', *Muc5ac* reverse primer: 5'-TGGGACAGCAGCAGTATTCAGT-3' (accession number AJ010792). β-actin was used as an internal control : mouse β-actin forward primer: 5'-GTGGGCCGCTCTAGGCACCA-3', mouse β-actin reverse primer: 5'-TGGCCTTAGGGTGCAGGGGG-3' (accession number

M12481). *Muc5ac* and *β-actin* PCR product sizes are 361 and 241 bp, respectively. Mouse TGF-*β*RII forward primer : 5'-CGTGTGGAGGAAGAACAACA-3'; reverse primer : 5'-TCTCAAACTGCTCTGAGGTG-3' (accession number S69114). PCR product is 560 bp long. Rat *β*-actin forward primer : 5'-ATATCGCTGCGCTCGTCGTCGACAA-3'; rat *β*-actin reverse primer : 5'-AACACAGCCTGGATGGCTACGTACAT-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 analyzed on 1.5% ethidium bromide-agarose gels run in 1X Tris-borate-EDTA buffer. 100 bp DNA ladder was purchased from Amersham Bioscience.

Transfections

Transfections and co-transfections experiments were performed using Effectene® reagent (Qiagen) as previously described using 1 μg of pGL3-Muc5ac deletion mutants [28]. Total cell extracts were prepared after a 48h incubation at 37°C using 1X 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 transcription factor of interest compared to 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

20 μg of nuclear proteins and 8 μl of prestained molecular weight markers (Life technologies) were loaded on a 10% SDS gel and electrophoresed for 1h15 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 1h at 100V using a BIO-RAD transfer apparatus. Immunostaining was performed as follows : the PVDF membrane was incubated with Tris-buffered saline-0.2% Tween20 (TBST) containing 20 % non-fat dry milk (w/v) for 2h at RT, then washed 3x5 min with TBST. Incubation with polyclonal anti-Sp1 PEP2 (sc-59) and anti-Sp3 (sc-644) antibodies (Santa Cruz laboratories) at 1:1,275 dilution in TBST was carried out for 2h at RT with shaking. The membrane was then washed 3x5 min at RT with TBST before adding alkaline phosphatase conjugated-anti-rabbit IgGs (Promega) for 30 min at RT. The membrane was washed 1x5 min with TBST and 1x5 min with substrate buffer (0.1M Tris-HCl, pH 9.5 containing 0.1M NaCl and 0.05M $MgCl_2$). Staining was developed by incubating the membrane with 10 ml of substrate buffer containing 0.33 mg/ml 4-nitroblue tetrazolium chloride and 0.16 mg/ml 5-bromo-4-chloro-3-indolyl-phosphate. The enzymatic reaction was stopped by soaking the membrane in distilled water.

Oligonucleotides and DNA probes

The sequences of the oligonucleotides used for gel-shift assays are indicated in Table II. They were synthesized by MWG-Biotech (Germany). Equimolar amounts of single-stranded oligonucleotides were annealed and radiolabeled using T4 polynucleotide kinase (Promega) and [γ^{32}P]-dATP. Radiolabeled probes were purified by chromatography on a Bio-Gel P-6 column (BIORAD, Ivry-sur-Seine, France).

Electrophoretic mobility shift assays (EMSA)

Incubation of nuclear proteins (8 μg) with radiolabeld probes, supershift analyses, and cold competitions were carried out as in [31]. Anti-Sp1, anti-Sp3, anti-Smad2, anti-Smad4, and anti-NF-κB p65 antibodies and consensus Smad4 oligonucleotide were purchased from Santa-Cruz laboratories (TEBU, France). Reactions were stopped by adding 2 μl of loading buffer. Samples were loaded onto a 4% non-denaturing polyacrylamide gel and electrophoresis conditions were as described in [29]. Gels were vacuum-dried and autoradiographed overnight at -80°C.

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 approximately 18 kb. After restriction mapping and Southern blot analysis, seven clones (pMS1-pMS7) were isolated and sequenced (see 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* mucin gene (Figure 1A). Sequence analysis of the pMS1 clone and alignment to human and rat *MUC5AC* 5'-flanking regions revealed a translational start codon at position 3211 identical to rat Muc5ac first codon and 3 short exons encoding a total of 72 amino acids with 52% identity to human MUC5AC and 79% identity to rat Muc5ac (Figure 1B).

Determination of the transcription start site of Muc5ac gene

To determine the transcription start site, 5'-RACE assays were performed on mouse gastric RNA using two nested antisense primers selected at about 300 bp 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). In conclusion, *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/RXR-α, -837/-832) or thyroid hormone (T3R-α, -634/-626) receptors were also found. In the 5'-UTR region, that is 33 nucleotides long, putative binding sites for AP-1 and Sp1 were found at +2/+10 and +14/+23, respectively. In the first exon, a putative binding site for 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 mucins was found in mucus granules of surface epithelial cells (Figure 3C). Labeling with *Muc5ac* antisense probe showed that *Muc5ac* mRNA is only expressed in the surface epithelium of the stomach (Figure 3D). The specificity of the labeling 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 4A. Absence of MUC5AC expression in KATO-III cells was previously published [8]. To define essential regions that drive transcription of *Muc5ac* promoter, ten deletion mutants, that cover 1.2 kb of the promoter, were constructed in 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 diagrams 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 portion of the distal region of the promoter. 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 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.

