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Maria van Der Sluis, Monique H M Melis, Nicolas Jonckheere, Marie-Paule Ducourouble, Hans A Bü Ller A, et al. The murine Muc2 mucin gene is transcriptionally regulated by the zinc-finger GATA-4 transcription factor in intestinal cells. *Biochemical and Biophysical Research Communications*, 2004, 325, pp.952 - 960. <10.1016/j.bbrc.2004.10.108>. <hal-02905675>

**HAL Id: hal-02905675**

**<https://hal.science/hal-02905675v1>**

Submitted on 29 Sep 2020

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Biochemical and Biophysical Research Communications 325 (2004) 952–960

www.elsevier.com/locate/ybbrc

## The murine *Muc2* mucin gene is transcriptionally regulated by the zinc-finger GATA-4 transcription factor in intestinal cells<sup>☆</sup>

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Received 14 October 2004

### 12 Abstract

MUC2, the major mucin in the intestine, is expressed early during development and shows an altered expression pattern in intestinal bowel diseases. However, the mechanisms responsible for MUC2 expression in the intestine during these events are largely unknown. Having found putative GATA binding sites in the murine *Muc2* promoter and that GATA-4 is expressed in *Muc2*-expressing goblet cells of the mouse small intestine, we undertook to study its regulation by this transcription factor. A panel of deletion mutants made in pGL3 vector and covering 2.2 kb of the promoter were used to transfect the murine CMT-93 colorectal cancer cell line. The role of GATA-4 on *Muc2* gene regulation was investigated by RT-PCR and co-transfections in the presence of expression vectors encoding either wild-type or mutated GATA-4 or by mutating the GATA-4 site identified within *Muc2* promoter. Four GATA-4 *cis*-elements were identified in the promoter by EMSA and *Muc2* promoter was efficiently activated when GATA-4 was overexpressed in the cells with a loss of transactivation when those sites were either mutated or a mutated form of GATA-4 was used. Altogether, these results identify *Muc2*, a goblet cell marker, as a new target gene of GATA-4 and point out an important role for this factor in *Muc2* expression in the intestine.

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**Keywords:** *Muc2*; Mucin; GATA-4; Intestine; Goblet cell; Differentiation; Transcription

The mucus layer in the lumen of the gut forms a physical barrier against microorganisms and insoluble material [1]. Goblet cells are a hallmark of the intestinal epithelium, and are known to secrete mucins that participate in mucus formation to protect the underlying mucosa. The secretory mucin *Muc2*, a very large *O*-glycoprotein, is particularly relevant for the study of goblet

cell biology as it is exclusively and abundantly expressed by intestinal goblet cells [2,3]. It is produced throughout the gastro-intestinal tract increasing from small intestine to colon.

*Muc2* is expressed early during embryonic development of the intestine [4], which suggests that *Muc2* transcription is under the influence of transcription factors responsible for intestinal development and cell differentiation. The early expression of mucin before mucus cell differentiation or during the process of differentiation indicates that they may be the targets of transcription factors responsible for those programs [5]. This hypothesis has been recently confirmed in a previous study

<sup>☆</sup> Abbreviations: bp, basepair; EMSA, electrophoretic mobility shift assay; kb, kilobase; LPH, lactase phlorizin hydrolase; SI, sucrase isomaltase.

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47 where we showed that the human *MUC2* mucin gene is  
48 regulated by homeobox *Cdx1* and *Cdx2* transcription  
49 factors [6], which are both involved in intestinal cell dif-  
50 ferentiation [7].

51 GATA transcription factors belong to another fam-  
52 ily of transcription factors involved in development  
53 and cell differentiation. GATA factors are zinc fingers  
54 and are classified into two subfamilies based on struc-  
55 tural features and expression patterns. GATA-1,  
56 GATA-2, and GATA-3 are involved in hematopoiesis  
57 and neurogenesis whereas GATA-4, GATA-5, and  
58 GATA-6 possess overlapping patterns in the develop-  
59 ing cardiovascular system and in endoderm-derived  
60 tissues including the liver, lungs, pancreas, stomach,  
61 and intestine [8].

62 During embryonic development GATA-4 mRNA is  
63 expressed in the primitive intestine [9,10] in which  
64 *Muc2* is also found [4]. Its expression in the intestine is  
65 sustained during adulthood with a distinct expression  
66 pattern along the crypt-villus axis, which strongly corre-  
67 lates with the status of differentiation of the cells. It is  
68 highly expressed on the tips of the villi and its expression  
69 decreases as we go downward towards the crypt [11].  
70 Moreover, GATA factors are known to regulate intes-  
71 tine-specific genes, which are considered as markers of  
72 enterocytes, such as fatty acid binding protein [11],  
73 intestinal lactase-phlorizin hydrolase (LPH) [11–13],  
74 and sucrase isomaltase (SI) [14]. However, no GATA-  
75 4 target gene, representative of goblet cells, which are  
76 responsible for mucus production/secretion and mainte-  
77 nance of a defense line and protective barrier in intes-  
78 tinal pathophysiology [1], has been identified so far.

79 Characterization of the 5'-regulatory regions of  
80 numerous genes has demonstrated that GATA factors  
81 interact with a DNA sequence element containing the  
82 core GATA motif (A/T)GATA(A/G) [8]. Computer  
83 analysis of the murine *Muc2* promoter sequence [15] re-  
84 vealed the presence of several putative GATA binding  
85 sites throughout the promoter region.

86 Altogether, these data are in favor of a role for  
87 GATA-4 in regulating *Muc2* gene expression. Having  
88 found binding sites for that factor in the promoter of  
89 *Muc2*, and because of the spatio-temporal restricted pat-  
90 tern of expression of *MUC2* and GATA-4 along the  
91 crypt-villus axis in the intestine, we undertook to study  
92 the regulation of the promoter of mouse *Muc2* by  
93 GATA-4 in a murine colorectal cancer cell line. Implica-  
94 tions for *MUC2* expression during intestine develop-  
95 ment and cell differentiation are discussed.

