GATA family transcription factors activate lactase gene promoter in intestinal Caco-2 cells

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Fang, Rixun, Lynne C. Olds, Nilda A. Santiago, and Eric Sibley. GATA family transcription factors activate lactase gene promoter in intestinal Caco-2 cells. Am J Physiol Gastrointest Liver Physiol 280: G58–G67, 2001.—The GATA family of transcription factors regulate tissue-specific patterns of gene expression during development. We have characterized the interaction between GATA proteins and the lactase gene promoter. Nuclear protein bound to the lactase gene GATA region cis element (~97 to ~73) was analyzed by electrophoretic mobility shift assays (EMSA) and supershift assays with GATA antibodies. Lactase promoter activities were assayed in Caco-2 cells transfected with wild-type and mutated luciferase promoter-reporter constructs and GATA-4/5/6 expression constructs. EMSA with the GATA region probe yields a specific DNA-protein complex that requires the GATA factor binding site WGA-TAR. The complex is recognized by GATA-4- and GATA-6-specific antibodies. GATA-4/5/6 expression constructs are able to activate transcription driven by the wild-type promoter, but not by a promoter in which the GATA binding site is mutated, in Caco-2 and nonintestinal QT6 cells. GATA factor binding to the lactase cis element correlates with functional promoter activation. We conclude that each of the GATA family zinc finger proteins expressed in the intestine, GATA-4, -5, and -6, can interact with the lactase promoter GATA element and can function to activate the promoter in Caco-2 cells.

GATA-4; GATA-5; GATA-6; enterocyte

THE INTESTINAL EPITHELIUM is comprised of four principal cell types that are derived from a proliferating stem cell population (see Ref. 10 for review). The stem cells, located in crypts near the intestinal villus base, undergo terminal differentiation as they migrate from the crypt to the villus tip, where they ultimately die and are sloughed off into the gut lumen. The four principal intestinal epithelial cell types have highly specialized functions. The absorptive enterocytes are the predominant intestinal cell type, and they function to digest and absorb luminal nutrients across the apical brush-border membrane. Goblet cells secrete mucus that provides a protective barrier lining the gut. The enteroendocrine cells secrete intestinal hormones involved in signaling gut motility. Paneth cells terminally differentiate during migration toward the base of the crypt and are lysozyme- and defensin-producing cells. The molecular mechanisms regulating terminal differentiation of the intestinal cell types have not been defined.

The GATA family of zinc finger transcription factors are important regulators of cell lineage differentiation during vertebrate development. Six GATA family members have been identified in vertebrate species. Each vertebrate GATA factor contains two conserved zinc fingers that bind to a consensus sequence (WGA-TAR) that is present in the transcriptional regulatory region of multiple lineage-specific genes (13, 17). The GATA-1, -2, and -3 subfamily proteins control critical steps in erythroid and lymphoid development (8, 22, 23, 29). GATA-4, -5, and -6 subfamily proteins are expressed in overlapping patterns in the developing heart and endoderm-derived organs of the gastrointestinal tract including the stomach, intestine, liver, and pancreas (1, 5, 16, 19, 20). The functional role of GATA-4, -5, and -6 proteins in regulating heart and gut development is largely unknown. Homozygous disruption of GATA-4 expression during embryogenesis results in the lack of a primitive heart tube and foregut in mice (15). Targeted mutagenesis of the GATA-4 gene in embryonic stem cells disrupts visceral endoderm differentiation (25). GATA binding sites have been identified in transcriptional regulatory elements of several cardiac-specific target genes. Overexpression of GATA-4 protein has been shown to transactivate these cardiac-specific cis elements in cell culture (11, 12, 18).

With respect to a role in regulating enterocyte differentiation, distinct patterns of expression for GATA-4, -5, and -6 were recently described by Gao et al. (9). The expression patterns of the GATA factors differ along the proximal-distal villus axis in the chicken intestine and during differentiation in culture. In situ transcript levels for GATA-4 are highest in the proliferating cell region of the crypts, whereas levels for GATA-4 and GATA-5 increase toward the villus tip. On stimulation to differentiate, intestinal HT-29 cells begin to express GATA-5 whereas GATA-6 transcript levels decline. Few intestinal target genes, however, have been identified for the GATA-4/5/6 factor subfamily. In the same report by Gao et al. (9), the gene encoding Xenopus intestinal fatty acid binding protein (IFABP) was identified as an in vitro target for the GATA family of transcription factors.
GATA-4/5/6 factors in intestinal cell culture. In addition, Fitzgerald et al. (7) recently reported that GATA-6 stimulates promoter activity of the human lactase gene promoter. We report here that GATA-4 and GATA-5, in addition to GATA-6, can activate the rat lactase promoter in intestinal cell culture. These studies provide compelling evidence for the potential of each member of the GATA-4/5/6 factor subfamily to regulate expression of multiple enterocyte-specific genes.

