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Transcription factor GATA-5 selectively up-regulates mucin gene expression

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Abstract Purpose: Overlapping expression patterns of epithelial mucins, MUC1-MUC6, and transcription factor GATA-5 were reported previously. However, the functional relationship between them is poorly understood. The aim of the current study is to elucidate whether or not expression of mucin genes is regulated by GATA-5. **Methods:** GATA-5 was transiently overexpressed in COS-7 and 293T cells by plasmid transfection and/or adenovirus infection. GATA-5 expression was confirmed by Western blot analysis. Expression of mucin genes was studied by reverse-transcription-PCR. Reporter gene assays were employed to analyze the effect of GATA-5 on the promoter activity of mucin genes. **Results:** mRNA levels of MUC2, MUC3, and MUC4 were increased, whereas those of MUC1, MUC5AC, MUC5B, and MUC6 remained unchanged upon the overexpression of GATA-5 in both COS-7 and 293T cells. By means of luciferase assay, GATA-5 was found to activate the promoters of the human MUC2 and MUC4 genes. GATA-5 lacking the zinc finger domain impaired these functions. **Conclusions:** These findings indicate that GATA-5 may play important roles in the regulation of mucin expression and gastrointestinal epithelial cell differentiation.

Keywords GATA-5 · Transcription factor · Mucin · Gut · Pepsinogen

Introduction

The epithelial surface of the gastrointestinal tract is coated by mucus, which plays a critical role in hydration and protection of the gastrointestinal epithelium.

Mucins are complex glycoproteins that represent the major structural proteins of mucous gels that cover respiratory, digestive, ocular, and reproductive tract surfaces. Mucins are thought to contribute to the physical barrier that protects underlying epithelial cells (Gendler and Spicer 1995). In humans, eight distinct mucin core polypeptide genes have been well characterized: MUC1, MUC2, MUC3A, MUC3B, MUC4, MUC5AC, MUC5B, and MUC6 (Gendler et al. 1990; Gum et al. 1994; Pratt et al. 2000; Porchet et al. 1991; Dufosse et al. 1993; Aubert et al. 1991; Toribara et al. 1993). Mucin gene expression is tissue- and cell-specific. MUC1, a membrane-bound mucin, is normally expressed in secretory epithelial cells of the respiratory, gastrointestinal, and female reproductive tracts as well as in lymphoid cells (Ho et al. 1993; Gipson et al. 1997). Expression of secreted mucin MUC2 and membrane-bound mucin MUC3 is primarily restricted to the intestinal tract. However, within the intestinal tract, MUC2- and MUC3-producing cells are distinct, that is, MUC2 is expressed in goblet cells of the small intestine and colon, whereas MUC3 is expressed in both goblet and absorptive cells of the small intestine. The expression level of MUC3 has been reported to increase along the crypt/villus axis of the intestinal epithelium (Pratt et al. 2000; Audie et al. 1993; Gambus et al. 1993; Chang et al. 1994), in a pattern similar to that previously reported for cGATA-4 and cGATA-5 in chick gut (Gao et al. 1998). A transmembrane mucin, MUC4, is widely distributed in human epithelial tissues including the trachea and bronchial area, cervix, stomach, small intestine, and colon (Porchet et al. 1991; Gipson et al. 1997; Audie et al. 1993). MUC5AC and MUC6 are mainly found in the stomach, while MUC6 is also expressed in the duodenal Brunner's glands (Ho et al. 1995; Bartman et al. 1998). MUC5B is essentially expressed in the trachea, bronchi, submaxillary glands, pancreas, gallbladder, and endocervix (Gipson et al. 1997; Audie et al. 1993; Balague et al. 1995; van Klinken et al. 1998). Numerous studies have demonstrated that the expression of mucin genes is altered during the

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pathogenesis of several diseases including cancer, which suggests that human mucin gene expression is tightly regulated, and that it may be related to cell differentiation and carcinogenesis (Ho et al. 1993; Audie et al. 1993; Carrato et al. 1994; Buisine et al. 1996). However, little is known about the molecular mechanisms that regulate mucin gene expression.

The 5'-regulatory regions of human mucin genes were studied for MUC1, MUC2, MUC4, MUC5AC, and MUC5B previously (Kovarik et al. 1993; Gum et al. 1997; Perrais et al. 2001; Li et al. 1998; Van Seuning et al. 2000). A number of transcription factor binding sites present in the promoter regions may be involved in the regulation of mucin gene expression. MUC1, MUC2, and MUC5AC are transcriptionally regulated by Sp1, a ubiquitous transcription factor (Kovarik et al. 1993; Gum et al. 1997; Li et al. 1998). However, transcription factors determining tissue-specific expression of mucin genes are poorly understood. Through a transcription factor binding site search, we found that several GATA and GATA-like consensus sequences are present in the 5'-flanking regions of the MUC1, MUC2, MUC5AC, and MUC5B genes. In addition, the human MUC4 gene promoter contains several GATA consensus sequences (Perrais et al. 2001). These findings suggest that mucin genes may be regulated by GATA factors.