## 96 Materials and methods

97 *Animals.* Adult specified pathogen-free Balb/c mice, obtained from  
98 Harlan (Zoetermeer, The Netherlands), were killed by cervical dislo-  
99 cation. The intestine was removed, fixed in 4% paraformaldehyde in

100 PBS, and subsequently processed for light microscopy as described  
101 previously [16]. The animal experiments were performed with the ap-  
102 proval of the Animal Studies Ethics Committee of the Erasmus MC  
103 (Rotterdam, The Netherlands).

104 *Immunohistochemistry.* Five micrometers of thick paraffin sections  
105 were cut and deparaffinized through a graded series of xylol-ethanol.  
106 Endogenous peroxidase activity was inactivated by 1.5% (v/v) hydro-  
107 gen peroxide in PBS for 30 min, followed by antigen retrieval in 0.01 M  
108 citrate buffer for 10 min at 100 °C. Thereafter, sections were incubated  
109 with TENG-T (10 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl, 0.25%  
110 (w/v) gelatin, and 0.05% (w/v) Tween 20) for 30 min to reduce non-  
111 specific binding. This was followed by overnight incubation with a  
112 1:2500 dilution of goat anti-GATA-4 antibody (sc-1237X, Santa Cruz  
113 Biotechnology, USA) in PBS containing 1% (w/v) bovine serum  
114 albumin and 0.1% (v/v) Triton X-100. Then, the sections were incu-  
115 bated for 1 h with biotinylated horse anti-goat IgG (diluted 1:2000,  
116 Vector Laboratories, England) followed by a 1 h incubation with  
117 ABC/PO complex (Vectastain Elite Kit, Vector Laboratories) diluted  
118 1:400. Binding was visualized after incubation in 0.5 mg/ml of 3,3'-  
119 diaminobenzidine (DAB), 0.02% (v/v) H<sub>2</sub>O<sub>2</sub> in 30 mM imidazole, and  
120 1 mM EDTA (pH 7.0). To visualize goblet cells, sections were stained  
121 with Alcian Blue 8GX (BDH, Brunschwig Chemie, Amsterdam, The  
122 Netherlands). Finally, sections were dehydrated and mounted.

123 *Muc2-pGL3 deletion mutant constructs.* The *Muc2*-pGL3 deletion  
124 mutants that cover 2.2 kb of the promoter (GenBank Accession No.  
125 AF221746) were constructed into promoterless pGL3 Basic vector  
126 (Promega) using a PCR-based method as described previously [17,18].  
127 PCRs were carried out on mouse genomic DNA using the primers  
128 depicted in Table 1. PCR products were then subcloned into pCR2.1  
129 vector (Invitrogen) before subcloning into *Bgl*II-*Mlu*I sites of the  
130 pGL3 Basic vector previously linearized with the same restriction  
131 enzymes. All clones were sequenced on both strands on an automatic  
132 LICOR sequencer using infrared-labeled RV3 and GL2 primers  
133 (Promega). Plasmids used for transfection studies were prepared using  
134 the Endofree plasmid Mega kit (Qiagen).

135 *Cell culture.* Murine rectal cancer cell line CMT-93 was cultured as  
136 described in [19]. IEC-6 cells were purchased from the European  
137 Collection of Animal Cell Cultures (ECACC). This cell line was  
138 established from rat small intestine crypt cells and was cultured in  
139 Dulbecco's modified essential medium containing 5% fetal bovine  
140 serum, 2 mM L-glutamine, 10 µg/ml insulin, 50 U/ml penicillin, and  
141 50 µg/ml streptomycin. All cells were cultured at 37 °C in a humidified  
142 5% CO<sub>2</sub> water-jacketed incubator. All reagents were from Invitrogen  
143 (Gibco) unless otherwise indicated.

144 *Transfections.* Transfection and co-transfection experiments were  
145 performed using Effectene reagent (Qiagen) as described previously  
146 [18] using 1 µg *Muc2*-pGL3 deletion mutants. Total cell extracts were  
147 prepared after a 48 h incubation at 37 °C using 1× Reagent Lysis Buffer  
148 (Promega) as described in the manufacturer's instruction manual.  
149 Luciferase activity (20 µl) was measured on a TD 20/20 luminometer  
150 (Turner Design). Total protein content in the extract (4 µl) was mea-  
151 sured using the bicinchoninic acid method in 96-well plates as de-  
152 scribed in manufacturer's instruction manual (Pierce, Bezons, France).  
153 In co-transfection experiments, 1 µg of the deletion mutant of interest  
154 was transfected with 0.25 µg of the expression plasmid encoding the  
155 transcription factor of interest (wild-type pcDNA-(WT) GATA-4 and  
156 mutated forms pcDNA-(Act) GATA-4 and pcDNA-(C290S)  
157 GATA-4) [13]. Results were expressed as fold activation of luciferase  
158 activity in samples co-transfected with the transcription factor of  
159 interest compared with the control co-transfected with the corre-  
160 sponding empty vector. To study the effect on endogenous *Muc2*  
161 expression, cells ( $0.5 \times 10^6$ ) were transfected with 4 µg of the expression  
162 vector of interest, and cultured for 48 h before being lysed and pro-  
163 cessed for total RNA preparation.

164 *RT-PCR.* Total RNA from CMT-93 cells was prepared using the  
165 RNeasy mini-kit from Qiagen. 1.5 µg of total RNA was used to  
166 prepare cDNA (Advantage RT-for-PCR kit, Clontech). PCR was

Table 1

Sequences of the pairs of primers used in PCR to produce deletion mutants covering the *Muc2* promoter

Position in the promoter	Oligonucleotide sequence (5' → 3')	Orientation
–221/+29	CGCACGCGTTTGGGGCTATGACATCCTGA CGCAGATCTGGTGGCTCACGAGGGTGGCAC	Sense Antisense
–563/+29	CGCACGCGTATGGGGTCAGACACCCGT CGCAGATCTGGTGGCTCACGAGGGTGGCAC	Sense Antisense
–729/+29	CGCACGCGTGAGGGGCTGCCAAGTTTAA CGCAGATCTGGTGGCTCACGAGGGTGGCAC	Sense Antisense
–1001/+29	CGCACGCGTGGCAAGCCAGGGACTGAAG CGCAGATCTGGTGGCTCACGAGGGTGGCAC	Sense Antisense
–1568/+29	CGCACGCGTGAGGTGGGAGGACTGGCTTC CGCAGATCTGGTGGCTCACGAGGGTGGCAC	Sense Antisense
–2213/+29	CGCACGCGTTCTTGGTCCTCAACCAAAGTT CGCAGATCTGGTGGCTCACGAGGGTGGCAC	Sense Antisense

*Bgl*II (AGATCT) and *Mlu*I (ACGCGT) sites (bold) were added at the end of the primers to direct subcloning into the pGL3 Basic vector.

