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

Maria van der Sluis^{a,b}, Monique H.M. Melis^a, Nicolas Jonckheere^c,
Marie-Paule Ducourouble^c, Hans A. Büller^a, Ingrid Renes^b,
Alexandra W.C. Einerhand^a, Isabelle Van Seuningen^{c,*}

^a Laboratory of Paediatrics, Department of Gastroenterology and Nutrition, Erasmus MC and Sophia Children Hospital, Rotterdam, The Netherlands

^b Laboratory of Paediatrics, Department of Neonatology, Erasmus MC and Sophia Children Hospital, Rotterdam, The Netherlands

^c Unité INSERM No. 560, Place de Verdun, 59045 Lille Cedex, France

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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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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.

* Corresponding author. Fax: +33 320 53 85 62.

E-mail address: isabelvs@lille.inserm.fr (I. Van Seuningen).

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

GATA transcription factors belong to another family of transcription factors involved in development and cell differentiation. GATA factors are zinc fingers and are classified into two subfamilies based on structural features and expression patterns. GATA-1, GATA-2, and GATA-3 are involved in hematopoiesis and neurogenesis whereas GATA-4, GATA-5, and GATA-6 possess overlapping patterns in the developing cardiovascular system and in endoderm-derived tissues including the liver, lungs, pancreas, stomach, and intestine [8].

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

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

Altogether, these data are in favor of a role for GATA-4 in regulating *Muc2* gene expression. Having found binding sites for that factor in the promoter of *Muc2*, and because of the spatio-temporal restricted pattern of expression of *MUC2* and GATA-4 along the crypt-villus axis in the intestine, we undertook to study the regulation of the promoter of mouse *Muc2* by GATA-4 in a murine colorectal cancer cell line. Implications for *MUC2* expression during intestine development and cell differentiation are discussed.

Materials and methods

Animals. Adult specified pathogen-free Balb/c mice, obtained from Harlan (Zoetermeer, The Netherlands), were killed by cervical dislocation. 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 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) hydrogen 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 incubated 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, Brunswick 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 *BglII-MluI* 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 measured using the bicinchoninic acid method in 96-well plates as described 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 corresponding empty vector. To study the effect on endogenous *Muc2* expression, cells (0.5×10^6) were transfected with 4 µg of the expression vector of interest, and cultured for 48 h before being lysed and processed 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

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	Sense
	CGCAGATCTGGTGGCTCACGAGGGTGGCAC	Antisense
–563/+29	CGCACGCGTATGGGGTCAGACACCCGT	Sense
	CGCAGATCTGGTGGCTCACGAGGGTGGCAC	Antisense
–729/+29	CGCACGCGTGAGGGCTGCCAAGTTTAA	Sense
	CGCAGATCTGGTGGCTCACGAGGGTGGCAC	Antisense
–1001/+29	CGCACGCGTGGCAAGCCCAGGGACTGAAG	Sense
	CGCAGATCTGGTGGCTCACGAGGGTGGCAC	Antisense
–1568/+29	CGCACGCGTGAGGTGGGAGGACTGGCTTC	Sense
	CGCAGATCTGGTGGCTCACGAGGGTGGCAC	Antisense
–2213/+29	CGCACGCGTTCTTGGTCCTCAACCAAAGTT	Sense
	CGCAGATCTGGTGGCTCACGAGGGTGGCAC	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.

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

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

Oligonucleotides and DNA probes. Oligonucleotides used as probes and competitors in EMSAs are shown in Table 2. They were synthesized by MWG-Biotech (Ebersberg, Germany). Equimolar amounts of single-stranded oligonucleotides were annealed and radiolabeled using T4 polynucleotide kinase (Promega) and [γ -³²P]dATP. Radiolabeled probes were purified by chromatography on a Bio-Gel P-6 column (Bio-Rad).

Electrophoretic mobility shift assay. The sequence of *Muc2* promoter was analyzed with MatInspector V2.2 and Alibaba2 software based on the genomix and gene-regulation databases, respectively, to 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 electrophoresis 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)	CACAGCTGTTTTCTCTGATAAAGTGGCA
Mutated T216	CACAGCTGTTTTCTCTTAAGTGGCA
Wild-type T168 (–168/–165, –158/–155)	TCATATAAAGATAAAGTCAAGTAACTG
Mutated T168	TCATATAAAGATAAAGTCAAGTAACTG
Wild-type T225 (–401/–398)	TCCTTATTCTATCTAGGCTGGGCT
Wild-type T169 (–1521/–1518)	TTGATCATTTTATCTCTGATGTCTTT
Mutated T169	TTGATCATTTTAAAGTCTGATGTCTTT
<i>Oligonucleotide used for site-directed mutagenesis</i>	
Double mutant (–168/–165; –158/–155)	CCAGGGAGTCATATAAAGATAAAGTCAAGTAACTGAACTCA

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.

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

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

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

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 identified 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*-elements by gel-shift assays.

