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The murine *Muc2* mucin gene is transcriptionally regulated by the zinc-finger GATA-4 transcription factor in intestinal cells[☆]

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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

[☆] 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

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

Immunohistochemistry. Five micrometers of thick paraffin sections
were cut and deparaffinized through a graded series of xylol-ethanol.
Endogenous peroxidase activity was inactivated by 1.5% (v/v) hydro-
gen peroxide in PBS for 30 min, followed by antigen retrieval in 0.01 M
citrate buffer for 10 min at 100 °C. Thereafter, sections were incubated
with TENG-T (10 mM Tris-HCl, 5 mM EDTA, 150 mM NaCl, 0.25%
(w/v) gelatin, and 0.05% (w/v) Tween 20) for 30 min to reduce non-
specific binding. This was followed by overnight incubation with a
1:2500 dilution of goat anti-GATA-4 antibody (sc-1237X, Santa Cruz
Biotechnology, USA) in PBS containing 1% (w/v) bovine serum
albumin and 0.1% (v/v) Triton X-100. Then, the sections were incu-
bated for 1 h with biotinylated horse anti-goat IgG (diluted 1:2000,
Vector Laboratories, England) followed by a 1 h incubation with
ABC/PO complex (Vectastain Elite Kit, Vector Laboratories) diluted
1:400. Binding was visualized after incubation in 0.5 mg/ml of 3,3'-
diaminobenzidine (DAB), 0.02% (v/v) H₂O₂ in 30 mM imidazole, and
1 mM EDTA (pH 7.0). To visualize goblet cells, sections were stained
with Alcian Blue 8GX (BDH, Brunschwig Chemie, Amsterdam, The
Netherlands). Finally, sections were dehydrated and mounted.

Muc2-pGL3 deletion mutant constructs. The *Muc2*-pGL3 deletion
mutants that cover 2.2 kb of the promoter (GenBank Accession No.
AF221746) were constructed into promoterless pGL3 Basic vector
(Promega) using a PCR-based method as described previously [17,18].
PCRs were carried out on mouse genomic DNA using the primers
depicted in Table 1. PCR products were then subcloned into pCR2.1
vector (Invitrogen) before subcloning into *Bgl*II-*Mlu*I sites of the
pGL3 Basic vector previously linearized with the same restriction
enzymes. All clones were sequenced on both strands on an automatic
LICOR sequencer using infrared-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 cultured as
described in [19]. IEC-6 cells were purchased from the European
Collection of Animal Cell Cultures (ECACC). This cell line was
established from rat small intestine crypt cells and was cultured in
Dulbecco's modified essential medium containing 5% fetal bovine
serum, 2 mM L-glutamine, 10 µg/ml insulin, 50 U/ml penicillin, and
50 µg/ml streptomycin. All cells were cultured at 37 °C in a humidified
5% CO₂ water-jacketed incubator. All reagents were from Invitrogen
(Gibco) unless otherwise indicated.

Transfections. Transfection and co-transfection experiments were
performed using Effectene reagent (Qiagen) as described previously
[18] using 1 µg *Muc2*-pGL3 deletion mutants. Total cell extracts were
prepared after a 48 h incubation at 37 °C using 1× Reagent Lysis Buffer
(Promega) as described in the manufacturer's instruction manual.
Luciferase activity (20 µl) was measured on a TD 20/20 luminometer
(Turner Design). Total protein content in the extract (4 µl) was mea-
sured using the bicinchoninic acid method in 96-well plates as de-
scribed in 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 (wild-type pcDNA-(WT) GATA-4 and
mutated forms pcDNA-(Act) GATA-4 and pcDNA-(C290S)
GATA-4) [13]. Results were 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 corre-
sponding empty vector. To study the effect on endogenous *Muc2*
expression, cells (0.5 × 10⁶) were transfected with 4 µg of the expression
vector of interest, and cultured for 48 h before being lysed and pro-
cessed for total RNA preparation.

