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Nicolas Jonckheere[1,2,3], Audrey Vincent[1,2,3], Hélène Franquet-Ansart[1,2, 4], Janneke Witte-Bouma[5], Anita Korteland-van Male[5], Emmanuelle Leteurtre[1,2,4], Ingrid B. Renes[5], and Isabelle Van Seuningen[1,2,3]

[1]Inserm, UMR837, Jean Pierre Aubert Research Center, Team #5 "Mucins, epithelial differentiation and carcinogenesis", rue Polonovski, 59045 Lille Cedex, France

[2]Université Lille Nord de France, 1 Place de Verdun, 59045 Lille cedex, France

[3]Centre Hospitalier Régional et Universitaire de Lille, Place de Verdun, 59037 LILLE cedex, France

[4]Centre de Biologie-Pathologie, Centre Hospitalier Régional et Universitaire de Lille, 2 avenue Oscar Lambret, 59037 Lille Cedex, France

[5]Erasmus Medical Center-Sophia, Laboratory of Pediatrics, Division of Neonatology, Dr. Molewaterplein 50, 3015 GE Rotterdam, the Netherlands.

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To whom correspondence should be sent:

Nicolas Jonckheere, Ph.D. Inserm UMR837, Rue Polonovski, 59045 Lille cedex, France

Phone : 33.320.29.88.50, FAX : 33.320.53.85.62, E-mail : nicolas.jonckheere@inserm.fr

Abbreviations

bp	basepair
ED	Embryonic Day
EMSA	Electrophoretic Mobility Shift Assay
HNF	Hepatocyte Nuclear Factor
kb	kilobase
PCR	Polymerase Chain Reaction
PND	Post Natal Day
RT	Reverse Transcriptase
TF	Transcription Factor

Abstract

During human embryonic and fetal development of the gastrointestinal tract, the gene encoding the *MUC5AC* mucin has a spatio-temporal pattern of expression restricted to the stomach. In order to better understand the molecular mechanisms responsible for this restricted pattern of expression, we have studied Muc5ac expression in the developing stomach of the mouse and correlated it to that of transcription factors known to be involved in cell differentiation programs during development. Our results indicate that GATA-6 and HNF-4α expression increased concomitantly with the induction of Muc5ac expression in embryonic stomach. We then studied *Muc5ac* transcriptional regulation by these transcription factors and showed that they all transactivate *Muc5ac* promoter. We also identified several active GATA-4/-5/-6 and HNF-1/-4 *cis*-elements using gel shift assays, chromatin immunoprecipitation and site-directed mutagenesis. Among all *Muc5ac* regulators, only GATA-6 and HNF-4*a* expression was concomitant to that of Muc5ac in the developing stomach. This is thus in favour of an important role for these two transcription factors as regulators of expression of the Muc5ac mucin during stomach development and in epithelial cancer cells.

1. Introduction

Mucins are large *O*-glycoproteins expressed either at the cell surface as transmembrane proteins or as secreted oligomeric molecules to form a protective mucous gel [1-3]. In the stomach, MUC5AC is one of the main secreted mucins where it plays a cytoprotective role against acid and pepsin in the gastric juice, against deleterious effects of exogenous agents (pathogens, drugs) and against mechanical damage [2, 4]. In humans, *MUC5AC* is encoded by a gene located on the p15 arm of chromosome 11 within a cluster of four mucin genes along with *MUC2*, *MUC5B* and *MUC6* [5]. In the mouse, the organization of the cluster and the structure of the genes are conserved and the mucin gene cluster is located in the syntenic region (murine chromosome 7) of the human chromosome 11 [6].

During human embryonic and fetal development of the gastrointestinal tract, *MUC5AC* has a spatially restricted pattern of expression, which suggests that this mucin may play a role in gastrointestinal cell differentiation and proliferation [7-9]. *MUC5AC* is expressed as early as 8 weeks of gestation in embryonic stomach [9]. In developing stomach it is first visible in the region destined to become the antrum and later it becomes visible in the fundus. In the normal adult, MUC5AC is expressed in the mucin producing gastric pit cells of the stomach [1, 4]. In the mouse, Muc5ac pattern of expression during development remains to be determined. Moreover, the molecular mechanisms that govern mouse *Muc5ac* expression are also unknown and most likely imply tight regulation by transcription factors (TF) involved in gastrointestinal differentiation [7, 8, 10].

The molecular mechanisms controlling cell differentiation in the gastro-intestinal tract are mediated by several TF and especially those belonging to the hepatocyte nuclear factor (HNF), GATA and Caudal-related (Cdx) families [11-16]. In the stomach, only HNF and GATA TF are expressed. The GATA family consists in six members, GATA-1 to -6, that bind to the 5'-(A/T)GATA(A/G)-3' response element via their zinc finger domains. GATA-4,

-5, and -6 are found mainly in heart and endoderm-derived tissues, including liver, lung, pancreas, stomach, and intestine [11]. Hepatocyte nuclear factors belong to a heterogeneous family of TF involved in a wide variety of biological pathways. They are involved in visceral endoderm differentiation and found in kidney, pancreas, stomach, small intestine, and colon [17]. HNF-1α and HNF-1β are homeodomain proteins that bind the consensus sequence 5'-GTTAATGATTAAC-3'. FOXA1 (HNF-3α) and FOXA2 (HNF-3β) belong to the forkhead/winged helix DNA binding domain family. They bind the consensus sequence 5'-GATTATTGACTT-3' [18] and are expressed in embryonic endoderm and in the adult intestine [19]. HNF-4α and HNF-4γ are members of the steroid hormone receptor family. They are zinc finger TF and bind the consensus sequence 5'-TGGACTTAG-3'.

Despite an increasing amount of data regarding the expression pattern of mucins in normal and pathological epithelia of human [1, 4, 10] and rodents [20, 21], the precise biological role of mucins as well as the mechanisms that govern their specific pattern of expression during embryonic development and adult differentiation in the gastrointestinal tract remain largely unknown. To this aim, we have previously cloned and characterized the 5'-flanking region of the mouse *Muc5ac* [22]. In this report, we have investigated the transcriptional regulation of the gastric Muc5ac mucin gene by gastric HNF and GATA TFs. We show that the expression of the murine Muc5ac mucin in developing stomach is correlated to that of HNF-4α and GATA-6. We also show that GATA-4 and HNF-1α/-1β may be important regulators of *Muc5ac* transcription.

2. Experimental

2.1. Animals

Adult specified pathogen free Balb/c mice, obtained from Harlan (Zoetermeer, The Netherlands), were sacrificed by cervical dislocation. Stomach from embryo (ED15.5, ED17.5 and ED18.5) and adult mice were removed and fixed in 4% (w/v) paraformaldehyde in phosphate-buffered saline and subsequently processed for light microscopy as previously described [23]. The animal experiments were reviewed and performed with the approval of the Animal Studies Ethics Committee of the Erasmus MC (Rotterdam, the Netherlands).