Regulation of Muc5ac promoter by TGF-β and Smads transcription factors

The high amount of putative binding sites for Smad transcription factors within *Muc5ac* promoter (see Figure 2) is in favor of 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*RII on the cell membrane. Thus, before studying TGF-*β* signaling pathway on *Muc5ac* expression in CMT-93 cells, we checked whether the cells expressed TGF-*β*RII. As shown in figure 5A, 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 *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 diagram 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, EMSA 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 II). Incubation of the radiolabeled probes with CMT-93 nuclear extracts resulted in one shifted band characteristic of Smad DNA complexes (Figure 6A, lanes 2, 7, 12) when compared with mobility of Smad4 consensus radiolabeled probe (lane 17). The specificity of the complexes were confirmed by total disappearance of the shifted bands when cold competition was performed (lanes 3, 8, 13, 18). Involvement of Smad4 in the DNA-protein shifted complex was then proven by inhibition of complex formation upon addition of Smad4 antibody (lanes 5, 10 and 14) whereas no effect was observed upon addition of Smad2 antibody (lanes 4, 9, and 15). The same result was obtained with -552/-529 and -92/-71 probes (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 (lane 21) when compared with untreated cells (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, that are co-factors of Smad4. As shown in figure 6B, Smad2 induces *Muc5ac* promoter activity with the strongest effect on fragment –

376/+3 (10 fold, gray bar). Smad3 transactivation of *Muc5ac* promoter is confined to the –376/+3 region of the promoter and is not as strong (4-5 fold activation, white bars, 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, black bars). Co-transfection experiments in the presence of Smad2 and Smad4 or Smad3 and Smad4 did not lead to synergistic activation of the promoter (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 (black bar) compared with cells mutated for Smad4 (white bar). The same result was obtained with fragment –376/+3 (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 neighboring 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 that Sp1/Sp3 are important regulators of mucin 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 radiolabeled probes (Table II), 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 but that could not be supershifted in the presence of anti-Sp1 or anti-Sp3 antibodies (not shown). On the other hand incubation of -83/-56 probe (Figure 7B), which contains CACCC box binding sites, with CMT-93 nuclear proteins produced three shifted complexes (lane 2, see arrows). Specificity of these complexes was confirmed by complete inhibition of complex formation when cold competition was performed with 50x excess of the cold probe (lane 3). Complex #1 was completely supershifted upon addition of specific anti-Sp1 antibody in the reaction mixture (lane 4, SS Sp1) whereas complex #2 was specifically supershifted upon addition of anti-Sp3 antibody (lane 5, SS Sp3). No supershift was observed upon addition of irrelevant NF-κB p65 antibody (lane 6). In conclusion, Sp1 and Sp3 bind to the same *cis*-element located at –76/-65 within *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 (pCMV4-Sp1, black bars) or Sp3 (pCMV4-Sp3, white bars) (Figure 7C). 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/+132) 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.

Cooperation 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 cooperative effect on the promoter. Co-transfection 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 (white bars). An additive effect was observed on the construct –376/+3 (black bars) whereas a synergistic transactivating effect was obtained on fragment –1171/+3 (gray bars). In conclusion, Sp1 appears to be the co-factor of Smad4 to activate *Muc5ac* transcription and essential elements that convey the synergistic effect are localized within the -1171/-377 region of the promoter.

Discussion

Organization, evolution, and regulation of expression of the four 11p15 mucin genes, *MUC2*, *MUC5AC*, *MUC5B* and *MUC6* have been extensively studied 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 harbor an altered profile of expression in human tumors of the respiratory, gastrointestinal and urogenital tracts [2,5,6,36] and such alterations (overexpression, repression, neoexpression) 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.

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 is less with human MUC5AC (52%). First ATG is identical to rat Muc5ac (Oinuma, accession number AB042530), which adds four amino acid residues (MLHS) when compared to human MUC5AC N-terminal amino acid sequence [33]. The TATA box sequence (TACAAAA) is conserved throughout evolution since both mouse (this report) 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 rats and shows typical features of genes with 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 what was previously described 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]. This difference is most likely due to histological differences between rodents and humans, since the epithelium of their conducting airways mostly consist of ciliated cells (upper tract) and Clara cells (lower tract) whereas mucous 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 tumor 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 out 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 favor of an important role for Sp1 in *MUC5AC* transcriptional regulation. Here, we confirmed that hypothesis by the 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 an 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.

Muc5ac promoter contains binding sites for Smad4 transcription factor throughout its sequence. Alignment of human [33] and mouse sequences (this report) 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 nontypeable *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 interact with Smad4 to induce transcription and this mechanism is TGF-β dependent [39,44,45]. The data presented in this manuscript are in favor of such a positive regulatory mechanism between Smad4 and Sp1 to explain the TGF-β-mediated

upregulation of *Muc5ac* expression in cancer cells. Since TGF-β is a pleiotropic cytokine with diverse function during development, 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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Figure legends

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 gray 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 *Muc5ac* promoter. The transcription start site +1 (bold and underlined) is located 22 nucleotides downstream of the TATA box (double underlined). The first ATG (+34) is bold, italicized and underlined. Nucleotides representing the first exon are italicized and underlined. Gray boxes indicate putative binding sites for transcription factors and boxed sequences indicate the sequences of oligonucleotides used in gel-shift assays. Broken arrows delimit the sequence of the deletion mutants used in this study.