The GATA family of transcription factors includes six proteins with a highly conserved zinc finger DNA binding domain that interacts with DNA regulatory elements containing consensus (A/T)GATA(A/G) sequence. Based on their tissue distributions and homology, six GATA family members are divided into two groups: GATA-1, -2, and -3, and GATA-4, -5, and -6. GATA-1, -2, and -3 are predominantly expressed in hematopoietic cells, where they are involved in the proliferation and differentiation of several hematopoietic cell lineages (Orkin 1992). GATA-4, -5, and -6 are expressed in various tissues of mesodermal and endodermal origin, including the heart, liver, lungs, gonads, and gut, where they play critical roles in regulation of tissue-specific gene expression (Molkentin 2000). GATA-5 belongs to the second group and is expressed in the adult small intestine, stomach, bladder, and lungs, whereas developmental GATA-5 expression is detected in the allantois, heart, outflow tract, lung bud, urogenital ridge, bladder and gut epithelium (Morrisey et al. 1997a). In *Xenopus*, xGATA-4, -5, and -6 are expressed in the foregut, and xGATA-5 transcripts are localized within epithelial cells of the stomach and intestine (Jiang and Evans 1996). In chick intestinal epithelium, the transcript pattern for cGATA-5 is consistent with a function in regulation of terminal differentiation, since increasing levels of cGATA-5 transcripts are found along the crypt/villus axis, with the highest levels toward the distal end (Gao et al. 1998). GATA-5 has been reported to up-regulate the embryonic chicken pepsinogen (ECPg) gene, which encodes a zymogen of the digestive enzyme pepsin specifically expressed in glandular epithelial cells of the embryonic proventriculus, through binding to the

GATA elements within the ECPg promoter (Sakamoto et al. 2000). The overlapping expression patterns of GATA-5 and gastrointestinal mucin genes, MUC1-MUC6, suggest that GATA-5 may be one of the transcriptional regulators of these mucin genes.

To examine the above, we studied the relationship between GATA-5 and gastrointestinal mucin genes, MUC1-MUC6, by using two expression vector systems, that is, plasmid DNA and adenovirus. We found that GATA-5 selectively up-regulated the expression of gastrointestinal mucin genes in both COS-7 and 293T cells. These findings imply an important role of GATA-5 in the regulation of mucin genes and the maturation of the gut.

Materials and methods

Plasmid construction

For expression vectors of GATA-5, full-length GATA-5 was amplified by PCR from cDNA of a human gastric cancer cell line, NUGC3, using primers 5'-GCG CAA GCT TAG ATC TAG GAT GTA CCA GAG CCT GGC GC-3' (sense) and 5'-CGC GGA ATT CTA GGC CAA GGC CAG CGC AC-3' (antisense), according to GenBank sequences (NM080473 and AL499627). To obtain non-functional GATA-5 expression plasmids termed 'ΔZF' ('lacks the zinc finger domain' codons 188-267) and 'ΔZF' ('lacks both the zinc finger domain and the basic domain' codons 188-293), a two-step PCR strategy was used as described previously (Higuchi et al. 1988). The PCR products were restricted with Bgl II and EcoR I, or Hind III and EcoR I, and then subcloned into a mammalian expression vector, pcDNA3-T7 (Miyake et al. 2003) or pcDNA3 (Invitrogen, Groningen, Netherlands).

The human MUC4 5'-flanking region (-3677 to -1) was generated by PCR amplification of human genomic DNA using primers 5'-GCG CGC TAG CGG TCG GCC CAG GTG ATT GCC-3' (sense) and 5'-GCG CAA GCT TGG CTG CGG CAA AAG TCC CCC-3' (antisense), according to the GenBank sequence (AF241535). The PCR product was restricted with Nhe I and Hind III, and then subcloned into a promoterless luciferase expression plasmid, pGL3-basic (Promega, Madison, Wis., USA). The human pepsinogen A (PGA) promoter construct (-905 to +31) was generated by PCR amplification of human genomic DNA using primers 5'-ATA TGG TAC CGA CAT ACC TGG CCT GTC TGA AG-3' (sense) and 5'-GCG CGC TAG CCT CGA GGG AGA AGG CAA GAC GG-3' (antisense), according to the GenBank sequence (Z14129). The PCR product was restricted with Kpn I and Nhe I, and then subcloned into the same vector. The human MUC2 promoter construct (-1131 to +20) was described previously (Yamamoto et al. 2003).