2.2. Histology - Immunohistochemistry

Five μm-thick sections of mouse stomach tissue were routinely stained with hematoxylin and eosin to study the morphology, or stained with Periodic Acid Schiff (PAS) reagent to stain for neutral mucins. Immunolocalization of mouse Muc5ac was carried out as previously described [22] using 45M1 monoclonal antibody (Novocastra). Anti-HNF-1α (sc-6547), FOXA2 (sc-6554), HNF-4α (sc-6556), HNF-4γ (sc-6558), GATA-4 (sc-1237), GATA-6 (sc-7244) antibodies were from Santacruz Laboratories (TEBU-BIO).

2.3. Cell culture

The murine rectal cancer cell line CMT-93 was cultured in Dulbecco's Modified Essential Medium containing 10% (v/v) fetal bovine serum, 4 mM L-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37°C in a humidified 5% CO_2 water-jacketed incubator as previously described [22].

2.4. RT-PCR

Total RNA from CMT-93 cells were prepared using the NucleoSpin® RNA II kit (Macherey Nagel) following the manufacturer's protocol. cDNAs were prepared as previously described [24]. Total RNA (1.5 μg) was used to prepare first strand cDNA (Advantage^{TM} RT-for-PCR kit, Clontech). PCR was performed on 2 μl of cDNA using specific pairs of primers as previously described [24]. The annealing temperature was 58°C. *Muc5ac* forward primer: 5'-GAGGGCCCAGTGAGCATCTCC-3', *Muc5ac* reverse primer: 5'-TGGGACAGCAGCAGTATTCAGT-3' (accession number AJ010792). β-actin was used as an internal control: mouse β-actin forward primer: 5'-GTGGGCCGCTCTAGGCACCA-3', mouse β-actin reverse primer: 5'-TGGCCTTAGGGTGCAGGGGG-3' (accession number M12481). *Muc5ac* and *β-actin* PCR product sizes are 361 and 582 bp, respectively. 100 bp DNA ladder was purchased from Amersham Bioscience. To study the effect of TF overexpression on endogenous *Muc5ac* mRNA level, cells (0.5×10^6) were transfected as before [25] with 2 μg of the expression vectors of interest and cultured for 48h before being lysed and processed for total RNA preparation and RT-PCR analysis. The *Muc5ac/β-actin* ratio was calculated by densitometric analysis of the DNA bands on the agarose gel by using the GelAnalyst-GelSmart software (Clara Vision, Paris, France). These experiments were performed in triplicate in three independent series.

2.5. Site-directed mutagenesis

QuickChange site-directed mutagenesis kit (Stratagene) was used to generate site-specific mutations in GATA and HNF binding sites in *Muc5ac* promoter as described previously in Mesquita *et al*. [24]. Oligonucleotides containing the desired mutations were

designed according to the manufacturer's instructions and their sequences are depicted in Table I.

2.6. Transfections

Transfections and co-transfections experiments were performed using Effectene® reagent (Qiagen) as previously described [26]. The *Muc5ac*-pGL3 deletion mutants covering 1.2 kb upstream of the first ATG were previously described [22]. Total cell extracts were prepared after a 48h incubation at 37°C using 1X Reagent Lysis Buffer (Promega) as described in the manufacturer's instruction manual. Luciferase activity (20 μl) was measured on a TD 20/20 luminometer (Turner Design). Total protein content in the extract (4 μl) was measured using the bicinchoninic acid method in 96-well plates as described in the manufacturer's instruction manual (Pierce, Bezons, France). In co-transfection experiments, 1 μg of *Muc5ac*-pGL3 constructs were transfected in the presence of 0.25 μg of the expression vector encoding the TF of interest. Relative luciferase activity was expressed as fold activation of luciferase activity in the sample with the expression vector compared to that with the empty vector (Ref.). Each combination was assayed in triplicate in three separate experiments.

2.7. Nuclear extract preparation

Nuclear extracts from the different cells were prepared as described by Van Seuningen *et al.* [27], and kept at -80°C until use. Protein content (2 μl of cell extracts) was measured using the bicinchoninic acid as described above.

2.8. Oligonucleotides and DNA probes

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

2.9. Electrophoretic mobility shift assays (EMSA)

Nuclear proteins (8 μg) were pre-incubated for 20 min on ice in 20 μl binding buffer with 1 μg of poly dI-dC (Sigma) and 1 μg sonicated salmon sperm DNA. Radiolabelled DNA probe was added (60,000 c.p.m.) and the reaction was left for another 20 min on ice. For super-shift analyses, 1 μl of the antibody of interest (anti-GATA-4, anti-GATA-6, anti-HNF-1α, anti-HNF-1β, anti-FOXA1, anti-FOXA2, anti-HNF4-α, anti-HNF-4γ, Santa-Cruz laboratories, TEBU-BIO, France) was added to the proteins and left for 30 min at RT before adding the radiolabelled probe. Cold competition were performed by preincubating nuclear proteins with 100X excess of unlabelled oligonucleotides before adding radioactive probe. Mutated oligonucleotides were also used under the same conditions to demonstrate the specificity of the binding. Reactions were stopped by adding 2 μl of loading buffer. Samples were loaded onto a 4% non-denaturing polyacrylamide gel and electrophoresis conditions were as described in [28]. Gels were vacuum-dried and autoradiographed overnight at -80°C. EMSA were performed independently at least three times.

2.10. Chromatin immunoprecipitation assays (ChIP)

The Chromatin Immuno Precipitation (ChIP) assay was carried out as previously described [29] using 4 mg of anti-GATA-4, anti-GATA-5 (R&D) and anti-GATA-6 (N18 from Santa Cruz Biotechnology), anti-HNF1α, anti-HNF1β, anti-HNF4α and HNF4γ (Santa Cruz Biotechnology) antibodies or normal rabbit IgGs (Upstate, Millipore, St Quentin en Yvelines, France). Immuno-precipitation was performed using Dynabeads® magnetic beads A and G (Invitrogen) with Dynabeads® rack (Invitrogen) following the manufacturer's protocol. The sequences of PCR primers covering DNA regions of interest are indicated in table I. PCR was carried out in a 30 µl volume containing 50 ng of DNA, 5U of AmpliTaq Gold (Applied Biosystems, Courtaboeuf, France), 0.5 mM of each primer, 2.5mM $MgCl_2$ and 5% (v/v) dimethylsulphoxide using the following protocol: 3 min at 95°C followed by ((95°C) 15 s, (55°C) 15 s, (72°C) 15 s) for 34 cycles, and 72°C for 5 min. The PCR products were analysed on a 1.2 % (w/v) agarose gel run in 1X tris borate-EDTA containing ethidium bromide.

2.11. Statistical analysis

Statistical analyses were performed using Graphpad Prism 4.0 software (Graphpad softwares Inc., La Jolla, USA). Data are presented as mean ±SEM. Differences in the mean of two samples were analysed by the student's t test or one way ANOVA test with selected comparison using tukey post-hoc test with differences less than 0.05 considered significant and were indicated with an *. ** indicates $p<0.01$.