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

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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 [γ -³²P]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 C TTAACTTGGCA
Wild-type T168 (–168/–165, –158/–155)	TCATATAAA GATA AACTCAGATAACCTG
Mutated T168	TCATATAAA C TTAAACT C TTAACTG
Wild-type T225 (–401/–398)	TCCTTATTCTATCTAGGCTGGGCT
Wild-type T169 (–1521/–1518)	TTGATCATT TTT TATCTCTGATGTCTTT
Mutated T169	TTGATCATT TTT <u>TAAG</u> CTGATGTCTTT
<i>Oligonucleotide used for site-directed mutagenesis</i>	
Double mutant (–168/–165; –158/–155)	CCAGGGAGTCATATAAA C TTAAACT C 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-

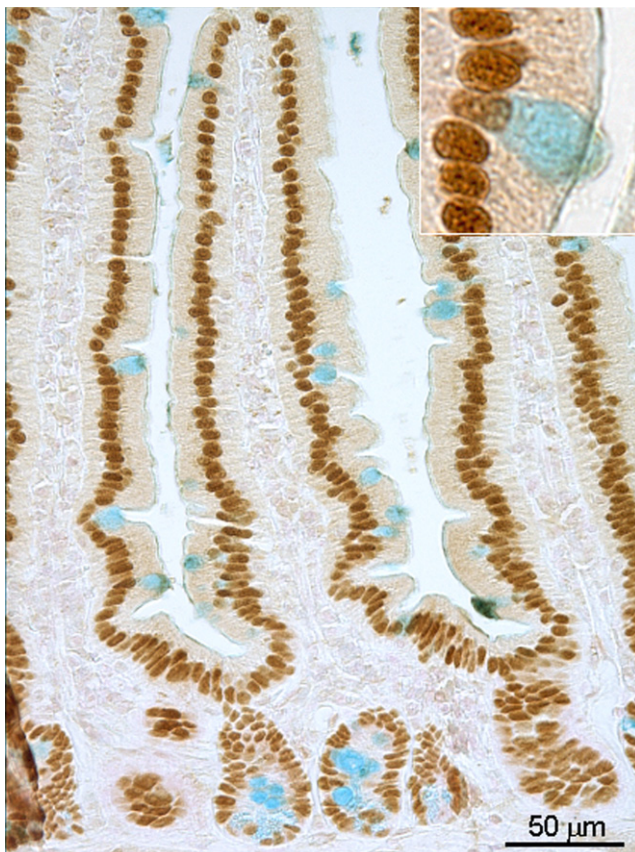


Fig. 1. Expression of GATA-4 transcription factor in mouse small intestine by immunohistochemistry. Alcian blue staining was performed as described in Material and methods. GATA-4 immunostaining was carried out on the same section of mid jejunum of an adult mice using a specific antibody for GATA-4.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

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

249 *Muc2 promoter activity in murine and rat intestinal cells*

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

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

276 Effect of GATA-4 overexpression on *Muc2* endoge-
 277 nous expression in CMT-93 cells was studied by RT-
 278 PCR (Fig. 3A). The result indicates that *Muc2* mRNA
 279 level is substantially increased when GATA-4 is overex-
 280 pressed in the cells (lane 2) when compared with cells
 281 transfected with the empty vector (lane 1). In order to
 282 identify GATA-4 responsive regions within *Muc2* pro-
 283 moter, co-transfection experiments were then carried
 284 out in the presence of pGL3-*Muc2* deletion mutant
 285 $-221/+29$ and GATA-4 expression vector (Fig. 3B,
 286 black bars). The luciferase diagram indicates that over-
 287 expression of GATA-4 resulted in a 10-fold increase of
 288 luciferase activity of the $-221/+29$ construct. When a