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)** : Hematoxylin and eosin staining of gastric mucosa, **(C)** : Alcian blue and PAS staining of gastric mucosa, **(D)** : Staining of gastric mucosa with *Muc5ac* digoxigenin-labeled antisense RNA probe, **(E)** : Staining of gastric mucosa with *Muc5ac* digoxigenin-labeled

sense probe, **(F)** : Staining of colon mucosa with *Muc5ac* digoxigenin-labeled antisense probe, **(G)** : Immunostaining of Muc5ac in gastric mucosa with monoclonal 45M1 antibody. Magnification x 200.

Figure 4 : Characterization of *Muc5ac* promoter activity in CMT-93, IEC-6 and KATO-III cell lines by transient transfection. (A) : Expression of *Muc5ac* mucin gene in CMT-93 (lane 3) and IEC-6 (lane 6) cells by RT-PCR. Mouse (lane 2) and rat (lane 5) *β-actins* (2 μl) were used as internal controls. 100 bp DNA ladder (lanes 1 and 4). **(B)** : Schematic representation of the different deletion mutants used to study *Muc5ac* promoter activity. Numbering refers to transcription initiation site designated as +1, **(C)** : Luciferase activity diagrams showing *Muc5ac* promoter activity in murine rectal CMT-93, rat colon IEC-6 and human gastric KATO-III cells. Results are expressed as fold activation of luciferase activity of the deletion mutant of interest compared with the activity of empty pGL3 basic vector (white bar). Standard deviation represents the means of values obtained in triplicate in three separate experiments.

Figure 5 : Regulation of *Muc5ac* mRNA expression and promoter activity by exogenous TGF-β in CMT-93 cells. (A) : Expression of *TGF-βRII* 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 (black bars) compared to the control (Ref., untreated cells, white bar).

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 radiolabeled DNA probes, -645/-622 (lanes 1-5), -1146/-1126 (lanes 6-10), -467/-444 (lanes 11-15) and consensus Smad4 (lanes 16-19) probes. Radiolabeled probes alone (lanes 1, 6, 11, 16), nuclear extract incubated with radiolabeled probe (lanes 2, 7, 12, 17), cold competition with x50 excess of cold probe (lanes 3, 8, 13, 18), supershift analysis by preincubating nuclear extract with 1 μl of anti-Smad2 (lanes 4, 9, 15) or anti-Smad4 (lanes 5, 10, 14, 19) antibodies before adding the radiolabeled probe. The arrow on the left hand side of the gel indicates the position of the DNA-protein complex engaging Smad4. 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 (gray 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.

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 weight markers (MWM). 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 radiolabeled DNA probe. Radiolabeled probe alone (lane 1). Incubation of -83/-56 probe with CMT-93 nuclear proteins (lane 2), cold competition with x50 excess of cold probe (lane 3), supershift analysis upon addition of anti-Sp1 (lane 4) or anti-Sp3 (lane 5) antibodies. Addition of

irrelevant NF-κB p65 antibody (lane 6). DNA-protein complexes are indicated by an arrow on the left hand-side of the autoradiogram. Asterisks indicate the position of Sp1 (SS Sp1) and Sp3 (SS Sp3) supershifts. 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. Standard deviation represents the means of values obtained in triplicate in three separate experiments.

Figure 8 : Cooperation between Smad4 and Sp1 to transactivate *Muc5ac* promoter. Co-transfection experiments were performed in CMT-93 cells in the presence of pCMV-Sp1, pCMV-Smad4 or both. Results are expressed as fold activation of the luciferase activity obtained in co-transfection with the expression vector encoding Smad4, Sp1 or both compared with cells transfected with corresponding empty vector (Ref.). Standard deviation represents the means of values obtained in triplicate in three separate experiments.

Position in the promoter	Oligonucleotide sequences (5’⟶ 3’)	Orientation
-199/+3	5'-CGC ***GAG CTC*** TGG GGG AGC CTC AGG GAA -3'	S
	5'-CGC ***ACG CGT*** GAA AGA CTC TAG TCA CCA -3'	AS
-199/+132	5'-CGC ***GAG CTC*** TGG GGG AGC CTC AGG GAA -3'	S
	5’-CGC ***ACG CGT*** ACT TTG AGT CTT ACC TGT GCA-3’	AS
-376/+3	5'-CGC ***GAG CTC*** CTC CTC TCC AGG GAA ATG -3'	S
	5'-CGC ***ACG CGT*** GAA AGA CTC TAG TCA CCA -3'	AS
-376/+132	5'-CGC ***GAG CTC*** CTC CTC TCC AGG GAA ATG -3'	S
	5’-CGC ***ACG CGT*** ACT TTG AGT CTT ACC TGT GCA-3’	AS
-1021/+3	5'-CGC ***GAG CTC*** CTC TCT TTC ACA CAC ACA -3'	S
	5'-CGC ***ACG CGT*** GAA AGA CTC TAG TCA CCA -3'	AS
-1021/+132	5'-CGC ***GAG CTC*** CTC TCT TTC ACA CAC ACA -3'	S
	5’-CGC ***ACG CGT*** ACT TTG AGT CTT ACC TGT GCA-3’	AS
-1171/+3	5'-CGC ***GAG CTC*** CAC TCC TGT GAT GTG TGA -3'	S
	5'-CGC ***ACG CGT*** GAA AGA CTC TAG TCA CCA -3'	AS
-1171/+132	5'-CGC ***GAG CTC*** CAC TCC TGT GAT GTG TGA -3'	S
	5‘-CGC ***ACG CGT*** ACT TTG AGT CTT ACC TGT GCA-3’	AS
-1021/-828	5'-CGC ***GAG CTC*** CTC TCT TTC ACA CAC ACA -3'	S
	5'-CGC ***ACG CGT*** AGA GAG GTC AAA GCT TAA -3'	AS
-1171/-828	5'-CGC ***GAG CTC*** CAC TCC TGT GAT GTG TGA -3'	S
	5'-CGC ***ACG CGT*** AGA GAG GTC AAA GCT TAA -3'	AS