167 performed on 5 µl cDNA using a specific pair of primers (MWG-  
168 Biotech, Germany) for mouse *Muc2* mucin gene (forward primer: 5'-  
169 TGTGGCCTGTGTGGGAACCTT-3'; reverse primer: 5'-CATA  
170 GAGGGCCTGTCCTCAGG-3'). Mouse β-actin (forward primer: 5'-  
171 TCACGCCATCCTGCGTCTGGACT-3'; reverse primer: 5'-CCG  
172 GACTCATCGTACTCCT-3') was used as the internal control. PCRs  
173 were carried out in 50 µl final solutions as previously described [6].  
174 Annealing temperature was 62 and 59 °C, respectively. PCR products  
175 were analyzed on a 1.5% agarose gel run in 1× Tris–borate–EDTA  
176 buffer. One hundred base pair DNA ladder was purchased from  
177 Amersham Biosciences. Expected sizes for *Muc2* and β-actin PCR  
178 products are 558 and 582 bp, respectively. RT-PCRs were carried out  
179 on cDNAs from three different sets of experiments.

180 *Nuclear extract preparation.* Nuclear extracts from CMT-93 cells  
181 were prepared as described by Van Seuning et al. [20] and kept at  
182 –80 °C until use. Protein content (2 µl of cell extracts) was measured  
183 using the bicinchoninic acid method as described above.

184 *Oligonucleotides and DNA probes.* Oligonucleotides used as probes  
185 and competitors in EMSAs are shown in Table 2. They were synthe-  
186 sized by MWG-Biotech (Ebersberg, Germany). Equimolar amounts of  
187 single-stranded oligonucleotides were annealed and radiolabeled using  
188 T4 polynucleotide kinase (Promega) and [ $\gamma$ -<sup>32</sup>P]dATP. Radiolabeled  
189 probes were purified by chromatography on a Bio-Gel P-6 column  
190 (Bio-Rad).

191 *Electrophoretic mobility shift assay.* The sequence of *Muc2* pro-  
192 moter was analyzed with MatInspector V2.2 and Alibaba2 software  
193 based on the genomix and gene-regulation databases, respectively, to  
194 determine the location of putative transcription factor binding sites

[21]. Electrophoretic mobility shift assay (EMSA) were performed as  
described previously by Mesquita et al. [6]. Supershift analyses were  
carried out using 1 µl of the anti-GATA-4 antibody (Santa Cruz, sc-  
1237X). Reactions were stopped by adding 2 µl loading buffer. Samples  
were loaded onto a 4% non-denaturing polyacrylamide gel and elec-  
trophoresis conditions were as described in [18]. Gels were vacuum-  
dried and autoradiographed overnight at –80 °C.

*Site-directed mutagenesis.* QuickChange site-directed mutagenesis  
kit (Stratagene) was used to generate site-specific mutations in the two  
proximal GATA sites found in the *Muc2* promoter at –168/–165 and  
–158/–155, respectively. The oligonucleotide containing the double  
mutation was designed according to manufacturer's instructions and  
its sequence is depicted in Table 2.

## Results and discussion

The GATA-4 transcriptions factor is expressed in mouse embryonic intestinal tissue as early as ED 9.5 [8]. Its importance in regulating several promoters of enterocyte-specific genes has been previously described [11–13]. The MUC2 mucin is a marker of another intestinal cell type, the goblet cell, and possesses a spatio-temporal pattern of expression in embryonic, fetal, and adult intestine [4,22]. However, regulation of MUC2 mucin expression during development is largely

Table 2

Sequences of the sense oligonucleotides used for EMSAs and for site-directed mutagenesis

	Sequence (5' → 3')
<i>Oligonucleotides used for EMSA</i>	
Wild-type T216 (–99/–96)	CACAGCTGTTTTCTGATAACTTGGCA
Mutated T216	CACAGCTGTTTTCT <b>C</b> TTAACTTGGCA
Wild-type T168 (–168/–165, –158/–155)	TCATATAAA <b>GATA</b> AACTCAGATAACCTG
Mutated T168	TCATATAAA <b>C</b> TTAAACT <b>C</b> TTAACTG
Wild-type T225 (–401/–398)	TCCTTATTCTATCTAGGCTGGGCT
Wild-type T169 (–1521/–1518)	TTGATCATT <b>TTT</b> TATCTCTGATGTCTTT
Mutated T169	TTGATCATT <b>TTT</b> <u>TAAG</u> CTGATGTCTTT
<i>Oligonucleotide used for site-directed mutagenesis</i>	
Double mutant (–168/–165; –158/–155)	CCAGGGAGTCATATAAA <b>C</b> TTAAACT <b>C</b> TTAACTGAATCA

Positions of the GATA binding sites, within the probe, relative to the *Muc2* transcription start site are indicated in parentheses. Putative GATA binding sites in the sequence are bold and in italics. Putative HNF binding site in T216 at –108/–104 is in italics. Mutated nucleotides are bold and underlined. Antisense oligonucleotides were also synthesized and annealed to the sense oligonucleotides to produce double-stranded DNA.

218 unknown and is mandatory to better understand intes-  
 219 tine differentiation and cell lineage establishment [23].  
 220 In this work we studied the transcriptional regulation  
 221 of murine *Muc2* by GATA-4 and mapped the cognate  
 222 *cis*-elements within the promoter. We focused on the  
 223 role of GATA-4 factor since it is known to play an  
 224 important role in directing cell lineage-specific gene  
 225 expression during development of the vertebrate gut.