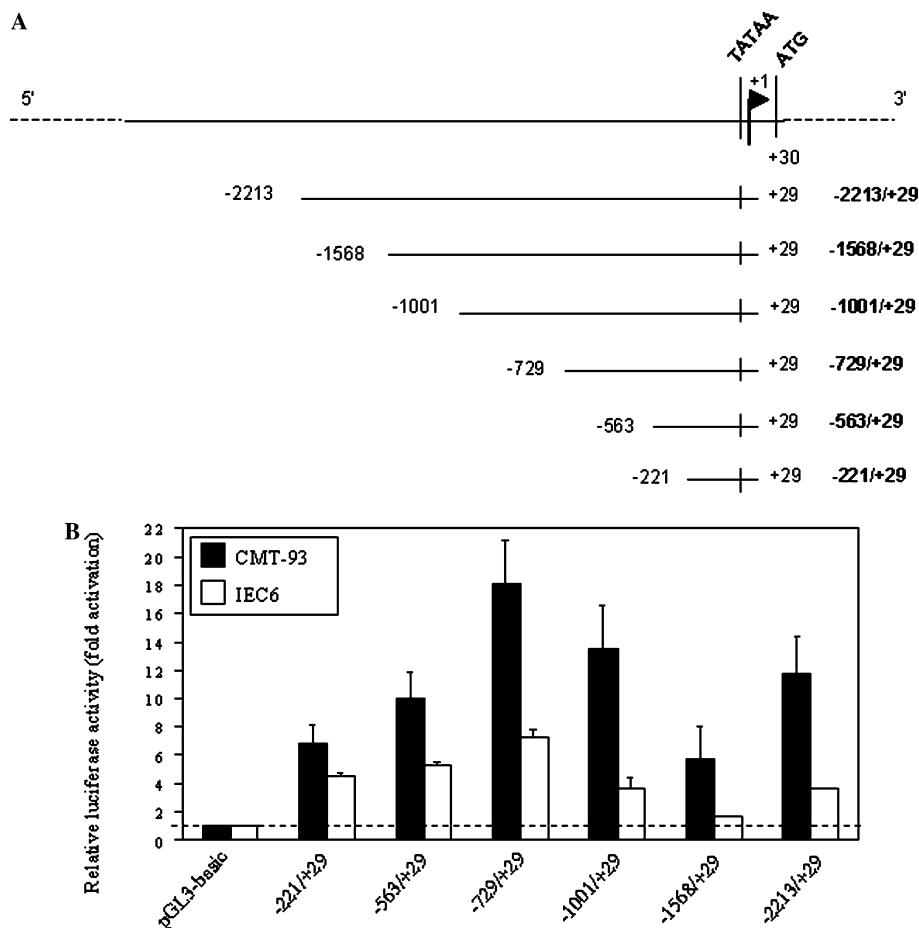


Fig. 2. *Muc2* promoter constructs and promoter activity in CMT-93 and IEC-6 intestinal cells. (A) Schematic representation of the different deletion mutants used to study *Muc2* promoter activity. Numbering refers to transcription initiation site designated +1. (B) Luciferase activity diagrams showing *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