Table I : Sequences of the pairs of oligonucleotides used in PCR to produce deletion mutants covering *Muc5ac* promoter. *Sac*I (GAGCTC) and *Mlu*I (ACGCGT) sites (bold and italicized) are added at the end of the primers to direct subcloning into pGL3 basic vector. S : sense, AS : antisense.

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Probe	Position of putative binding site	Sequence (5' ⟶ 3')
-57/-34	Sp1 (-51/-42)	GTCACT*GGGGCTGGAG*CCAGCTCT
-83/-56	Sp1/CACCC (-76/-65)	AGACCTG*CTCCACCCACCC*ACGTGAAG
-113/-81	Sp1 (-100/-90)	GCCACTGTTTACC*TGGGTGAGGGG*AACCACAGA
-92/-71	Smad (-84/-80)	GGGAACCA*CAGAC*CTGCTCCAC
-467/-444	Smad (-458/-454)	CTCACTGGG*GTCTG*GGAACTTGAA
-552/-529	Smad (-538/-534)	TCTGTCCATCCCAG*CAGAC*ATGAA
-645/-622	Smad (-641/-637)	CCTG*GTCTG*GGCAAAGGTCCATGC
-1146/-1126	Smad (-1131/-1128)	TCTCTCTCTCTCCTC*TCTG*TC

Table II : Sequences and positions within the promoter of *Muc5ac* of the sense oligonucleotides used for EMSAs. Antisense oligonucleotides were also synthesized and annealed to the sense oligonucleotides to produce double-stranded DNA. Putative binding sites in the sequences are italicized and underlined.

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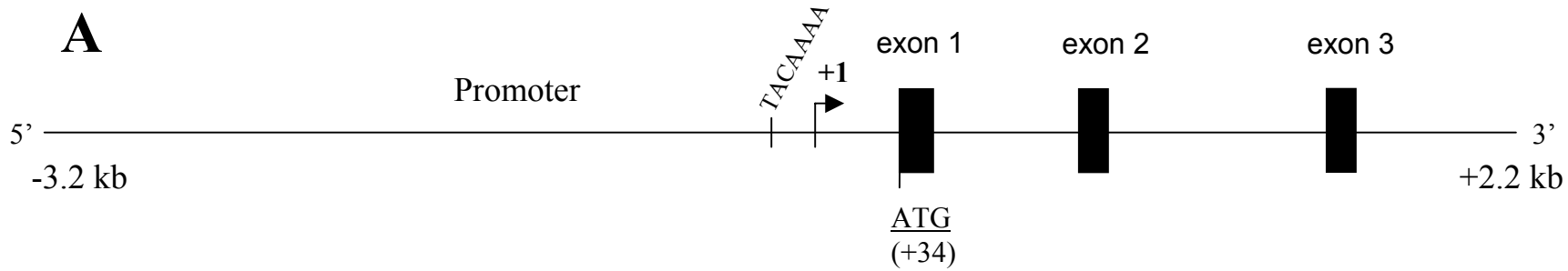
Figure 1 : Jonckheere *et al.*