226 *GATA-4 is expressed in goblet cells of the mouse small*  
 227 *intestine*

228 *Muc2* is the main mucin expressed by intestinal gob-  
 229 let cells [2]. Alcian blue staining of the mouse small  
 230 intestine indicates the presence and location of these  
 231 goblet cells (Fig. 1). Alcian blue-positive goblet cells  
 232 are present both along the villi and in the crypts. Immu-  
 233 nohistochemical staining of the same section with a  
 234 GATA-4-specific antibody showed staining of nuclei of  
 235 all the cells along the crypt-villus axis, including goblet  
 236 cells (Fig. 1). We can thus conclude that GATA-4 tran-

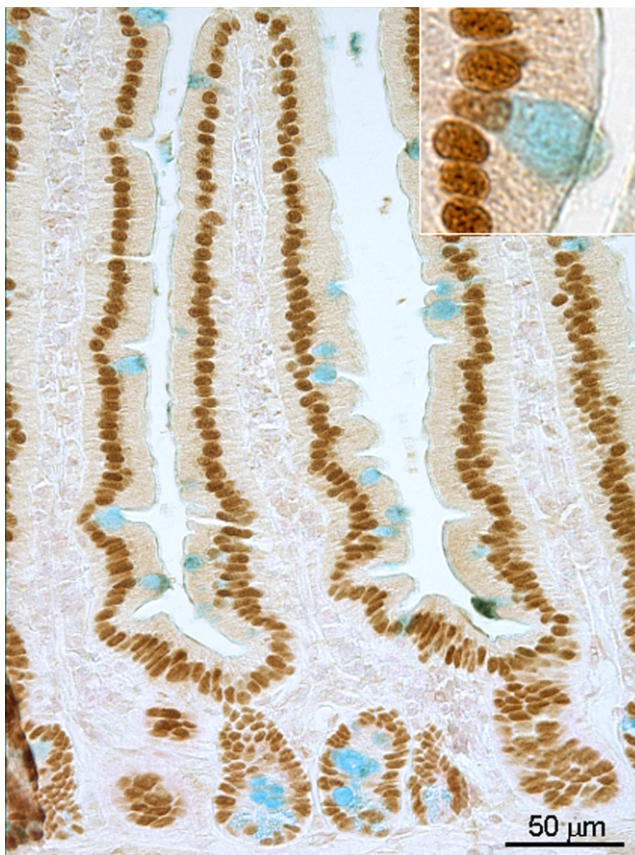


Fig. 1. Expression of GATA-4 transcription factor in mouse small intestine by immunohistochemistry. Alcian blue staining was performed as described in Material and methods. GATA-4 immunostaining was carried out on the same section of mid jejunum of an adult mice using a specific antibody for GATA-4. Sections were counterstained with hematoxylin. Inset: close-up of one GATA-4 expressing goblet cell.

scription factor is expressed in *Muc2*-expressing goblet  
 cells in the small intestine. The fact that GATA-4 has  
 been shown to play an important role in the regulation  
 of genes expressed in the intestine [11–13] and its co-  
 localization with *Muc2* in the goblet cells suggests an  
 important role for GATA-4 in the regulation of *Muc2*  
 gene. In order to confirm this hypothesis we first identi-  
 fied GATA-4 responsive regions within *Muc2* promoter  
 by co-transfection experiments in the presence of wild-  
 type or mutated forms of either *Muc2* promoter or  
 GATA-4 factor and then identified GATA-4 *cis*-ele-  
 ments by gel-shift assays.

*Muc2 promoter activity in murine and rat intestinal cells*

To define essential regions that drive *Muc2* transcrip-  
 tion, six deletion mutants covering 2.2 kb of the pro-  
 moter were constructed in the promoterless pGL3  
 Basic vector (Fig. 2A). Numbering refers to the tran-  
 scription start site designated as +1 that was previously  
 described [24]. These constructs were then transfected  
 into two different cell lines, a goblet-like cell line  
 CMT-93 [25] and a crypt-like cell line IEC-6 [26], that  
 both express *Muc2* mRNA. The luciferase diagram  
 shown in Fig. 2B, indicates that *Muc2* promoter activity  
 is the strongest in the CMT-93 cell line (black bars). In  
 both cell lines, the highest luciferase activity was ob-  
 tained with fragment  $-729/+29$ . Since the activity in-  
 creased from the fragment  $-563/+29$  to  $-729/+29$ , it  
 indicates that the  $-729/-564$  region possesses essential  
 positive regulatory elements that confer maximal activi-  
 ty to the promoter. In both cell lines, a decreased activi-  
 ty was seen with fragment  $-1568/+29$  when compared  
 with fragments  $-1001/+29$  and  $-2213/+29$ . This indi-  
 cates that there are inhibitory elements present within  
 the  $-1568/-1002$  region of the promoter. In conclusion,  
 despite being active in both cell lines, *Muc2* promoter  
 appears more active in goblet-like CMT-93 cells than  
 in crypt-like IEC-6 cells.

*GATA-4 regulates Muc2 expression at the transcriptional level*

Effect of GATA-4 overexpression on *Muc2* endoge-  
 nous expression in CMT-93 cells was studied by RT-  
 PCR (Fig. 3A). The result indicates that *Muc2* mRNA  
 level is substantially increased when GATA-4 is overex-  
 pressed in the cells (lane 2) when compared with cells  
 transfected with the empty vector (lane 1). In order to  
 identify GATA-4 responsive regions within *Muc2* pro-  
 moter, co-transfection experiments were then carried  
 out in the presence of pGL3-*Muc2* deletion mutant  
 $-221/+29$  and GATA-4 expression vector (Fig. 3B,  
 black bars). The luciferase diagram indicates that over-  
 expression of GATA-4 resulted in a 10-fold increase of  
 luciferase activity of the  $-221/+29$  construct. When a