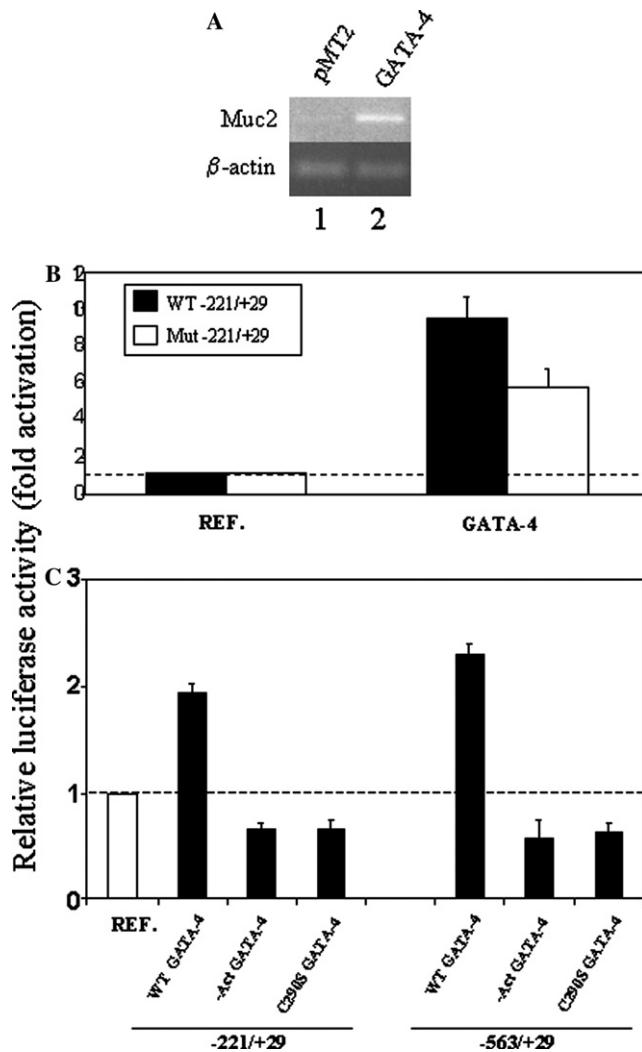


Fig. 3. Activation of *Muc2* mRNA level and promoter activity by GATA-4 in CMT-93 cells by RT-PCR and transfection assays, respectively. (A) Endogenous expression of *Muc2* mRNA after co-transfection with GATA-4 expression vector (lane 2) or corresponding empty vector (lane 1). 10 μ l (*Muc2*) and 2 μ l (β -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. 332
 333 4B and C three GATA-4 binding sites were identified 333
 334 in the T216 (one GATA site at -99/-96) and T168 (two 334
 335 GATA sites at -168/-165 and -158/-155) probes, 335
 336 respectively. When radiolabeled probes were incubated 336
 337 with nuclear extracts, two specific shifted bands were 337
 338 visualized with T216 probe (Fig. 4B, lane 2) whereas 338
 339 only one was seen with T168 probe (Fig. 4C, lane 2). 339
 340 Specificity of the protein-DNA complexes was con- 340
 341 firmed by the loss of the bands when cold competi- 341
 342 tions were performed with a 50 \times excess of the cold 342
 343 probes (Figs. 4B and C, lanes 3). As expected, cold 343
 344 competition with a 50 \times excess of a cold T168 mutated 344
 345 probe (Fig. 4C, lane 4) did not modify the pattern of the 345
 346 shift. Moreover, when the mutated T168 probe was radiola- 346
 347 beled and incubated with nuclear extracts, no shift cor- 347
 348 responding to the GATA binding was visualized (Fig. 348
 349 4C, lane 7). The same result was obtained with radiola- 349
 350 beled mutated T216 (not shown). Finally, implication of 350
 351 GATA-4 in the binding to the T216 and T168 probes 351
 352 was proven by the supershifts (ssGATA-4) obtained 352
 353 when nuclear extracts and radiolabeled probes were 353
 354 incubated with an anti-GATA-4 antibody (Figs. 4B, 354
 355 lane 4 and 4C, lane 5, respectively). The identifica- 355
 356 tion of another GATA-4 *cis*-element at -99/-96 may thus 356
 357 explain the result shown in Fig. 3B in which GATA-4 357
 358 transactivation was not completely lost when GATA 358
 359 sites at -168/-165 and -158/-155 were mutated. That 359
 360 third proximal GATA-4 *cis*-element is therefore most 360
 361 likely involved in the regulation of proximal *Muc2* 361
 362 promoter by GATA-4. Of interest, this element (-99/-96) 362
 363 is conserved in the promoter of human *MUC2* [24,27], 363
 364 which makes it central in the regulation of *MUC2* by 364
 365 GATA-4 between species. When EMSA was performed 365
 366 with radiolabeled probe T225 containing the fourth 366
 367 putative GATA binding site at -401/-398, no binding 367
 368 was observed, indicating that no GATA factor is bind- 368
 369 ing to that site (not shown). 369