To generate a human anti-GATA-5 antibody, a partial GATA-5 expression vector was constructed. A DNA fragment of human GATA-5 (codons 305-397) was amplified by RT-PCR, and then the PCR product was ligated into the BamHI/PstI sites of the pQE30 vector containing a 6xHis-tag sequence (Qiagen, Hilden, Germany). All constructs were verified by DNA sequencing.

Cell culture, transfection, and adenovirus infection

COS-7 and 293T cells were grown in Dulbecco's Modified Eagle's medium containing 10% fetal bovine serum and 50 µg/ml of kanamycin. For overexpression of the wild type or a deletion mutant of GATA-5, COS-7 and 293T cells were seeded at 1.25×10^6 per 10-mm dish. Twenty-four hours after cell plating, the cells were transfected with 10 µg of pcDNA3-GATA5, pcDNA-T7-GATA5, pcDNA-T7-GATA5ΔZF, pcDNA3-T7-GATA5ΔZF&B or the

empty pcDNA3 vector, using TransIT-LT1 transfection reagent (Mirus, Madison, Wis., USA) according to the manufacturer's recommendations. The wild type GATA-5 was also overexpressed in COS-7 cells using an adenovirus system (Akiyama et al. 2003). The same amounts of COS-7 cells were seeded 24 h before infection, and the cells were then infected with Ad-GATA5 (MOI 2.4) or Ad- β -Gal (MOI 12), which shows at least 90% green fluorescent protein (GFP) reactive cells with minimal to no cytotoxicity as compared with parental cells. In addition, cells without transfection or infection were cultured in parallel as a control.

Production of antibodies

Plasmid pQE30-GATA5 was introduced into competent *Escherichia coli* JM109 cells and then expression was induced with 0.1 mol/l isopropyl- β -D-thiogalactopyranoside. The 6xHis-tagged GATA-5 protein was purified from a soluble fraction of cells using Ni-NAT matrices. Rabbits were injected subcutaneously with 100 μ g of 6xHis-tagged GATA-5 protein conjugated in Freund's incomplete adjuvant five times (every 2 weeks). The sera were found to contain GATA-5 antibodies, as judged from their ability to recognize the bacterially produced GATA-5 protein on immunoblotting analysis and enzyme-linked immunosorbent assay (data not shown).

Western blot analysis

Protein was extracted from harvested cells according to the protocol described previously (Harlow and Lane 1999). Protein concentrations were determined by the Bradford method (Bio-Rad, Hercules, Calif., USA). Twenty micrograms of the whole protein extracts were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The separated proteins were then transferred to Immobilon-P transfer membranes (Millipore, Bedford, Mass., USA). Each membrane was blocked in 5% powdered milk in Tris-buffered saline containing 0.05% Tween 20 for 1 h at room temperature prior to incubation with the primary antibody. The membrane was then incubated with anti-GATA-5 serum at 1:200 or anti-T7 (Novagen, Darmstadt, Germany) at the concentration of 0.2 μ g/ml or anti- α -tubulin (B-5-1-2; Sigma, St. Louis, Mo., USA) at 1:2,000 for 1 h at room temperature. Following four washes with Tris-buffered saline containing 0.05% Tween 20, the bound antibodies were detected with alkaline phosphatase-conjugated secondary antibodies and with Immuno-Star (Bio-Rad) according to the manufacturer's instructions.

Reverse-transcription-PCR (RT-PCR) analysis

Total RNA was isolated 48 h after DNA transfection or adenovirus infection using an RNeasy Mini Kit (Qiagen) according to the manufacturer's recommendations. The isolated RNA (1 μ g) was preincubated with 0.4 μ g of 12–18 mer oligo (dT) at 70 °C for 10 min, and then with 10 mmol/l dNTP, 0.1 mol/l DTT, and 1 μ l of Superscript II (RNase H [-] reverse transcriptase; Invitrogen) at 42 °C for 1 h.

RT-PCR analysis was performed for amplification of the mucins and PGA mRNAs with the specific primer pairs for human MUC1, MUC2, MUC3A, MUC3B, MUC4, MUC5AC, MUC5B, MUC6, and PGA listed in Table 1. All these primers were designed to cross over one or more intron/exon boundaries according to the sequences of these genes. Each PCR cycle consisted of 94 °C for 1 min, 57–61 °C for 1 min, and 72 °C for 1 min, followed by final extension at 72 °C for 10 min. We carried out PCR with 25, 28, 30 and 35 cycles to semiquantitatively analyze expression of these genes. The PCRs were performed in a 25- μ l mixture comprising 1 μ l of cDNA, 5% dimethyl sulfoxide, 2.5 μ l of 10 \times PCR buffer, 4 μ l of 1.25 mmol/l dNTP (Biotect International, Bentley, Australia), 10 pmol of each oligonucleotide primer pair, and 0.5 units of Taq DNA polymerase (Biotect International). The glyceraldehyde-3-phosphate dehydrogenase