B

```
                    *         20        *         40
mMuc5AC : MLHSMGVGRRKLVPFWVLALALACSQCTGQAQQDSLKSYH : 40
rMuc5AC : MLHSMGVGRRKLAPFWVLALALTFNQHTGQALEDTRKSHL : 40
hMuc5AC : ----MSVGRRKLALLWALALALACTRHTGHAQDGSSESSY : 36
          mlhsMgVGRRKLapfWvLALALac qhTGqAq ds kS

                    *         60        *         80
mMuc5AC : EHRSDV-P-PQGHVGTPLNRVTIIPPLKTIPVVR------ : 72
rMuc5AC : EHYSDLSQ-PQGHVGTPLNRVTIIPPLKTIPVVRAFNPAH : 79
hMuc5AC : KHHPALSPIARGPIGVPLRGATVFPSLRTIPVVRASNPAH : 76
          eH sdlsp pqGhvGtPLnrvTiiPpLkTIPVVRa npah
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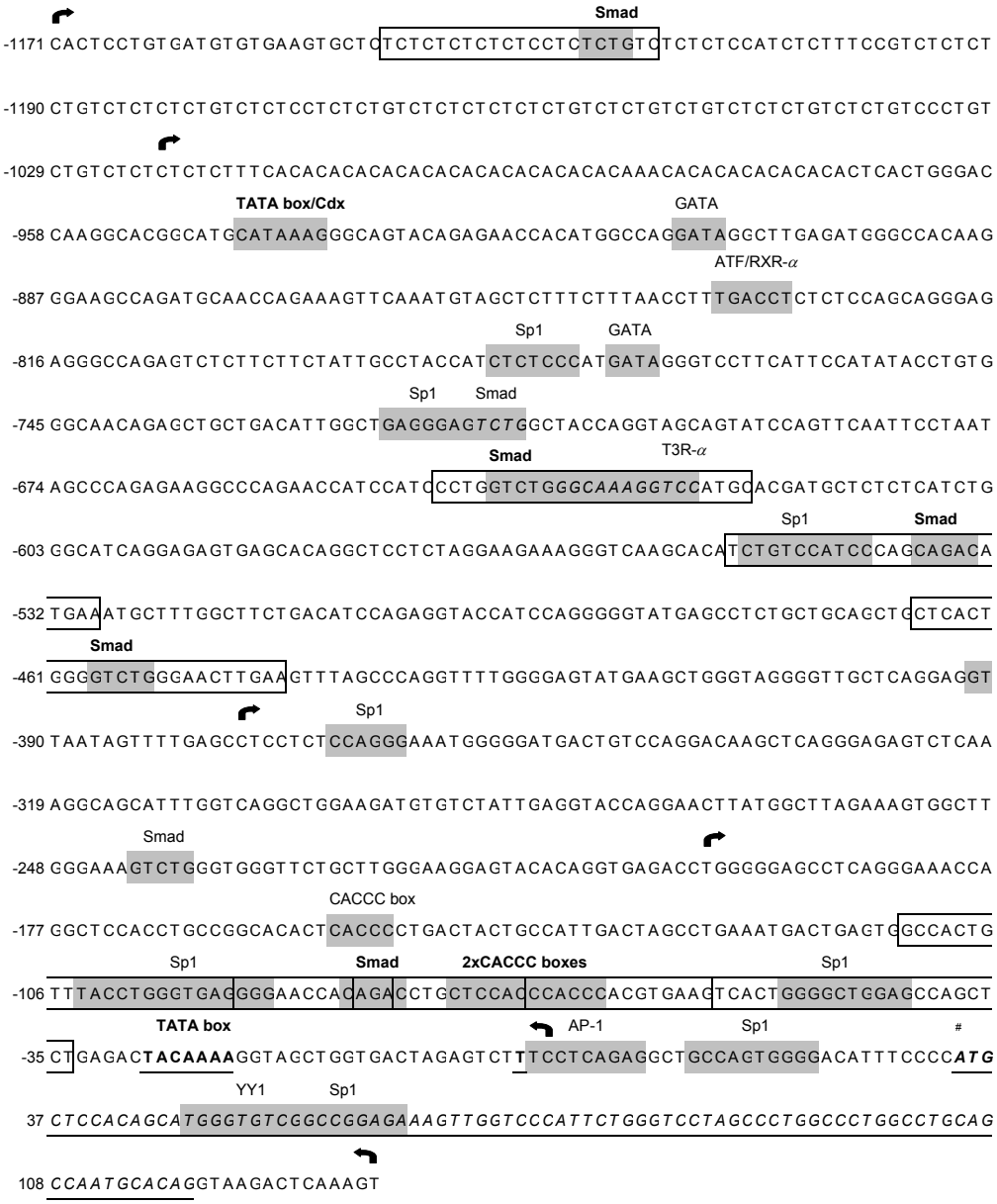

Figure 2 : Jonckheere*et al* .

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Figure 3 : Jonckheere *et al*.