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

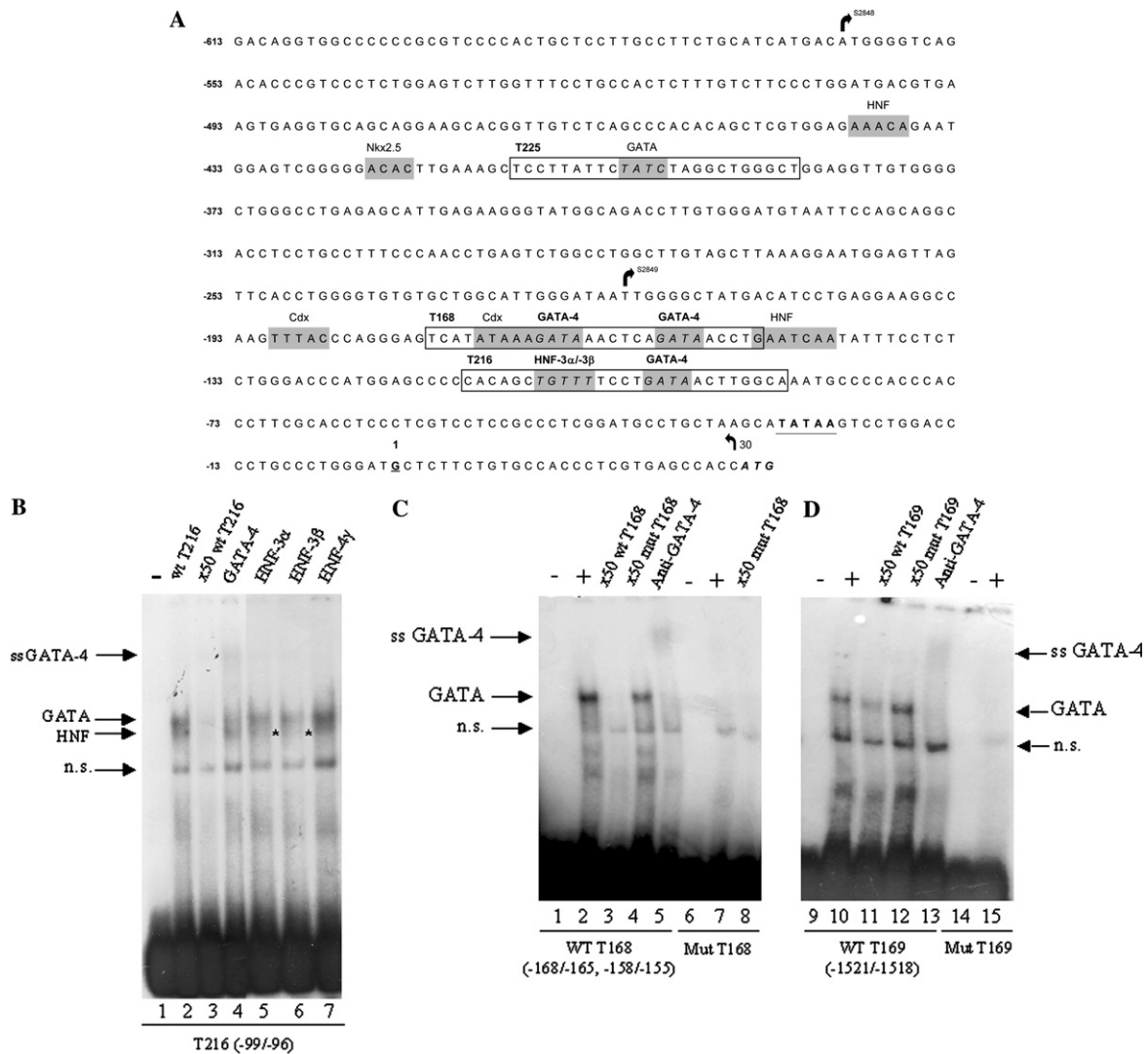


Fig. 4. Sequence of the proximal part of *Muc2* promoter and identification of GATA-4 *cis*-elements by EMSA. (A) The transcription site +1 (bold and underlined) is located 29 nucleotides downstream of the TATA box. Gray boxes indicate putative binding sites for transcription factors and boxed sequences 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- 398
 formed supershift analysis with specific anti-HNF-3α 399
 and anti-HNF-3β antibodies, and one irrelevant anti- 400
 HNF-4γ antibody, respectively. As can be seen in Fig. 401
 4B, incubation with anti-HNF-3α (lane 5) and anti- 402
 HNF-3β (lane 6) resulted in the disappearance of the 403
 high mobility complex (HNF), indicated by an asterisk, 404
 whereas nothing happened with anti-HNF-4γ (lane 7) 405
 antibody. This result indicates that HNF-3α and 406
 HNF-3β factors bind to their cognate element at 407

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 β 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 α to upre-
 425 gulate LPH [14,31]. Our result suggests that such a
 426 cooperation may exist between GATA-4 and HNF-3 α
 427 or HNF-3 β 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 α /3 β *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 α and HNF-3 β 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.

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