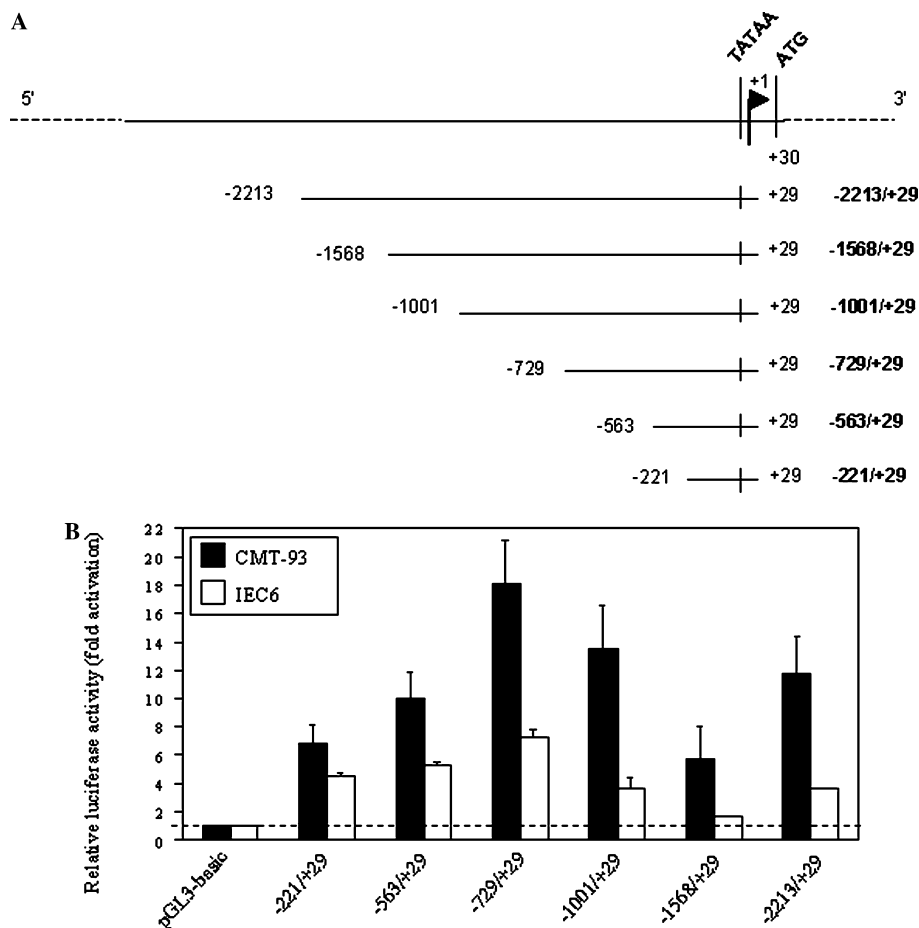


Fig. 2. *Muc2* promoter constructs and promoter activity in CMT-93 and IEC-6 intestinal cells. (A) Schematic representation of the different deletion mutants used to study *Muc2* promoter activity. Numbering refers to transcription initiation site designated +1. (B) Luciferase activity diagrams showing *Muc2* promoter activity in murine rectal CMT-93 (black bars) and rat colon IEC-6 cells (white bars). Results are expressed as fold activity of the deletion mutant of interest compared with the activity of empty pGL3 Basic vector. Results represent means  $\pm$  SD obtained in triplicate in three separate experiments.

289 mutated form of the  $-221/+29$  construct was used, in  
 290 which the GATA sites at  $-168/-165$  and  $-158/-155$   
 291 were mutated (GATA to CTTA, see Table 2), 30% of  
 292 the transactivating activity was lost (white bars). The  
 293 fact that the luciferase activity did not return to basal  
 294 levels suggests that either other GATA sites are present  
 295 with the  $-221/+29$  region and contribute to the GATA-  
 296 4-mediated transactivating activity or that GATA-4 acts  
 297 indirectly on the promoter via interactions with co-factors.  
 298 In order to investigate the latter hypothesis, we  
 299 co-transfected cells in the presence of the  $-221/+29$   
 300 deletion construct with either wild-type GATA-4 vector  
 301 or mutated forms ( $-Act$  and C290S) (Fig. 3C). The  
 302  $-Act$  GATA-4 vector encodes a mutated form of  
 303 GATA-4 in which the activation domains were deleted  
 304 whereas C290S mutant encodes a GATA-4 form bearing  
 305 a point mutation at amino acid position 290 (cysteine to  
 306 serine) in the COOH-terminal zinc finger [13]. Both  
 307 forms are not able to bind DNA cognate element. Co-  
 308 transfusions with the wild-type vector (WT GATA-4)

led to a twofold activation of both fragments tested 309  
 ( $-221/+29$  and  $-563/+29$ ). When the same experiment 310  
 was performed with the mutated GATA-4 vectors 311  
 ( $-Act$  and C290S), the luciferase activity decreased 312  
 and returned to basal levels. This implicates that the 313  
 transcription activation of the *Muc2* promoter by 314  
 GATA-4 is mediated by a direct interaction of GATA- 315  
 4 with the DNA sequence. In conclusion, these experi- 316  
 ments demonstrate that (i) GATA-4 induces *Muc2* tran- 317  
 scription, (ii) responsive elements are present within the 318  
 $-221/+29$  region of the promoter, and (iii) GATA-4 319  
 binds directly to the promoter to transactivate *Muc2* 320  
 promoter. 321

#### Identification of GATA-4 cis-elements within *Muc2* promoter 322 323

Analysis of the proximal part of *Muc2* promoter se- 324  
 quence with MatInspector V2.2 and Alibaba2 software 325  
 indicated the presence of four several putative GATA 326