Table 1 Primer pairs used for PCR reactions

Gene	Primer pair		Antisense	Annealing temperature	Size of product (bp)	GenBank accession no.
	Sense	Antisense				
MUC1	5'-GAACTACGGGACGCTGGACATC-3'	5'-GGGCTCTTGGGCCAGTCTCC-3'	5'-GGGCTCTTGGGCCAGTCTCC-3'	65 °C	451	NM_002456
MUC2	5'-GACATTTGTCATGACTCGGC-3'	5'-GCAAGGACTGAACAAAGACTC-3'	5'-GCAAGGACTGAACAAAGACTC-3'	57 °C	358	M194132
MUC3A/B	5'-GCCCTGGAGAACGCTGGACACC-3'	5'-GGAGGGGATGGGTTTAAAGGC-3'	5'-GGAGGGGATGGGTTTAAAGGC-3'	56 °C	1082	AB038784 (A)
MUC4	5'-GAACGTGAGCACGCTGAAGGCTTAC-3'	5'-CAAGGCAAGGCCTCAGGTGATTC-3'	5'-CAAGGCAAGGCCTCAGGTGATTC-3'	60 °C	1213	AB038783 (B)
MUC5AC	5'-CGACCTGTGTGTACCATAG-3'	5'-GGACAGGGGCACAAAGTCCAC-3'	5'-GGACAGGGGCACAAAGTCCAC-3'	58 °C	470	AJ001402
MUC5B	5'-GTGGACAACCTGCACCGTGTACC-3'	5'-GTAGGTGTGCAGGATGGCTGAG-3'	5'-GTAGGTGTGCAGGATGGCTGAG-3'	58 °C	362	XM_039877
MUC6	5'-ACCTTGTGCCTACCACACCG-3'	5'-CACCTGCAGGTGAGTACGAG-3'	5'-CACCTGCAGGTGAGTACGAG-3'	61 °C	350	U977698
PGA	5'-CCATGACGGAGAGCCATCG-3'	5'-GGAAAGAACAGTCAGGAGAATTAG-3'	5'-GGAAAGAACAGTCAGGAGAATTAG-3'	60 °C	480	NM_014224

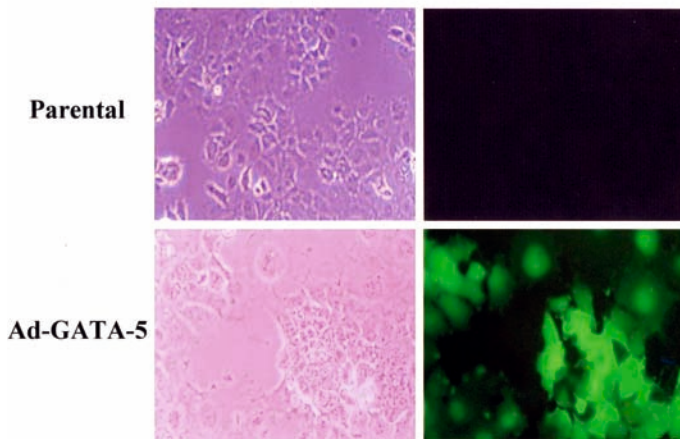
A

Fig. 1A, B Expression of GATA-5 in COS-7 cells. **A** Expression of GATA-5 in COS-7 cells using the adenovirus system. The *left panels* show the morphological appearance and the *right ones* show GFP expression in the same fields; **B** Western blot analysis of GATA-5 protein expression in COS-7 cells. The levels of GATA-5 expression were determined with an antibody against human GATA-5. Anti- α -tubulin is shown at the bottom as a control

(GAPDH) gene was amplified as an internal control for RT-PCR (Bai et al. 2000). The PCR products (10 μ l) were electrophoresed in 2% agarose gels. We confirmed the nucleotide sequences of the PCR products by sequencing.