3. Results

3.1. Expression of Muc5ac and transcription factors during development of the mouse stomach

Expression of the Muc5ac mucin during developing stomach of the mouse was studied by immunohistochemistry (IHC) at embryonic days (ED) 15.5, 17.5, 18.5, post-natal day 1.5 (PND1.5) and in adult mice. As shown in figure 1, Muc5ac was expressed in embryonic stomach as of ED18.5. The staining was localized in the surface epithelial cells. Muc5ac staining was conserved at PND1.5 (supplemental figure 1) and in adult mouse (Figure 1). A similar pattern was observed with PAS staining. Expression of TF known to be involved in epithelial cell differentiation during gastrointestinal development was then studied in order to correlate their expression with that of Muc5ac. GATA-6 and HNF-4α expression in the stomach was only observed as of ED17.5, shortly before the outgrowth of the hindstomach into a glandular stomach (not shown). Their expression remained weak until ED18.5 but increased substantially after birth (Figure 1). GATA-4, HNF-1α, HNF-4γ and FOXA2 are expressed as early as ED15.5 in the pseudostratified epithelium of the hindstomach and throughout development, after birth and in adult mice (not shown). As of ED17.5 all of the TF studied were expressed in epithelial cells from the bottom to the top of the glands. Among all the TF studied, only GATA-6 and HNF-4α were expressed right before the induction of Muc5ac expression. IHC performed on serial sections of PND1.5 mouse stomach showed that Muc5ac, GATA-6 and HNF-4α were expressed in the surface epithelial cells of the stomach (supplemental figure 1).

3.2. Muc5ac gene is transcriptionally regulated by HNF-1/-4 and GATA-4/-5/-6 transcription factors

Having shown that the Muc5ac mucin is co-expressed with HNF and GATA TF during stomach development we undertook to study their effect on *Muc5ac* transcription. To this end, co-transfection experiments were performed with two deletion fragments of *Muc5ac* promoter (-3070/+3 and –1021/+3) in the presence of expression vectors encoding the TF of interest. The longest fragment contains two putative GATA (T127 and T191) and two putative HNF-4 (T122 and T126) binding sites, respectively (Figure 2A). The Muc5ac promoter does not contain any HNF-1 5'-TAGTTAC-3' consensus binding site. Promoter activity and mRNA expression were evaluated by measurement of luciferase activity and by RT-PCR, respectively.

Co-transfection studies in CMT-93 cells (Figure 2A), indicated that among the three GATA TF, GATA-5 had the strongest effect on *Muc5ac* promoter activity (8 fold activation on the –3070/+3 fragment, black bars), whereas GATA-4 and GATA-6 were less active (4 fold activation). The transactivation profile was the same on the shortest fragment (-1021/+3, gray bars) with a slightly weaker activation (2- and 6- fold, respectively).

Regulation of the *Muc5ac* promoter by HNF factors indicated that HNF-1β is the most potent transactivator (10 fold on fragment –3070/+3). The transactivating effect decreased substantially when the shortest fragment was used indicating that distal HNF binding sites may be important in mediating HNF-1β effects. HNF-1α, HNF-4α and HNF-4γ also transactivated the *Muc5ac* promoter but to a lesser extent (2 fold activation). Members of the FOXA family (FOXA1, FOXA2, FOXA3) did not induce *Muc5ac* promoter activity.

At the mRNA level, we observed an increase of *Muc5ac* expression with GATA-4 (1.8 fold, p=0.0015, **) GATA-5 (4.7 fold, p=0.0064, **) and GATA-6 (2.8 fold, p=0.0291, *) once we had overexpressed these TF in CMT-93 cells. Overexpression of HNF-1α, HNF-1β,

HNF-4α and HNF-4γ also induced an increase of *Muc5ac* mRNA level in CMT-93 cells (2.6, 3.8, 1.3 and 1.5 fold, respectively, p=0.0324 *, p=0.0449 *, p=0.46 and p=0.023 *, respectively), while no effect was observed with FOXA1, FOXA2 and FOXA3 (Figure 2B).

Since these TF are known to act in synergy, this possibility was investigated on *Muc5ac* promoter activity by transient co-transfection experiments. The results indicate additive effects for GATA-6 and HNF-1β and GATA-6 and HNF-4α (Figure 2C).

In conclusion, we have identified GATA-4/-5/-6 and HNF-1α/-1β as strong trans-activators of *Muc5ac* promoter activity and mRNA expression and HNF-4α/-4γ as weaker transactivators.

3.3. Muc5ac promoter contains GATA-4/-5//-6 and HNF-1/-4 cis-*elements*

The sequence upstream of the TATA box is characterized by the presence of putative binding sites for members of the HNF (T122 and T126) and GATA (T127 and T191) TF families (Figure 2A). In order to identify which factor binds, if any, to these sites, EMSA and ChIP assays were carried out.

Using EMSA (Figure 3A), a GATA binding site was identified at -774/-771 as two shifted bands appeared when the double-stranded radiolabelled probe T127 was incubated with nuclear proteins from CMT-93 cells (*lane 2*). The specificity of the binding was confirmed by cold competition that abolished formation of the two complexes (*lane 3*). The binding of GATA-4 and GATA-6 to this site was confirmed by preincubation of the extract with anti-GATA-4 and anti-GATA-6 antibodies that led to the disappearance of the upper shifted band for GATA-4 (*lane 4*) and disappearance of the lower shifted band and formation of a supershift for GATA-6 (ssGATA-6, *lane 5*). Incubation with the T191 (-2521/-2518 distal GATA site) probe did not result in any shifted band (not shown).

Incubation with the T126 and T122 radiolabelled probes that contain the proximal (-634/-630) and distal (-2614/-2610) HNF-4 binding sites produced retarded bands (*lanes 7 and 14*). The binding was specific since it disappeared when cold competition was performed with 100x excess of wild-type T126 or T122 probes (*lanes 8 and 15*) and was maintained when a mutated version of the probes was used (*lanes 9 and 16*). Incubation with anti-HNF-1α (*lanes 10 and 17*), anti-HNF-1β (*lanes 11 and 18*), anti-FOXA1 (*lanes 12 and 19*), anti-FOXA2 (*lane 20*) did not produce supershifts but only slightly reduced the intensity of the shifted bands (22, 29, 31 and 31% decrease for T122 binding site and 49 and 46% for T126 binding site, respectively). Incubation with antibodies for HNF-4α (*lanes 13 and 21*) and HNF-4γ (*lane 22*) led to supershifted bands.

In order to confirm these interactions *in cellulo*, ChIP assays were carried out on the -1019/-723 region of *Muc5ac* promoter containing the T127 (-774/-771) GATA-4/-6 binding site and on the -2904/-2608 and -769/-544 regions containing the T122 and T126 HNF-4 binding sites, respectively (Figure 3B). The results indicate that GATA-6 and to a lesser extent GATA-4 and GATA-5 bind to the -1019/-723 *Muc5ac* promoter region (Figure 3B). HNF-1α, HNF-1β, HNF-4α and HNF-4γ TF were found to strongly bind to the -769/-544 promoter region (T126) (Figure 3C). They also bound with less intensity to the -2904/-2608 (T122) region. Specificity of the bindings in all ChIPs was confirmed by absence of PCR amplification in the presence of IgGs.