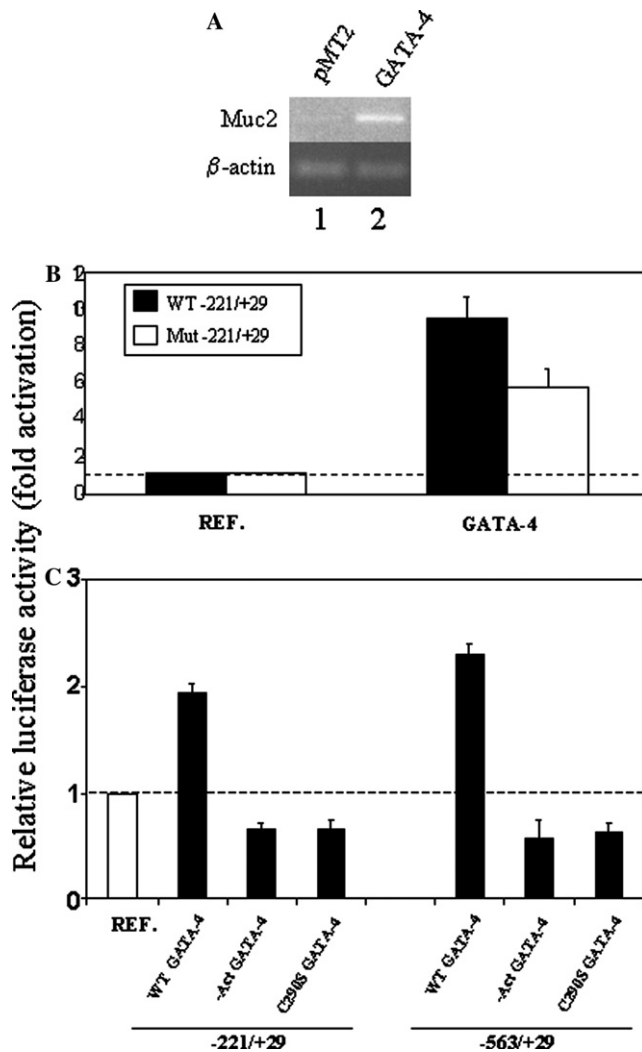


Fig. 3. Activation of *Muc2* mRNA level and promoter activity by GATA-4 in CMT-93 cells by RT-PCR and transfection assays, respectively. (A) Endogenous expression of *Muc2* mRNA after co-transfection with GATA-4 expression vector (lane 2) or corresponding empty vector (lane 1). 10  $\mu$ l (*Muc2*) and 2  $\mu$ l ( $\beta$ -actin) of the PCR products were loaded on a 1.5% agarose gel and electrophoresed in a 1 $\times$  Tris-borate buffer in the presence of ethidium bromide. (B) Luciferase diagram showing the effects of overexpression of GATA-4 on wild-type deletion construct -221/+29 (black bars) or deletion construct -221/+29 with two mutated GATA-4 sites at -168/-165; -158/-155 (white bars). (C) Luciferase diagram showing the effects of co-transfection of wild-type -221/+29 or -563/+29 pGL3-*Muc2* deletion mutants with wild-type GATA-4 (wt GATA-4) or mutated GATA-4 expression vectors (-Act GATA-4 and C290S GATA-4). Results are expressed as fold activation of luciferase activity in samples co-transfected with the transcription factor of interest compared with the control co-transfected with the corresponding empty vector (REF). Results represent means  $\pm$  SD obtained in triplicate in three separate experiments.

327 binding sites at -99/-96, -168/-165, -158/-155, and  
 328 -401/-398, respectively (Fig. 4A). They all contain the  
 329 conserved consensus sequence required for GATA bind-  
 330 ing. To confirm the binding of GATA-4 to these sites,  
 331 EMSAs were carried out in the presence of nuclear

332 extracts prepared from CMT-93 cells. As shown in Figs.  
 333 4B and C three GATA-4 binding sites were identified  
 334 in the T216 (one GATA site at -99/-96) and T168 (two  
 335 GATA sites at -168/-165 and -158/-155) probes,  
 336 respectively. When radiolabeled probes were incubated  
 337 with nuclear extracts, two specific shifted bands were  
 338 visualized with T216 probe (Fig. 4B, lane 2) whereas  
 339 only one was seen with T168 probe (Fig. 4C, lane 2).  
 340 Specificity of the protein-DNA complexes was con-  
 341 firmed by the loss of the bands when cold competitions  
 342 were performed with a 50 $\times$  excess of the cold probes  
 343 (Figs. 4B and C, lanes 3). As expected, cold competi-  
 344 tion with a 50 $\times$  excess of a cold T168 mutated probe  
 345 (Fig. 4C, lane 4) did not modify the pattern of the shift.  
 346 Moreover, when the mutated T168 probe was radiola-  
 347 beled and incubated with nuclear extracts, no shift cor-  
 348 responding to the GATA binding was visualized (Fig.  
 349 4C, lane 7). The same result was obtained with radiola-  
 350 beled mutated T216 (not shown). Finally, implication of  
 351 GATA-4 in the binding to the T216 and T168 probes  
 352 was proven by the supershifts (ssGATA-4) obtained  
 353 when nuclear extracts and radiolabeled probes were  
 354 incubated with an anti-GATA-4 antibody (Figs. 4B,  
 355 lane 4 and 4C, lane 5, respectively). The identifica-  
 356 tion of another GATA-4 *cis*-element at -99/-96 may thus  
 357 explain the result shown in Fig. 3B in which GATA-4  
 358 transactivation was not completely lost when GATA  
 359 sites at -168/-165 and -158/-155 were mutated. That  
 360 third proximal GATA-4 *cis*-element is therefore most  
 361 likely involved in the regulation of proximal *Muc2*  
 362 promoter by GATA-4. Of interest, this element (-99/-96)  
 363 is conserved in the promoter of human *MUC2* [24,27],  
 364 which makes it central in the regulation of *MUC2* by  
 365 GATA-4 between species. When EMSA was performed  
 366 with radiolabeled probe T225 containing the fourth  
 367 putative GATA binding site at -401/-398, no binding  
 368 was observed, indicating that no GATA factor is bind-  
 369 ing to that site (not shown).