Muc2 promoter activity in murine and rat intestinal cells

To define essential regions that drive *Muc2* transcription, six deletion mutants covering 2.2 kb of the promoter were constructed in the promoterless pGL3 Basic vector (Fig. 2A). Numbering refers to the transcription 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 obtained with fragment –729/+29. Since the activity increased from the fragment –563/+29 to –729/+29, it indicates that the –729/–564 region possesses essential positive regulatory elements that confer maximal activity to the promoter. In both cell lines, a decreased activity was seen with fragment –1568/+29 when compared with fragments –1001/+29 and –2213/+29. This indicates 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* endogenous 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 overexpressed 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* promoter, 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 overexpression of GATA-4 resulted in a 10-fold increase of luciferase activity of the –221/+29 construct. When a

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.

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-fac-
 298 tors. 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 transfections with the wild-type vector (WT GATA-4)

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

Identification of GATA-4 cis-elements within *Muc2* promoter

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

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 (- Δ 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.

extracts prepared from CMT-93 cells. As shown in Figs. 4B and C three GATA-4 binding sites were identified in the T216 (one GATA site at -99/-96) and T168 (two GATA sites at -168/-165 and -158/-155) probes, respectively. When radiolabeled probes were incubated with nuclear extracts, two specific shifted bands were visualized with T216 probe (Fig. 4B, lane 2) whereas only one was seen with T168 probe (Fig. 4C, lane 2). Specificity of the protein-DNA complexes was confirmed by the loss of the bands when cold competitions were performed with a 50 $\times$ excess of the cold probes (Figs. 4B and C, lanes 3). As expected, cold competition with a 50 $\times$ excess of a cold T168 mutated probe (Fig. 4C, lane 4) did not modify the pattern of the shift. Moreover, when the mutated T168 probe was radiolabeled and incubated with nuclear extracts, no shift corresponding to the GATA binding was visualized (Fig. 4C, lane 7). The same result was obtained with radiolabeled mutated T216 (not shown). Finally, implication of GATA-4 in the binding to the T216 and T168 probes was proven by the supershifts (ssGATA-4) obtained when nuclear extracts and radiolabeled probes were incubated with an anti-GATA-4 antibody (Figs. 4B, lane 4 and 4C, lane 5, respectively). The identification of another GATA-4 *cis*-element at -99/-96 may thus explain the result shown in Fig. 3B in which GATA-4 transactivation was not completely lost when GATA sites at -168/-165 and -158/-155 were mutated. That third proximal GATA-4 *cis*-element is therefore most likely involved in the regulation of proximal *Muc2* promoter by GATA-4. Of interest, this element (-99/-96) is conserved in the promoter of human *MUC2* [24,27], which makes it central in the regulation of *MUC2* by GATA-4 between species. When EMSA was performed with radiolabeled probe T225 containing the fourth putative GATA binding site at -401/-398, no binding was observed, indicating that no GATA factor is binding to that site (not shown).

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

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

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).

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

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

implication of HNF-3 factors in the binding, we performed 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

–108/–104, located next to the GATA-4 *cis*-element at –99/–96 within *Muc2* proximal promoter. Previous studies have shown that transcription factors from HNF and GATA families are able to act synergistically to transactivate target genes [28–30]. These mechanisms imply either direct binding to DNA or physical interactions between HNF and GATA factors. It is of importance as HNF3 factors are also involved in the formation of mammalian gut endoderm, early lineage specification, and intestinal cell differentiation [28,29]. Preliminary results indicate that HNF-3 β is also expressed in *Muc2*-expressing goblet cells in the murine small intestine (I. Renes, unpublished data), which makes it an important factor to consider in *Muc2* regulation as well. Cooperation between factors of the HNF and GATA families has already been described such as the interaction between GATA-5 and HNF-1 α to upregulate LPH [14,31]. Our result suggests that such a cooperation may exist between GATA-4 and HNF-3 α or HNF-3 β to regulate *Muc2*. In conclusion, the binding studies allowed us to identify three GATA-4 *cis*-elements within the proximal part of *Muc2* promoter at –99/–96, –168/–165, and –158/–155, respectively, and one in its distal part at –1521/–1518. Moreover, a HNF-3 α /3 β *cis*-element was identified at –108/–104.

In conclusion, we have shown that GATA-4, which co-localizes with *Muc2* in intestinal goblet cells, is a strong transactivator of *Muc2* gene expression. GATA-4 induces *Muc2* transcription by directly binding to its cognate *cis*-elements within the promoter. We also showed that HNF-3 α and HNF-3 β bind to a *cis*-element within the proximal promoter. All these data are in favor of an important role for GATA-4 factor in *Muc2* spatio-temporal expression pattern observed in embryonic, fetal, and adult small intestine, and identifies for the first time *Muc2*, a gene that is a marker of goblet cells, as a direct target of transcription factors involved in intestinal development and cell differentiation. The identification of GATA-4 as a main regulator of *Muc2* is not only important for intestine differentiation but also in other tissues where both GATA-4 and MUC2 are co-expressed. The gastric epithelium in which GATA-4 is also expressed [32] is a good example, especially in gastric cancers associated with development of intestinal metaplasia which are characterized by the ectopic expression of *Muc2* [33]. Thus, GATA-4 appears as an important general regulator of *Muc2* expression and identifies *Muc2* as a target gene of GATA-4 in differentiated intestinal mucosa and metaplastic stomach.

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