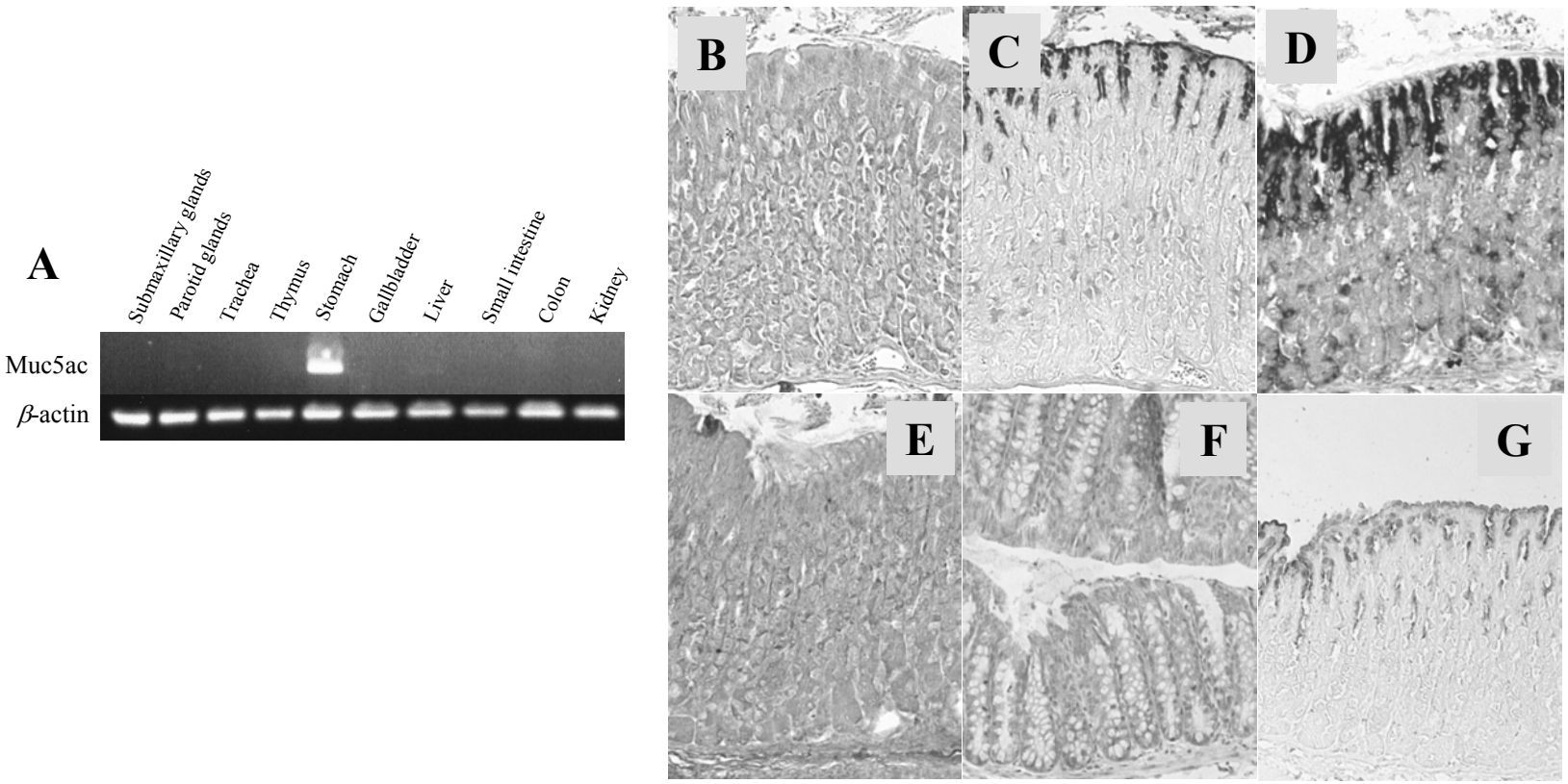

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Figure 4 : Jonckheere *et al.*

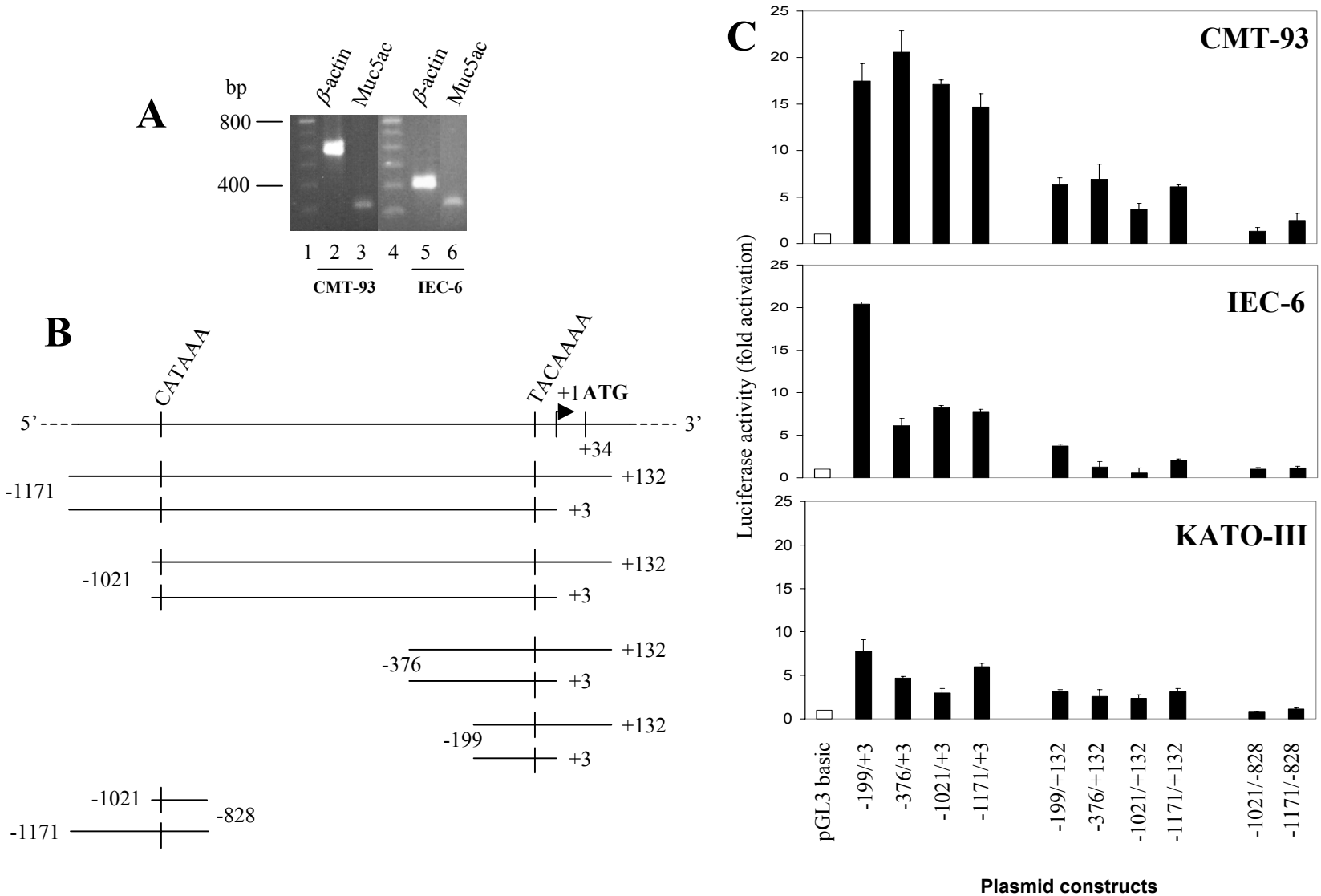

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Figure 5 : Jonckheere *et al.*

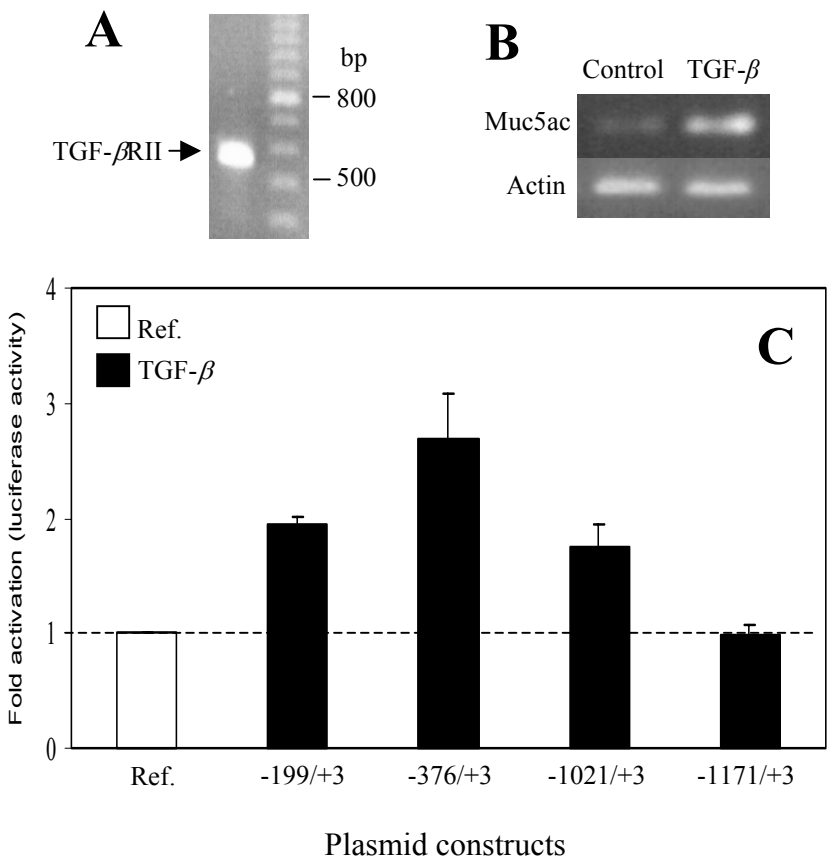

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Figure 6 : Jonckheere *et al.*