370 Having found that GATA-4 transactivates the 2.2 kb  
 371 fragment of *Muc2* promoter (not shown) and that a  
 372 putative GATA binding site was present at -1521/  
 373 -1518, we undertook to study whether GATA-4 was  
 374 binding to that site. The result is shown in Fig. 4D. As  
 375 for T168, when radiolabeled T169 was incubated with  
 376 CMT-93 nuclear extracts one specific retarded band  
 377 was visualized (GATA, lane 10). The binding was lost  
 378 when cold competition was carried out in the presence  
 379 of a 50 $\times$  excess of the cold T169 probe (lane 11) whereas  
 380 nothing happened when cold mutated probe was used in  
 381 the competition (lane 12). Involvement of the GATA  
 382 consensus sequence in the binding was confirmed by ab-  
 383 sence of complex formation when the mutated probe  
 384 was radiolabeled and incubated with nuclear extracts  
 385 (lane 15). Finally, binding of GATA-4 on that element  
 386 was confirmed when supershift analysis was performed  
 387 in the presence of an anti-GATA-4 antibody in the reac-

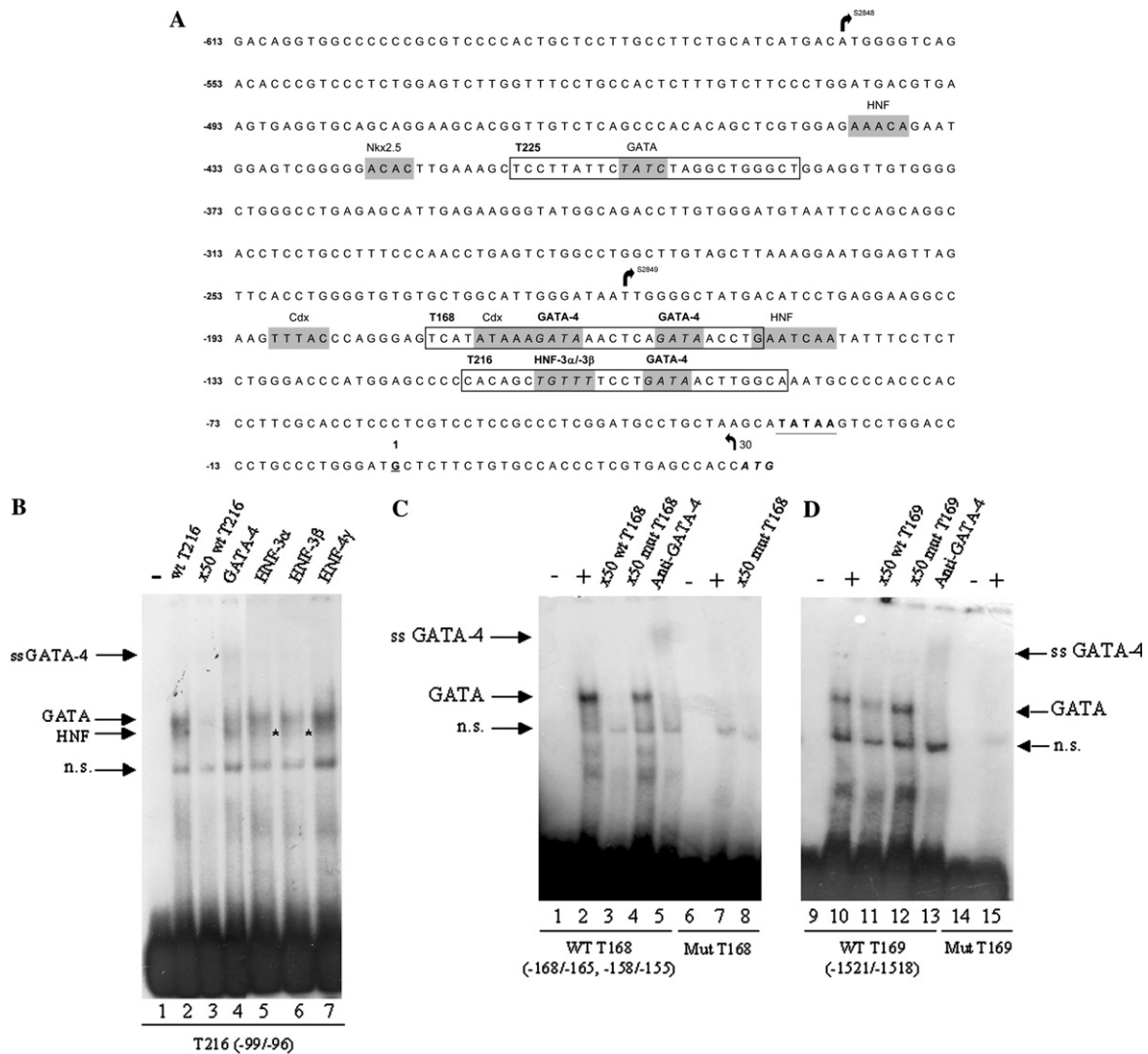


Fig. 4. Sequence of the proximal part of *Muc2* promoter and identification of GATA-4 *cis*-elements by EMSA. (A) The transcription site +1 (bold and underlined) is located 29 nucleotides downstream of the TATA box. Gray boxes indicate putative binding sites for transcription factors and boxed sequences indicate the sequences of oligonucleotides used in gel-shift assays. Arrows delineate the sequence of the deletion mutants used in this study. (B) Nuclear extracts were incubated with radiolabeled probe T216 (lanes 1–7). Probe alone (lane 1), radiolabeled T216 with nuclear extract (lane 2), and cold competition with 50× excess of wt T216 probe (lane 3). Supershift analysis was performed by preincubating the nuclear extract with 1 μl of anti-GATA-4 (lane 4), anti-HNF-3α (lane 5), anti-HNF-3β (lane 6), or anti-HNF-4γ (lane 7) antibodies, respectively. (C) Nuclear extracts were incubated with wild-type (lanes 1–5) and mutated (lanes 6–8) radiolabeled T168 probe, respectively. Probe alone (lanes 1 and 6), radiolabeled probes with nuclear extract (lanes 2 and 7), cold competition with 50× excess of wt T168 probe (lane 3), and cold competition with 50× excess of mutated T168 probe (lanes 4 and 8). Supershift analysis with 1 μl anti-GATA-4 antibody (lane 5). (D) Nuclear extracts were incubated with wild-type (lanes 9–13) and mutated (lanes 14 and 15) radiolabeled T169 probe, respectively. Probe alone (lanes 9 and 14), radiolabeled probes with nuclear extract (lanes 10 and 15), cold competition with 50× excess of wt T169 probe (lane 11), and cold competition with 50× excess of mutated T169 probe (lane 12). Supershift analysis with 1 μl anti-GATA-4 antibody (lane 13).