In conclusion of these binding assays, we have identified one GATA-4/-5/-6 *cis*-element at -774/-771 (T127) and two HNF-4α/-4γ *cis*-elements at -634/-630 (T126) and -2614/-2610 (T122) within the *Muc5ac* promoter. Moreover, -769/-544 and -2904/-2608 promoter regions are also able to bind HNF-1α/-1β on *cis*-elements that remain to be identified.

We have also investigated whether HNF-1 may modulate the levels of HNF-4α/-4β or GATA-6. We thus performed RT-PCR on RNA extracts from CMT-93 cells transfected with expression vectors encoding either HNF-1α or HNF-1β. Although HNF-1α/-1β expression was increased as expected, HNF-4α, HNF-4γ or GATA-6 mRNA levels were not modified (Supplemental figure 2). These results are thus not in favour of a regulatory mechanism of HNF4α/4γ and GATA-6 by HNF-1α/-1β.

3.4. HNF-1/-4 and GATA-4/-6 cis-elements mediate Muc5ac transcriptional activity

In order to confirm the importance of the two GATA and HNF *cis*-elements in the transcriptional regulation of *Muc5ac* promoter, site-directed mutagenesis of these sites was performed. The luciferase diagram indicates that mutation of the proximal HNF-1/-4 *cis*-element at -634/-630 (T126) led to a total loss of the transactivating effect mediated by HNF-1α and HNF-1β that was statistically significant ($p<0.05$) (grey bars) (Figure 4A). Loss of transactivation by HNF-4α and HNF-4γ was also observed but remained statistically non significant. A total loss in *Muc5ac* promoter transactivation, significant for HNF-1α, HNF-1β and HNF-4α was also observed when the distal *cis*-element at -2614/-2610 (T122) was mutated (black bars).

Mutation of the GATA T127 (-774/-771) *cis*-element in the -1021/+3 construct induced a statistically significant ($p<0.05$) decrease of *Muc5ac* promoter activity in the presence of GATA-4 and GATA-6 (grey bars) when compared to the activity with the non-mutated construct (white bar) (Figure 4B). Transactivation by GATA-5 was not affected. As expected mutagenesis of the distal GATA element located at -2521/-2518 (T191), where no binding was observed did not alter transactivation of the -3070/+3 construct (not shown).

Altogether, these results indicate that -634/-630 (T126) and -2614/-2610 (T122) HNF *cis*-elements are necessary to convey *Muc5ac* promoter activity by HNF-1α/-1β and HNF-4α/–4γ. Similarly, the -777/-771 GATA *cis*-element (T127) is necessary to convey *Muc5ac* promoter activation by GATA-4 and GATA-6.

4. Discussion

The Human *MUC5AC* mucin is expressed very early during human embryonic development before any mucous cytodifferentiation has started in the stomach [9]. In the present work, we show that the murine Muc5ac mucin is expressed at ED18.5 in the mouse embryonic stomach and maintained during adulthood. These results have led us to hypothesize that mucin genes expressed early during development may play important roles in establishment of cell lineages derived from endoderm and cells committed to become mucus-secreting [1, 7, 10]. Genes specifically regulated during those events are often targets of TF expressed during the same key events of cytodifferentiation. TF involved in gastrointestinal differentiation have been identified [11, 14, 15, 30-32] and belong notably to the GATA and HNF families. These factors also have a spatio-temporal pattern of expression during development. They regulate several enterocyte-specific genes such as sucrase isomaltase [33, 34], intestinal fatty acid binding protein [35], lactase-phlorizin hydrolase [33, 36] but also human and mouse *Muc2, Muc5b* and *MUC4* mucin genes [25, 29, 37, 38]. In this paper, we show for the first time that the murine *Muc5ac* mucin gene is also regulated at the transcriptional level by GATA-4/-5/-6, HNF-1α/-1β, HNF-4α and HNF-4γ transcription factors.

In relation with the role of GATA-4/-5/-6 TF in gastrointestinal development [11, 31, 32], we studied their effect on *Muc5ac* regulation. We found that the three TF up-regulate *Muc5ac*. These factors are all expressed in the visceral endoderm, developing gut and are found in cells lining the lumen of the primitive stomach at E11.5-12.5 of the mouse [39]. It is also striking that GATA-4/-5/-6 TF and *Muc5ac* follow the same pattern of expression during development of the gut. They are transcribed in the small intestine only during embryogenesis but are not maintained in the adult whereas in stomach, their expression is maintained in the

adult in specialized surface epithelial cells. GATA-5 also regulates genes important in the earlier commitment step of specialized cell types, since it was found in predifferentiated crypt cells of the gut epithelium [40], a cell type in which *MUC5AC* is also present at that stage of development [8]. However, from our site-directed mutagenesis data *Muc5ac* regulation by GATA-5 is most likely indirect. Altogether, these data and ours reveal a new mechanism of regulation of *MUC5AC* by GATA factors during stomach development and early commitment of future specialized mucus-secreting cells.

HNF factors belong to a structurally heterogeneous family of TF and are known to be involved in the development and differentiation of the gastrointestinal tract [12, 14, 15]. In order to delineate which HNF transcription factors are important in *Muc5ac* regulation we used a large panel of expression vectors encoding HNF-1α/1β, FOXA1/A2/A3 and HNF-4α/-4γ. Our data show that HNF-1α and HNF-1β are strong activators of *Muc5ac* transcription both at the promoter and RNA levels. These TF are known to regulate several intestine-specific genes [33, 34, 36], that are markers of enterocytes. We show now for the first time similar mechanisms of regulation at the transcriptional level for *Muc5ac*, a specific marker of gastric mucus-secreting cells.

In the present work, we also show that the Muc5ac mucin is expressed concomitantly with GATA-6 and HNF-4α TF during stomach development and that *Muc5ac* gene is transcriptionally regulated by these two TF. This suggests that the spatio-temporal pattern of expression of Muc5ac, which participates in mucus-secreting lineage establishment and epithelial differentiation of tissues derived from primitive gut, especially in embryonic stomach is probably under the control of these two TF. HNF-4 and GATA-6 are also known to control the expression of genes specifically expressed in other territories of the digestive tract such as the intestine. However, Muc5ac is not expressed in the normal gut suggesting that other regulatory mechanisms are involved. In the laboratory, recent works in humans

have indeed confirmed this hypothesis as transient expression of MUC5AC in colon carcinoma was found to be correlated with demethylation of the CpG island found in its 5'-flanking region [41]. Since the same pattern of expression is observed for Muc5ac in the mouse intestine, one may hypothesize that epigenetic regulation of Muc5ac also occurs.