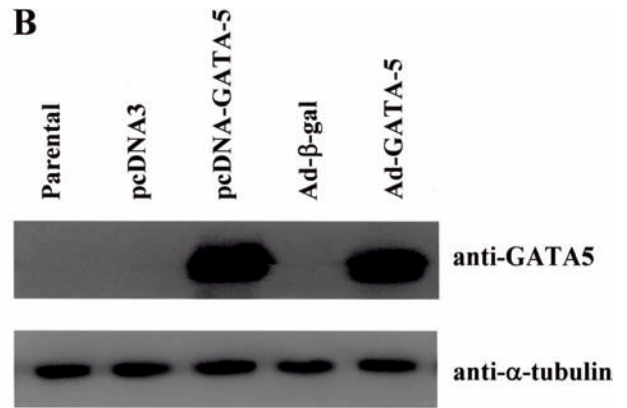
Reporter gene assays

For luciferase assays, transfection was performed using TransIT transfection reagent (Mirus) according to the manufacturer's recommendations. COS-7 cells were plated at 5×10^4 cells per well (24-well plate) 1 day before transfection. The cells (50%–80% confluent) were co-transfected with 400 ng of pcDNA3-T7-GATA5, pcDNA3-T7-GATA5 Δ ZF, pcDNA3-T7-GATA5 Δ ZF&B or pcDNA3 empty vector, 100 ng of MUC2, MUC4 or PGA reporter plasmid, and 10 ng of pRL-SV40 vector. The pcDNA3 vector was used as a negative control. The pRL-SV40 vector containing the SV40 early promoter upstream of renilla luciferase was co-transfected as an internal control. Cells were harvested after 48 h of transfection, and then luciferase activity was measured with a Dual-Luciferase Reporter Assay System (Promega) as described by the manufacturer with a Lumicounter 700 (Microtech Niti-On, Chiba, Japan). Each transfection was performed in triplicate and experiments were repeated three times. The results were expressed as fold activation, i.e., the ratio of normalized luciferase activity of the GATA-5 expression construct to that of the empty vector.

Results

Analysis of transgene expression

To study the effect of GATA-5 on the endogenous expression of several mucin genes *in vitro*, COS-7 cells, which do not express GATA-5, were transiently transfected with either a GATA-5 expression vector or a pcDNA3 empty vector. Adenovirus-mediated infection was also performed in this study because of the high infection efficiency, and at least 90% of the cells expressed GFP, which was determined under a fluorescent microscope at 48 h post-infection for both Ad-GATA-5-

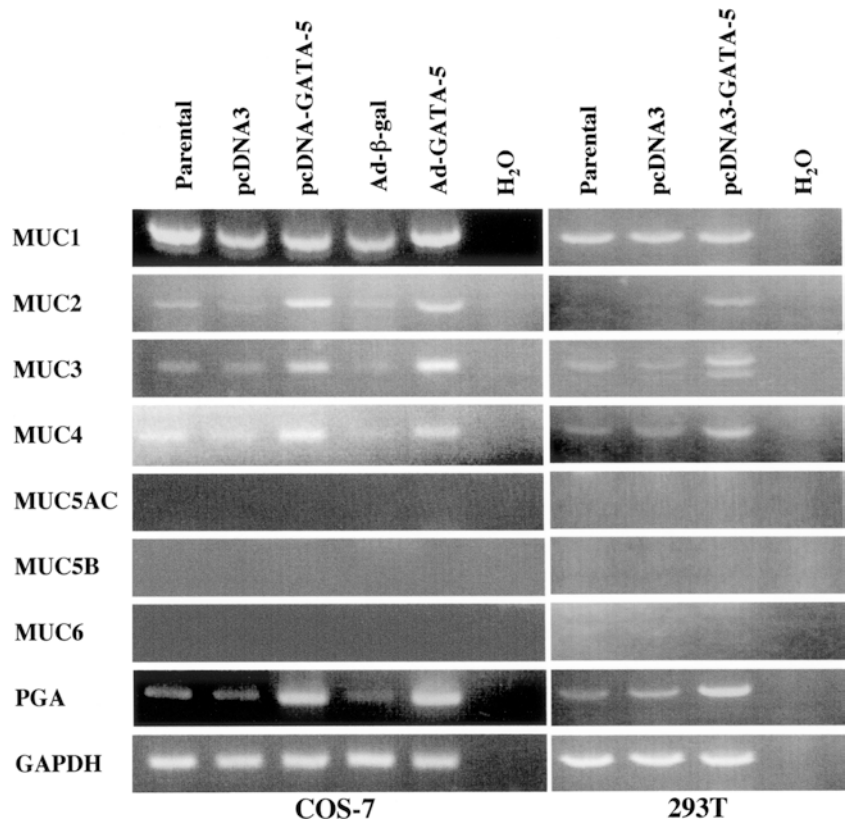
B

and Ad- β -gal-infected COS-7 cells (Fig. 1A). Ectopic expression of the GATA-5 protein was confirmed by Western blot analysis (Fig. 1B), GATA-5 being abundantly expressed in both DNA-transfected and adenovirus-infected COS-7 cells. In contrast, the GATA-5 protein was undetectable in parental cells or empty vector-treated cells. Similar results were obtained in 293T cells after transfection of the GATA-5 expression vector (data not shown). There were no obvious morphological changes in the GATA-5-expressing COS-7 or 293T cells during the experiment.