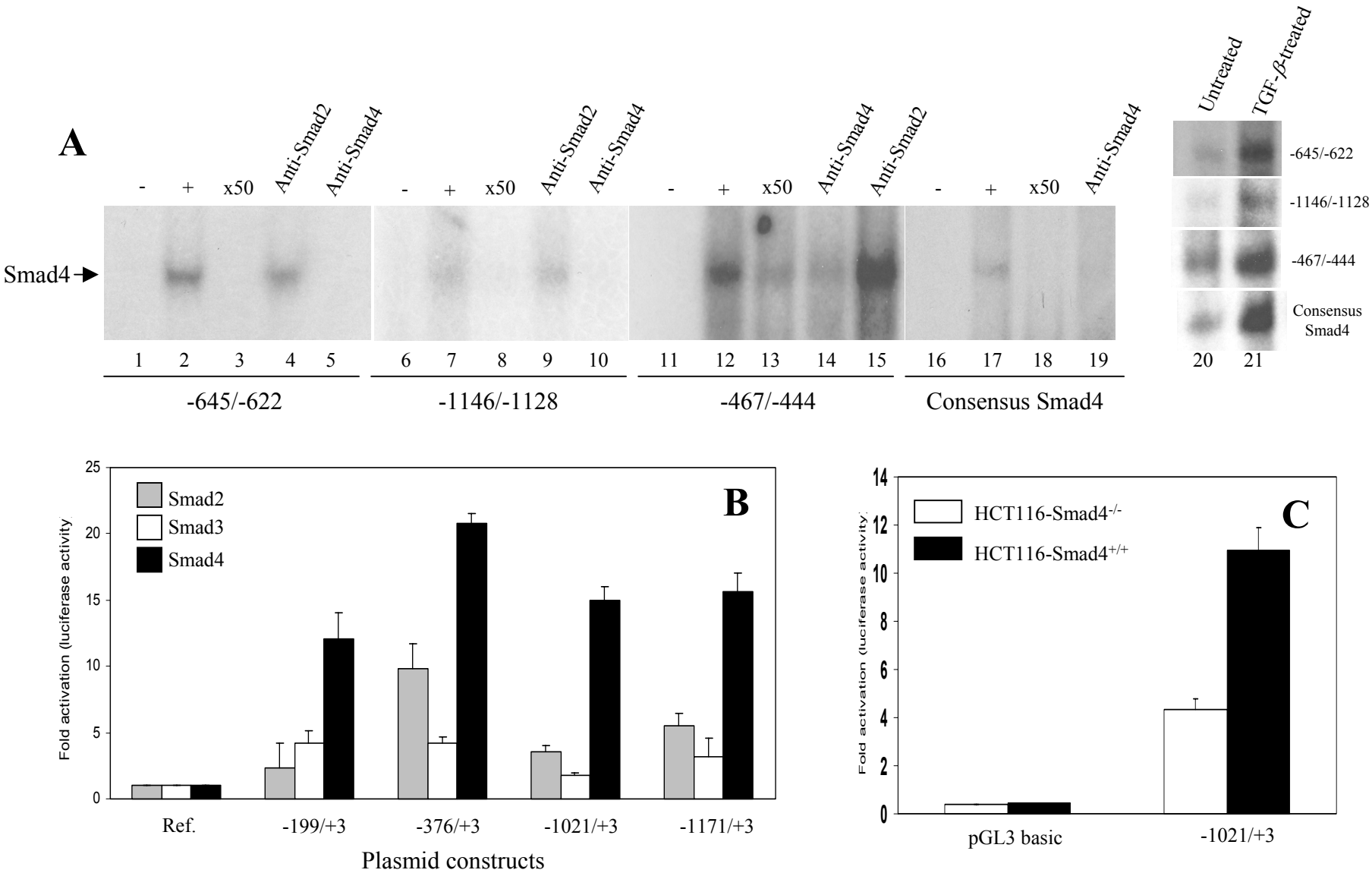

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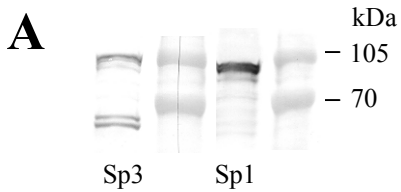
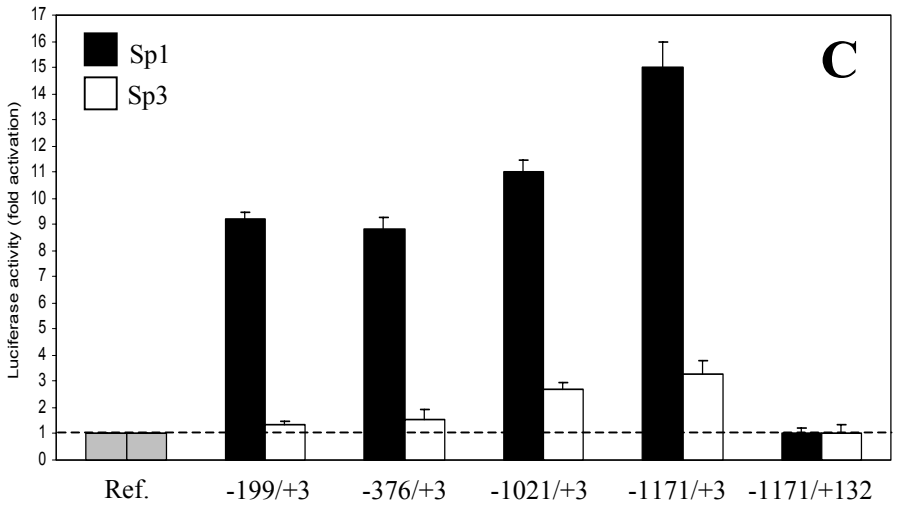
Figure 7 : Jonckheere *et al*.





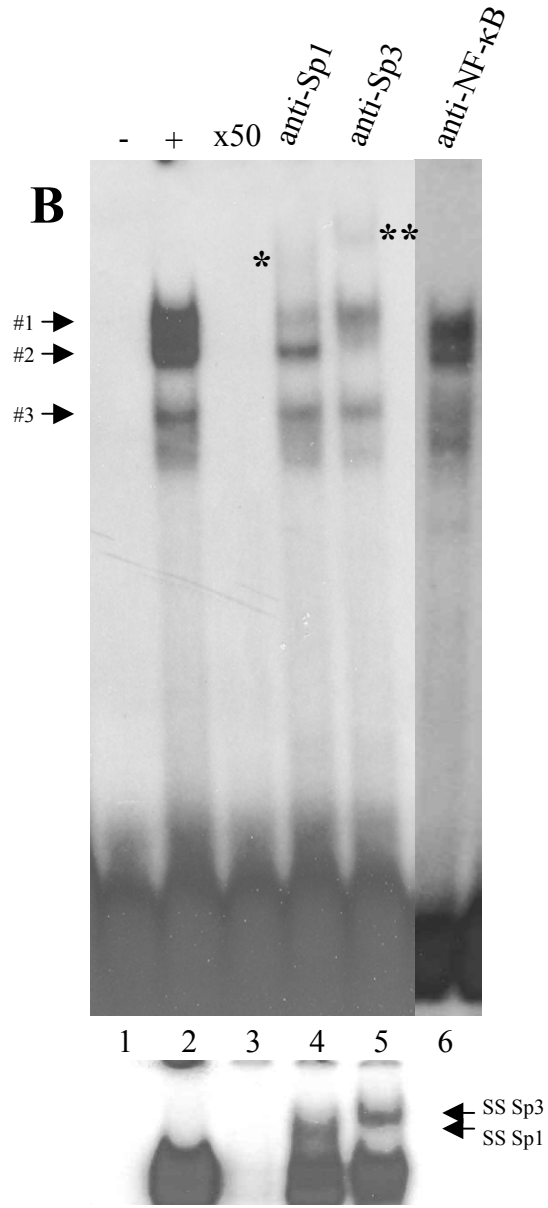


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Figure 8 : Jonckheere *et al.*