388 tion mixture and resulted in a complete supershifting of  
 389 the protein–DNA complex (ssGATA-4, lane 13).

390 Of interest, we noticed that when the radioactive  
 391 probe T216 was incubated with nuclear extracts (Fig.  
 392 4B, lane 2) two specific shifted bands were visualized.  
 393 As described above, the low mobility complex corre-  
 394 sponds to a GATA-4 *cis*-element. Analysis of the se-  
 395 quence showed that the T216 probe also contained a  
 396 putative HNF-3 binding site (see Fig. 4A), that may  
 397 be responsible for the second shifted band. To confirm

implication of HNF-3 factors in the binding, we per-  
 formed supershift analysis with specific anti-HNF-3α  
 and anti-HNF-3β antibodies, and one irrelevant anti-  
 HNF-4γ antibody, respectively. As can be seen in Fig.  
 4B, incubation with anti-HNF-3α (lane 5) and anti-  
 HNF-3β (lane 6) resulted in the disappearance of the  
 high mobility complex (HNF), indicated by an asterisk,  
 whereas nothing happened with anti-HNF-4γ (lane 7)  
 antibody. This result indicates that HNF-3α and  
 HNF-3β factors bind to their cognate element at

408 –108/–104, located next to the GATA-4 *cis*-element at  
 409 –99/–96 within *Muc2* proximal promoter. Previous  
 410 studies have shown that transcription factors from  
 411 HNF and GATA families are able to act synergistically  
 412 to transactivate target genes [28–30]. These mechanisms  
 413 imply either direct binding to DNA or physical interac-  
 414 tions between HNF and GATA factors. It is of impor-  
 415 tance as HNF3 factors are also involved in the  
 416 formation of mammalian gut endoderm, early lineage  
 417 specification, and intestinal cell differentiation [28,29].  
 418 Preliminary results indicate that HNF-3 $\beta$  is also ex-  
 419 pressed in *Muc2*-expressing goblet cells in the murine  
 420 small intestine (I. Renes, unpublished data), which  
 421 makes it an important factor to consider in *Muc2* regu-  
 422 lation as well. Cooperation between factors of the HNF  
 423 and GATA families has already been described such as  
 424 the interaction between GATA-5 and HNF-1 $\alpha$  to upre-  
 425 gulate LPH [14,31]. Our result suggests that such a  
 426 cooperation may exist between GATA-4 and HNF-3 $\alpha$   
 427 or HNF-3 $\beta$  to regulate *Muc2*. In conclusion, the binding  
 428 studies allowed us to identify three GATA-4 *cis*-ele-  
 429 ments within the proximal part of *Muc2* promoter at  
 430 –99/–96, –168/–165, and –158/–155, respectively,  
 431 and one in its distal part at –1521/–1518. Moreover,  
 432 a HNF-3 $\alpha$ /3 $\beta$  *cis*-element was identified at –108/–104.

433 In conclusion, we have shown that GATA-4, which  
 434 co-localizes with *Muc2* in intestinal goblet cells, is a  
 435 strong transactivator of *Muc2* gene expression.  
 436 GATA-4 induces *Muc2* transcription by directly binding  
 437 to its cognate *cis*-elements within the promoter. We also  
 438 showed that HNF-3 $\alpha$  and HNF-3 $\beta$  bind to a *cis*-element  
 439 within the proximal promoter. All these data are in fa-  
 440 vor of an important role for GATA-4 factor in *Muc2*  
 441 spatio-temporal expression pattern observed in embry-  
 442 onic, fetal, and adult small intestine, and identifies for  
 443 the first time *Muc2*, a gene that is a marker of goblet  
 444 cells, as a direct target of transcription factors involved  
 445 in intestinal development and cell differentiation. The  
 446 identification of GATA-4 as a main regulator of *Muc2*  
 447 is not only important for intestine differentiation but  
 448 also in other tissues where both GATA-4 and MUC2  
 449 are co-expressed. The gastric epithelium in which  
 450 GATA-4 is also expressed [32] is a good example, espe-  
 451 cially in gastric cancers associated with development of  
 452 intestinal metaplasia which are characterized by the ec-  
 453 topic expression of *Muc2* [33]. Thus, GATA-4 appears  
 454 as an important general regulator of *Muc2* expression  
 455 and identifies *Muc2* as a target gene of GATA-4 in dif-  
 456 ferentiated intestinal mucosa and metaplastic stomach.

#### 457 Acknowledgments

458 We thank Dr. S. Cereghini (INSERM U423, Hôpital  
 459 Necker, Paris) for the kind gift of the pMT2-GATA-4  
 460 expression vector and Dr. S.D. Krasinski (Harvard Med-

ical School, Boston, MA, USA) for the gift of pcDNA3  
 (WT)-GATA-4 vector and its mutated forms pcDNA3  
 (–Act)-GATA-4 and pcDNA3 (C290S)-GATA-4. We  
 are grateful to Dr. D. Podolsky (Massachusetts General  
 Hospital, Boston, MA, USA) for providing us with the  
 murine rectal cancer cell line CMT-93. This work was sup-  
 ported by grants from the Sophia Foundation for Medical  
 Research (M.V.D.S. and A.W.C.E.), from INSERM/  
 ZonMw-NWO (I.V.S. and A.W.C.E.), from l'Associa-  
 tion de Recherche sur le Cancer (No. 5785, I.V.S.), from  
 la Ligue Régionale du Pas de Calais Contre le Cancer  
 (I.V.S.), from l'Association François Aupetit (I.V.S.),  
 and from the European Regional Development Fund.  
 Nicolas Jonckheere is the recipient of a Conseil Régional  
 Nord-Pas de Calais and Centre Hospitalier and Univers-  
 itaire de Lille Ph.D. fellowship.

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