The relatively modest transactivating effect of HNF-4α is not surprising as it is well-known that this TF is a weak transactivator [42] which often acts in synergy or in combination with other TF. We previously showed that GATA and HNF TF act synergistically to regulate *MUC4* gene expression in epithelial cells [38]. One can envision a similar mechanism involving HNF-4α and GATA-6 regarding *Muc5ac* regulation similarly to what was shown for ATP-Binding Cassette Sterol Transporters *ABCG5* and *ABCG8* for which HNF-4α contributes to maintaining the basal transcriptional activities and acts synergistically with GATA TF to activate transcription [42]. Co-transfection experiments carried out in the presence of the two TF showed additive effects for GATA-6 and HNF-1β or GATA-6 and HNF-4α.

In conclusion, we have shown that *Muc5ac* is regulated at the transcriptional level by TF involved in gastrointestinal development and goblet cell differentiation (GATA-4/-5/-6, HNF-1α/-1β, HNF-4α/-4γ). These results open new avenues of research in animal models that will aim at demonstrating the importance of *Muc5ac* in maintaining gastrointestinal homeostasis and correlate *Muc5ac* expression during development of the epithelium with mucus surface cell differentiation [21, 43]. In the long run, a better understanding of the role of mucins in health and disease may allow the development of novel therapeutic targets and preventive strategies.

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Figure legends

Figure 1: Expression of Muc5ac, GATA-6 and HNF-4α transcription factors during stomach development of the mouse. PAS staining and IHC for Muc5ac, GATA-6 and HNF-4α were performed as described in the material and methods section on embryonic day (ED) 18.5 and adult stomach tissue. PAS and Muc5ac stainings were observed in embryonic stomach as of ED18.5 and expression was conserved in adult stomach. GATA-6 and HNF-4α expression was induced in the stomach as of ED18.5 and expression increased after birth.

Figure 2: Regulation of *Muc5ac* promoter by HNF and GATA transcription factors. (A) Co-transfection experiments were performed in CMT-93 cells in the presence of 1 μg of *Muc5ac* pGL3-deletion mutant -3070/+3 (black bars) or -1021/+3 (grey bars) and 0.25 μg of the expression vector as indicated. Ref. refers to the normalized luciferase activity of the pGL3-deletion mutants of interest co-transfected with the corresponding empty expression vector. The data are displayed as averages of values obtained in triplicate in three separate experiments ± standard error of the mean. Differences in the mean were analysed by the one way ANOVA test with selected comparison using tukey post-hoc test with differences less than 0.05 considered significant and were indicated with an *. ** indicates $p<0.01$. **(B)** Measurement of *Muc5ac* mRNA level by RT-PCR in CMT-93 cells transfected with either 2 μg of plasmid encoding GATA-4, GATA-5, GATA-6, HNF-1α, HNF-1β, FOXA1, FOXA2, FOXA3, HNF-4α and HNF-4γ, expression vectors or corresponding empty vector (Ref.). The diagram represents the calculated ratio of Muc5ac/β-actin. Standard deviation represents the means of values obtained from three separate experiments. **(C)** Study of the synergistic effects between GATA-6, HNF-1α and HNF-4α on the promoter activity of *Muc5ac*. Co-transfection experiments were performed in CMT-93 cells in the presence of 1 μg of *Muc5ac* pGL3-

deletion mutant -1021/+3 and 0.125 μg of the each expression vector as indicated. Ref. refers to normalized luciferase activity of the pGL3 plasmid with the corresponding empty vector. The data are displayed as average ± standard error of the mean.

Figure 3: Identification of HNF and GATA *cis*-elements in the promoter of *Muc5ac*. (A) Identification of GATA and HNF *cis*-elements by EMSA. 8 μg of nuclear extracts prepared from CMT-93 cells were incubated with the radiolabelled T127, T126 and T122 DNA probes (+, *lanes 2, 7* and *14*). *Lanes 1-5*, T127, GATA-binding site at -774/-771 ; *lanes 6-12*, T126, HNF binding site at -634/-630 ; *lanes 13-22*, T122, HNF binding site at -2614/-2610. Supershift experiments were carried out by adding 1 μl of the antibody of interest : anti-GATA-4 (*lane 4*), anti-GATA-6 (*lane 5)*, anti-HNF-1α *(lane 10 and 17)*, anti-HNF-1β *(lane 11 and 18)*, anti-FOXA1 *(lane 19)*, anti-FOXA2 *(lane 20)*, anti-HNF4-α *(lane 12 and 21)*, anti-HNF-4γ (*lane 22*). Cold competitions were performed by preincubating the nuclear extracts with 100x excess of cold T127 (*lane 3*), cold T126 *(lane 8*), cold mutated T126 (*lane 9*), cold T122 (*lane 15*), and cold mutated T122 (*lane 16*) probes, respectively. Radiolabelled probe alone were loaded in the first lane of each series (*lanes 1, 6* and *13*). **(B)** Binding of GATA-4, -5, and -6 to chromatin of CMT-93 cells by ChIP. PCRs were carried out with specific pairs of primers covering the -1019/-723 DNA region including T127. PCR products (10 μl) were analyzed on 1.2 % (w/v) agarose gels. IgGs, negative control with rabbit IgGs. Input, positive control with total chromatin. **(C)** Binding of HNF-1α, HNF-1β, HNF-4α, HNF-4γ to chromatin by ChIP. PCRs were carried out with specific pairs of primers covering -769/-544 (including T126) and -2904/-2608 (including T122) DNA regions.

Figure 4: Site-directed mutagenesis of HNF and GATA *cis*-elements in *Muc5ac* promoter. (A) Transient transfection experiments were performed in the presence of 1 μg of

T122 or T126 wild type (wt.) or mutated (mut.) forms of -3070/-1 and -1021/-1 *Muc5ac* promoter constructs, and 0.5 μg of HNF-1α, HNF-1β, HNF-4α, HNF-4γ expression vectors. **(B)** Transient transfection experiments were performed in the presence of 1 μg of T127 wild-type (wt.) or mutated (mut.) forms of -1021/-1 *Muc5ac* promoter constructs and 0.5 μg of GATA-4, GATA-5 and GATA-6 expression vectors Results are expressed as fold activation of relative luciferase activity obtained when co-transfected with the expression vector encoding the TF of interest compared with cells transfected with corresponding empty vectors. The transactivating activity obtained with the corresponding empty vector was arbitrarily set to 1. The data are displayed as averages of values obtained in triplicate in three separate experiments ± standard error of the mean. *, $p < 0.05$.

Supplemental figure 1: Expression of Muc5ac mucin, GATA-6 and HNF-4α transcription factors at PND 1.5. IHC for Muc5ac, GATA-6 and HNF-4α was performed as described in the material and methods section on postnatal day (PND) 1.5 serial sections. Muc5ac, GATA-6 and HNF-4α stainings were observed in gastric cell population of PND 1.5 mouse embryo.