Overexpression of GATA-5 up-regulates endogenous MUC2, MUC3, MUC4, and PGA mRNA expression

To study the effects of GATA-5 on the regulation of endogenous MUC1, MUC2, MUC3A, MUC3B, MUC4, MUC5AC, MUC5B, and MUC6 gene expression, a semi-quantitative RT-PCR assay was used for GATA-5-expressing COS-7 and 293T cells using a specific primer pair for each gene (listed in Table 1). The pcDNA-3 empty vector-transfected and Ad- β -gal-infected cells were used as negative controls. As shown in Fig. 2, the internal standard GAPDH mRNA level remained constant in parental cells, pcDNA3 empty vector- or pcDNA3-GATA-5-transfected cells, and Ad- β -gal- or Ad-GATA-5-infected cells (refer to the GAPDH panel). Among these MUC genes, COS-7 and 293T cells expressed a high level of MUC1, but undetectable levels of MUC5AC, MUC5B, and MUC6, and the expression of these genes was remained constant in empty vector- or GATA-5 expression vector-treated cells. In contrast, these two cells expressed low levels of MUC2 and MUC4 endogenously, and the mRNAs of MUC2 and MUC4 were significantly up-regulated in GATA-5-expressing cells compared with in parental cells or empty vector-treated cells (Fig. 2). As for MUC3, one and two bands were detected in COS-7 and 293T cells, respectively (Fig. 2). Sequencing analysis revealed that MUC3A was amplified in COS-7 cells, while MUC3A (1082 bp, lower band) and MUC3B (1213 bp, upper band) were amplified in 293T cells (data

Fig. 2 Increase in MUC2, MUC3, MUC4, and PGA gene expression caused by GATA-5 in COS-7 and 293T cells. Semiquantitative RT-PCR was performed as described under Materials and methods. The number of PCR cycles was 28 for MUC1 and 35 for MUC2, MUC5AC, MUC5B, and MUC6 in COS-7 and 293T cells. As for MUC3 and MUC4, the number of PCR cycles was 35 in COS-7 and 33 in 293T. GAPDH (21 cycles) was used as a control



not shown). The difference may be explained by cell origins, that is, COS-7 and 293T cells were derived from monkey and human tissues, respectively. In COS-7 cells, MUC3A was up-regulated in GATA-5-expressing cells. MUC3A was abundantly found in GATA-5-expressing 293T cells compared with in parental cells or empty vector-treated cells, which showed a very low level of MUC3A expression. Although basal MUC3B expression was seen in 293T cells, GATA5 slightly up-regulated MUC3B (Fig. 2). These results indicate that overexpression of GATA-5 in both COS-7 and 293T cells up-regulates the endogenous expression of MUC2, MUC3A, and MUC4, but not that of MUC1, MUC5AC, MUC5B, and MUC6.

In addition, GATA binding sites are present in the promoter region of the human PGA gene as well as chicken ECPg, therefore we also studied whether or not overexpression of GATA-5 up-regulates endogenous expression of the PGA gene. A faint band of PGA was detected for parental COS-7 and 293T cells, whereas the mRNA level of PGA was strongly up-regulated in GATA-5-expressing cells compared with in empty vector-treated cells. Thus GATA-5 up-regulates endogenous PGA expression.

Mutant type GATA-5 lacking the zinc finger domain abrogate up-regulation of endogenous MUC2, MUC3A, MUC4, and PGA

To confirm the effects of GATA-5 on up-regulation of MUC2, MUC3A, MUC4, and PGA gene expression,

plasmids encoding two GATA-5 deletion mutants, pcDNA3-T7-GATA5 Δ ZF and pcDNA3-T7-GATA5 Δ ZF&B (Fig. 3A), were transfected into COS-7 cells, the pcDNA3 empty vector being transfected as a negative control. Cells were harvested after 48 h of transfection, and then the abundant production of the wild type GATA-5 and mutant GATA-5 proteins were confirmed by Western blotting using anti-T7 (Fig. 3B) or anti-GATA-5 antibodies (data not shown). Endogenous expression of MUC2, MUC3A, and MUC4 was determined by means of RT-PCR, as shown in Fig. 3C. Up-regulation of MUC2, MUC3A, and MUC4 was detected in cells treated with wild type GATA-5, but not in cells expressing mutant type GATA-5. The same results were obtained for the human PGA gene, suggesting that the zinc finger DNA binding domain is required for GATA-5 to regulate its target genes.