Supplemental figure 2: HNF-4α, HNF-4γ and GATA-6 mRNA expression is not up-regulated by HNF-1α, or HNF-1β transcription factors. HNF-4α, HNF-4γ and GATA-6 mRNA level were assessed by RT-PCR in CMT-93 cells transfected with either 2 μg of plasmid encoding HNF-1α, or HNF-1β expression vectors or corresponding pSG5 empty vector.

Figure 1: Jonckheere *et al.*

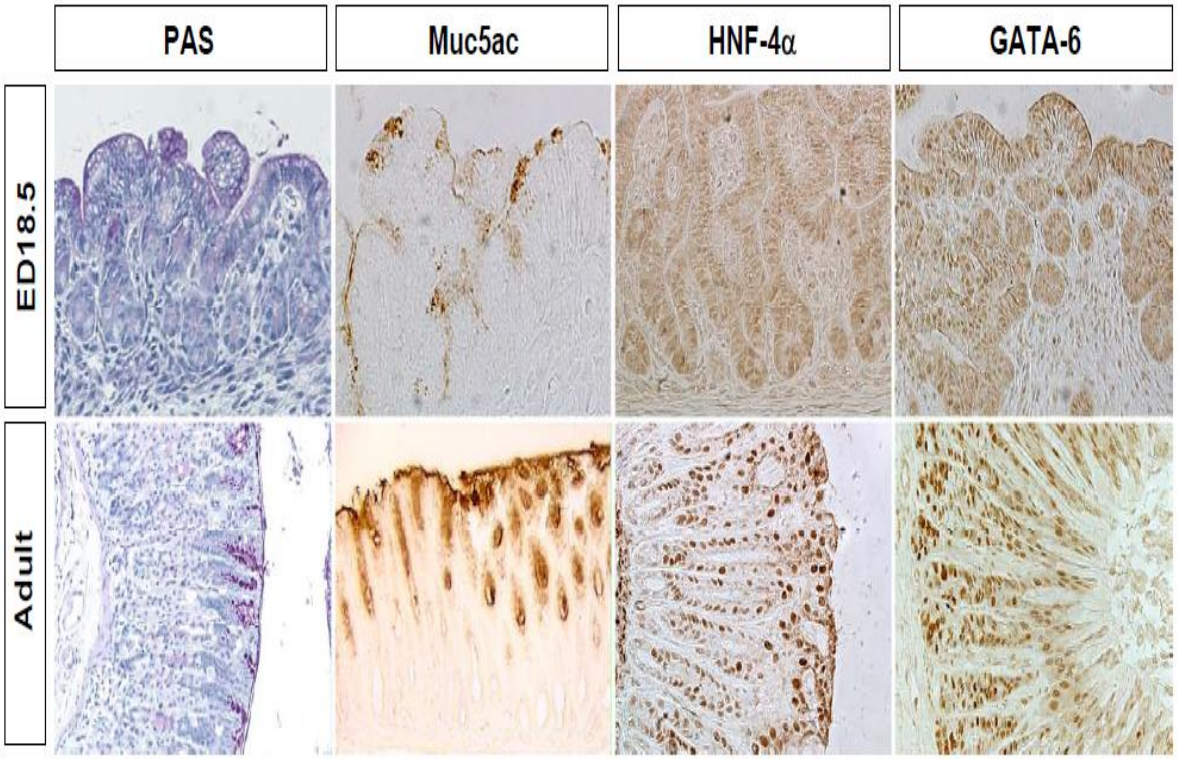


Figure 2 : Jonckheere *et al.*

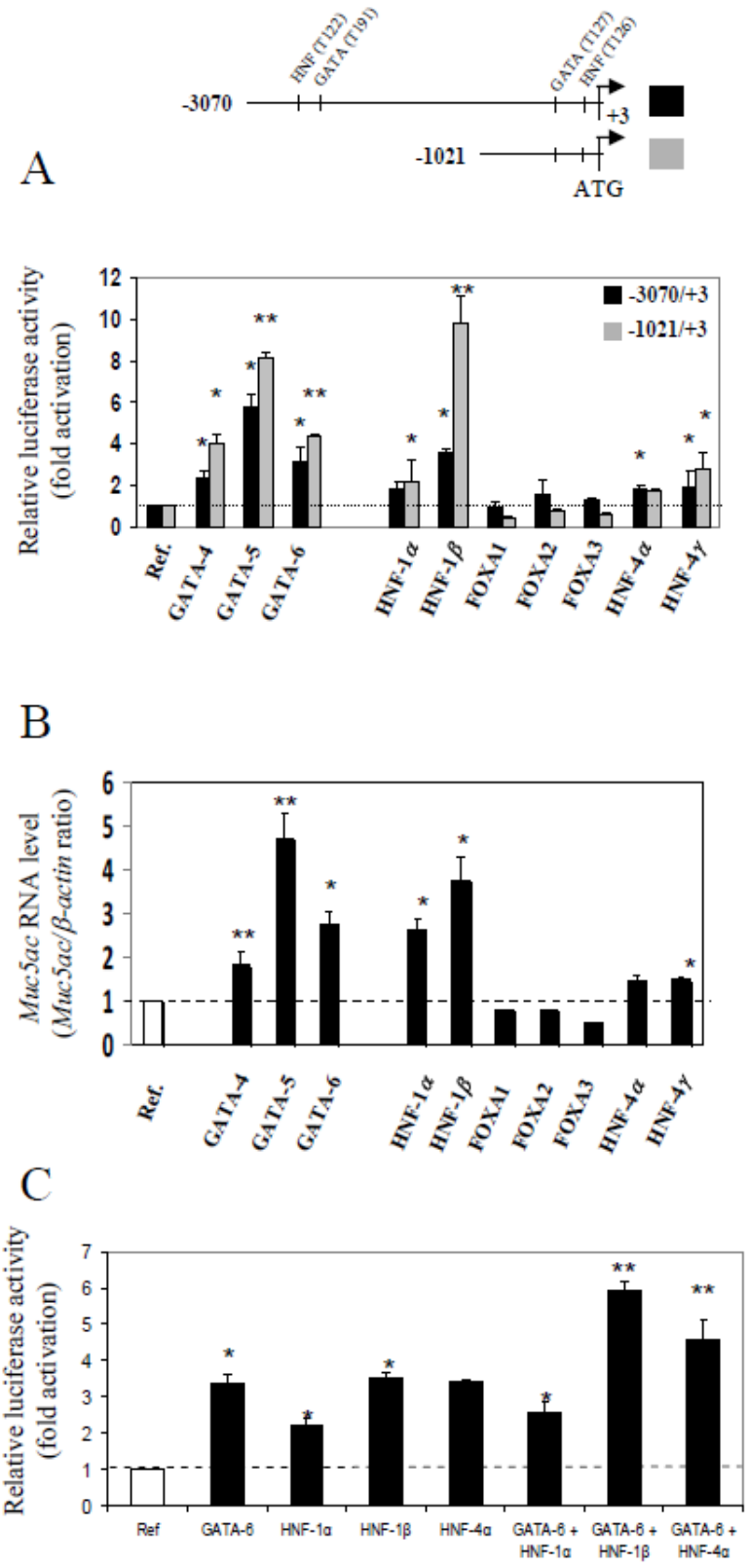


Figure 3 : Jonckheere *et al.*

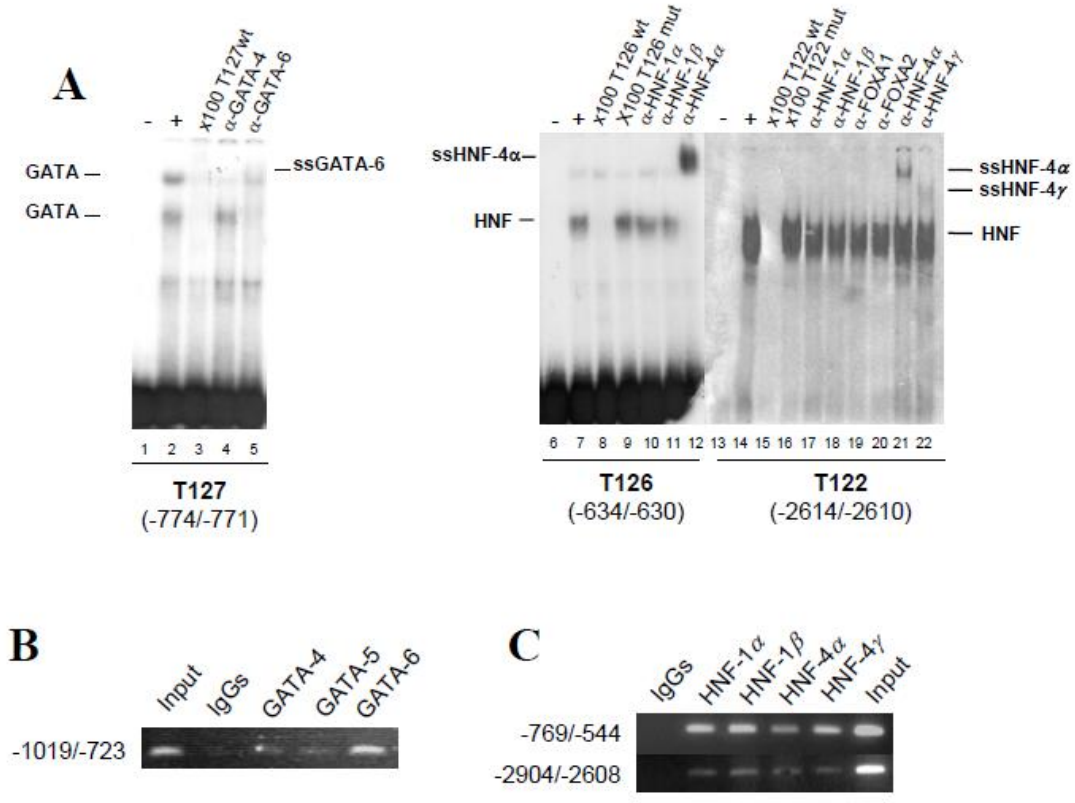


Figure 4 : Jonckheere *et al.*

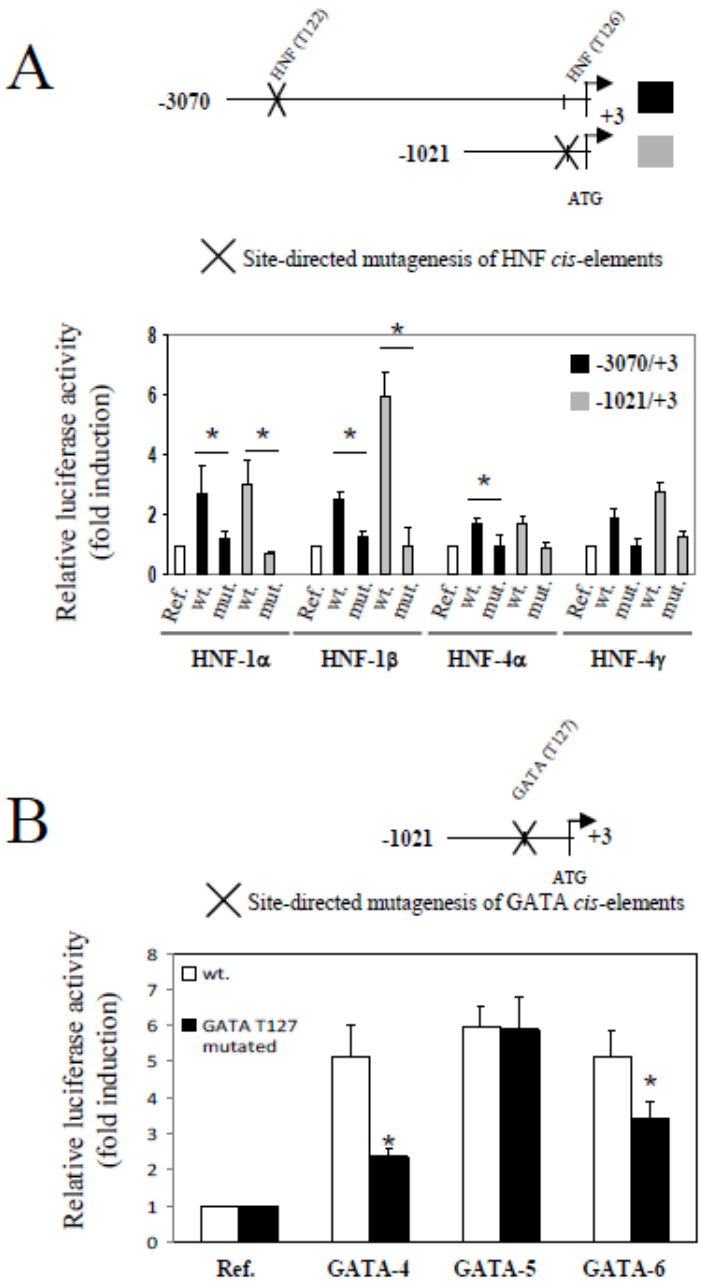


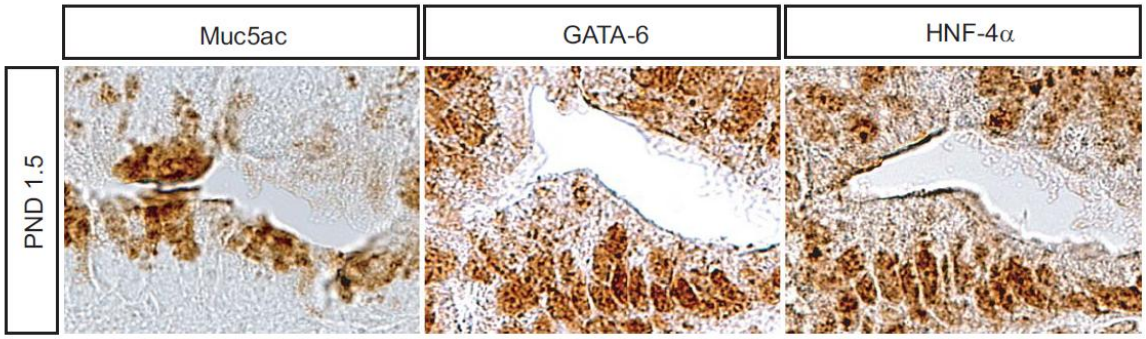
Table I : Sequences of the sense oligonucleotides used for EMSAs, ChIP and site-directed mutagenesis. For EMSA studies, antisense oligonucleotides were also synthesized and annealed to the sense oligonucleotides to produce double-stranded DNA. Mutated nucleotides are italicized and underlined. Positions of the putative binding sites in the promoter are indicated and are bold.