Activation of MUC2, MUC4, and PGA reporter constructs by GATA-5

To determine whether or not GATA-5 is capable of transactivating the promoters of the MUC2, MUC3, MUC4, and PGA genes, reporter gene assays were performed on COS-7 cells. Among these four genes, the 5'-flanking regions of the MUC2, MUC4, and PGA genes have been identified. Therefore promoter constructs of human MUC2 (Yamamoto et al. 2003), MUC4 (-3677 to -1), and PGA (-905 to +31) were co-transfected with either the wild type or a mutant type

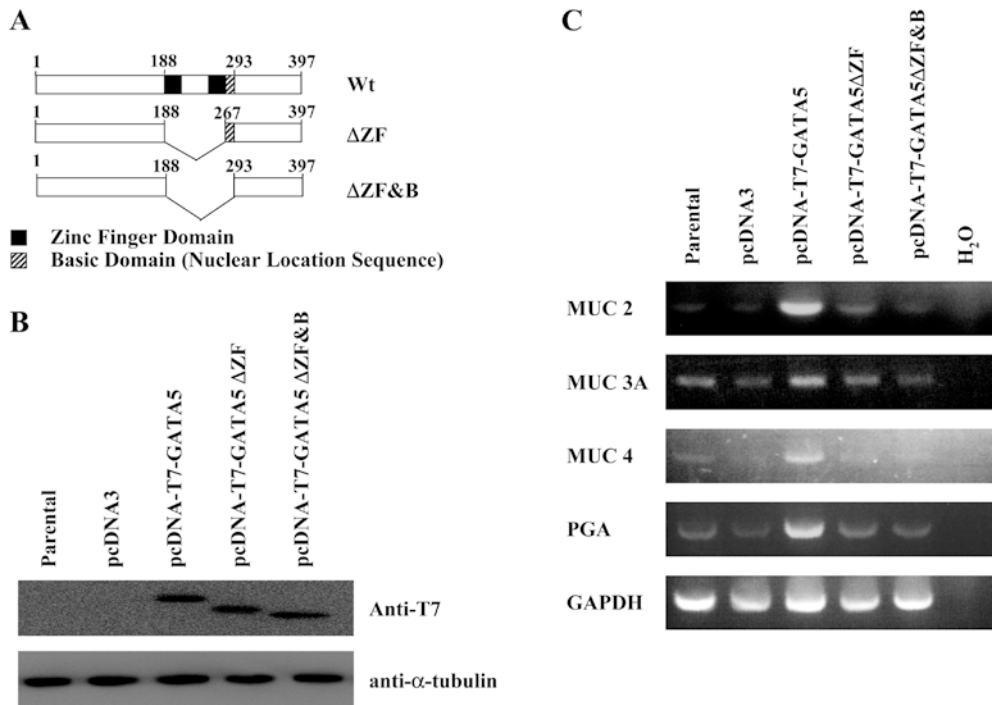


Fig. 3A–C The wild type GATA-5, but not the GATA-5 deletion mutants, up-regulates MUC2, MUC3A, MUC4, and PGA expression. **A** Structures of the wild type (Wt) and mutant GATA-5 (Δ ZF and Δ ZF&B) cDNAs. COS-7 cells were transfected with pcDNA3-T7 expression vectors encoding either the wild type GATA-5, or GATA-5 Δ ZF or GATA-5 Δ ZF&B. **B** The GATA-5 constructs are abundantly expressed in COS-7 cells. Western blot analyses of cell extracts was performed with an anti-T7 antibody; **C** RT-PCR analysis of endogenous MUC2, MUC3A, MUC4, and PGA mRNA expression in COS-7 cells transfected with a plasmid encoding either the wild or mutant type GATA-5. The number of PCR cycles was 35 for each gene. Expression of GAPDH (21 cycles) was used as a control

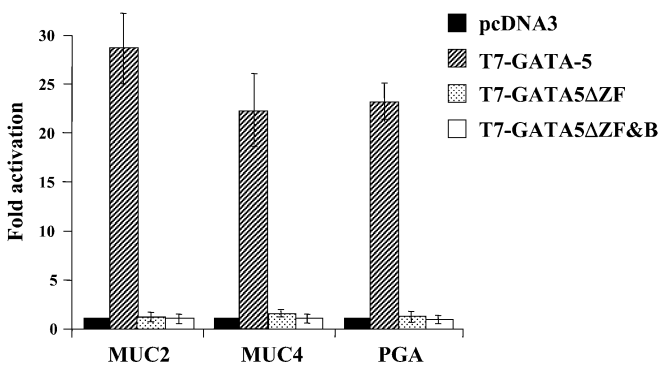


Fig. 4 GATA-5 transactivates the promoters of the human MUC2, MUC4, and PGA genes. Transfection was carried out as described under Materials and methods. Transcriptional activation by GATA-5 is expressed as fold activation. The experiments were repeated three times, and the results are presented as means \pm SD

GATA-5 expression vector. Wild type GATA-5 stimulated the transcription of MUC2 28-fold, MUC4 22-fold, and PGA 24-fold, respectively (Fig. 4). In contrast, transcription was not enhanced for MUC2, MUC4 or PGA by GATA-5 Δ ZF or GATA-5 Δ ZF&B, suggesting that GATA-5 is a transcriptional regulator of these genes.

Discussion

Understanding of molecular mechanisms that govern tissue-specific gene expression often leads to the identification of transcription factors responsible for tissue specialization and maturation. In the present study, we found that transcription factor GATA-5 up-regulated the endogenous expression of gastrointestinal mucin genes MUC2, MUC3A, and MUC4 in both COS-7 and 293T cells. In 293T cells, MUC3B was also slightly up-regulated in GATA-5-expressing cells. Since we used two GATA-5 expression vector systems, that is, plasmid DNA and adenovirus in COS-7 cells, and obtained the same results, our results are more reliable. The reporter gene assay performed for MUC2 and MUC4 genes revealed that the up-regulation is possibly due to the activation of their promoters. Recently, the 5'-regulatory region of the human MUC3A gene was reported to contain GATA binding sites (Gum et al. 2003), which strongly suggested that GATA-5 also activates the promoter of human MUC3A gene. In contrast, GATA-5 lacking the zinc finger domain impaired the functions of GATA-5. Our results strongly indicated that GATA-5 plays critical roles in the regulation of mucin gene expression.

Each GATA protein contains highly conserved zinc finger DNA binding domains, which interact with GATA regulatory elements present in a number of tissue-specific genes. Protein domain analyses of GATA-4 and GATA-6 indicated that the zinc-finger is necessary for DNA binding (Morrisey et al. 1997b; Perlman et al. 1998). Here we revealed that deletion of the zinc finger domain impaired the ability of GATA-5 to both up-regulate endogenous MUC2, MUC3A,

MUC4, and PGA expression, and activate promoter constructs of the MUC2, MUC4, and PGA genes. The loss of function of GATA-5 may be due to the lack of ability to bind to GATA elements within their promoters. These results indicated that the zinc-finger DNA binding domain is necessary for GATA-5 as well as for other GATA factors for the regulation of their target genes.

Previous studies demonstrated that GATA-5 modulates the promoter functions of several intestine-specific genes, including the rat and human lactase-phlorizin hydrolase (LPH) (Fang et al. 2001; Krasinski et al. 2001), human sucrase-isomaltase (SI) (Krasinski et al. 2001), and *Xenopus* intestinal fatty acid-binding protein (xIFABP) genes (Gao et al. 1998). Here we showed that GATA-5 up-regulated intestine-specific mucin genes MUC2 and MUC3, which supports the concept that GATA-5 plays critical roles in the regulation of intestinal gene expression.

GATA-5 has also been implicated in activation of gastroprotective trefoil factor genes TFF1 and TFF2 (Akiyama et al. 2003). Our results showed that GATA-5 activated both the endogenous expression and promoter activity of human PGA, a differentiation marker of chief cells of the gastric glands. Thus, our findings indicated that GATA-5 may also function in the regulation of stomach-specific gene expression.

The transcriptional activity of GATA proteins is modulated through interactions with other transcription factors and transcriptional coactivators. The combinatorial interaction of GATA-4 with homeoprotein CDX2 and transcription factor HNF-1 α to up-regulate intestinal gene SI has been reported (Boudreau et al. 2002). Expression of CDX2 was observed in the intestinal epithelium and CDX2 has been reported to transcriptionally regulate intestinal mucin MUC2 (Yamamoto et al. 2003; Yuasa 2003). We found that GATA binding sites are very close to the CDX binding sites in the 5'-flanking region of the human MUC2 gene, so it is possible that GATA-5 may synergistically interact with CDX2 to up-regulate the expression of the MUC2 gene. In addition, putative CDX binding sites are present in the 5'-flanking region of the human MUC4 gene, and the recently identified human MUC3A promoter also contains both GATA and CDX binding sites (Gum et al. 2003). It would be interesting to determine whether or not GATA-5 interacts with the other transcription activators of MUC2, MUC3A, and MUC4, such as CDX2.

GATA-4 and GATA-6 are members of the GATA transcription factor family that has been reported to be expressed in the gastrointestinal epithelium, like GATA-5. They also recognize DNA sequence elements containing a core GATA motif. They have been implicated in modulating the expression of intestinal genes including xIFABP (Gao et al. 1998), human LPH (Krasinski et al. 2001), and SI (Boudreau et al. 2002). The overlapping expression patterns and functions of GATA-4/-5/-6 suggest that GATA-4 and GATA-6 are also candidate transcription regulators of gastrointestinal mucin

genes. Therefore, additional studies are needed to determine the distinct roles of GATA-4, -5, and 6 in the regulation of gut-specific gene expression.

Conclusions

Gastrointestine-specific genes MUC2, MUC3, MUC4, and PGA were identified as direct downstream targets for GATA-5. Further investigations are necessary to determine the role of GATA-5 in the differentiation of gastrointestinal cells.

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