Sequences of oligonucleotides for EMSA		
Probe	Putative binding site	Sequence (5'→3')
T126	HNF (-634/-630)	CCTGGTCTGGG**CAAAG**GTCCATGC
T164	T126 mutated	CCTGGTCTGGG*TGCGT*GTCCATGC
T127	GATA (-774/-771)	CTCTCCCAT**GATA**GGGTCCTT
T187	T127 mutated	CTCTCCCAT*CT*TAGGGTCCTT
T191	GATA (-2521/-2518)	CCATTCACT**TATC**AGCTCCCA
T122	HNF (-2614/-2610)	TATGGCCATGAC**CTTTGC**CCTATA
T159	T122 mutated	TATGGCCATGACC*GCGTG*CCTATA

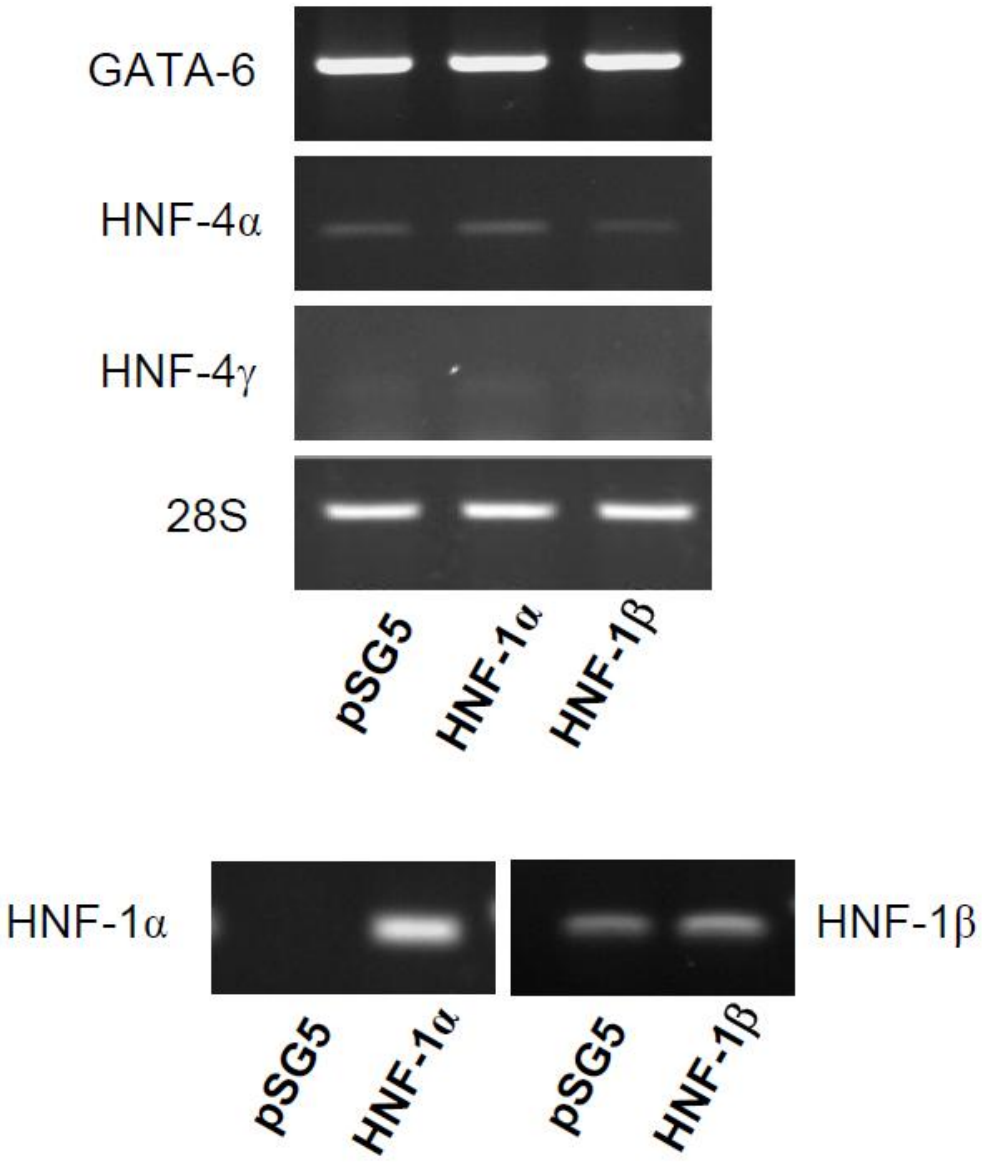
Sequences of oligonucleotides for ChIP		
Amplified DNA region	TF binding site	Sequence (5'→3')
-1019/-723	GATA T127	F : 5'-CACACACACACACACTCAACT-3' R : 5'- CCAATGTCAGCAGCTCTGT-3'
-2904/-2608	HNF T122	F : 5' TAATCTCCACTGAGTCACCAG-3' R : 5'-GGACTGGGTCTGGTCTGA-3'
-769/-544	HNF T126	F : 5'-GCTGCTGACATTGGCTGA-3' R : 5'-GTCAGAAGCCAAAGCATT-3'

Sequences of oligonucleotides for site-directed mutagenesis		
HNF	T122 mutated	GAATATGGCCATGACC*GCGTG*CCTATACTGGTCCCT
HNF	T126 mutated	TCCCTGGTCTGG*C*C*CGC*GGTCCATGCACG
GATA	T127 mutated	GCCTACCATCTCCCAT*CTTA*GGGTCCTTCATTCC
GATA	T191 mutated	CCAGATTGTAGTAAGCCATTCA*CTTA*AGAGCTCCCA AAAGG

Jonckheere *et al.*, Supplemental figure 1



Supplemental figure 2 : Jonckheere *et al.*



Highlights :

- Muc5ac, GATA-6 and HNF-4α are expressed concomitantly in the developing mouse stomach
- GATA-4/-5/-6 and HNF-1α/-1β/-4α/-4γ TF promote Muc5ac transcription
- Muc5ac promoter contains one active GATA cis-element.

- Two active HNF cis-elements were identified within Muc5ac promoter
- HNF and GATA TF are regulators of Muc5ac mucin expression in the developing stomach