Intestinal lactase-phlorizin hydrolase (LPH, lactase) is the absorptive enterocyte membrane glycoprotein essential for digestive hydrolysis of lactose in milk. Lactase is present predominantly along the brush-border membrane of differentiated enterocytes lining the villi of the small intestine. Expression of the lactase gene is spatially restricted around the brush border membrane of differentiated enterocytes lining the villi of the small intestine. Expression of the lactase gene is expressed maximally in the proximal small intestine and declines significantly in the distal segments of the intestine. Lactase gene expression is also temporally restricted in the gut during intestinal maturation. Enzyme activity is maximal in the small intestine of preweaned mammals and declines markedly during maturation (3, 14, 21). The mechanisms regulating the spatial restriction and maturational decline in lactase activity have not been fully defined. In vitro binding studies showed that the lactase gene promoter interacts with specific nuclear proteins from intestinal cells (28). The homeodomain protein Cdx-2 binds to a distinct cis element, CE-LPH1, of the lactase 5’-flanking region and is capable of activating transcription of the lactase promoter (6, 27). Regulation of intestine-specific differentiation is likely to involve additional transcription factors that control expression of terminal differentiation genes such as LPH. Here we identify a GATA binding site element in the promoter region of the lactase gene, identify the GATA proteins interacting with the element, and characterize transcriptional activation mediated by GATA-4, -5, and -6 proteins in intestinal Caco-2 cell culture.

MATERIALS AND METHODS

Materials and reagents. Restriction endonucleases and modifying enzymes were purchased from New England Biolabs (Beverly, MA). Radioisotopes were purchased from DuPont NEN. Oligonucleotides were synthesized in the Protein and Nucleic Acid facility of the Stanford University Beckman Center.

Subcloning of deletion and mutant lactase promoter-reporter constructs. The lactase promoter deletion and mutant reporter constructs were generated from pgLac (6), a previously described plasmid in which a 200-bp 5’-flanking region of the rat lactase gene (nt −200 to +13) was cloned upstream of the firefly luciferase reporter gene in the vector pGL3-Basic (Promega). Deletion promoter-reporter constructs were generated by initial PCR amplification of pgLac using forward oligonucleotides corresponding to nt −100 to −80, nt −73 to −53, or nt −58 to −38 (each with an added 5’ Bgl II site) and GL primer 2 (Promega), the reverse oligonucleotide proximal to the luciferase gene in pGL3-Basic. The Bgl II-Hind III fragments for each of the PCR products were then cloned into pGL3-Basic to generate the corresponding −100, −73, and −58 bp deletion promoter-reporter constructs.

To clone mutant lactase promoter-reporter constructs, a fragment of the wild-type pgLac construct was amplified by PCR using a forward mutant GATA region oligonucleotide (Mut-A, Mut-B, Mut-C, or Mut-D, sequences shown in Fig. 1) and GL primer 2. The mutant PCR product and a forward lactase gene oligonucleotide corresponding to nt −265 to −240 were used in a second PCR reaction to amplify a 0.3-kb segment of the pgLac template. The internal 0.2-kb Bgl II-Xho I fragment of the second PCR product was cloned into pGL3-Basic to generate pMutA, pMutB, pMutC, and pMutD. Incorporation of the correct deletion and mutated base pairs was confirmed by sequencing.

Electrophoretic mobility shift assay and supershift assay. Nuclear extracts were prepared from Caco-2 or QT6 cells according to a modification of a previously described procedure (2). Cells were harvested by scraping and resuspended in 5 vol of phosphate-buffered saline followed by centrifugation (400 g for 10 min at 4°C). The cells were resuspended in five packed cell volumes of cold buffer A [in mM: 10 HEPES (pH 7.9), 10 KCl, 1.5 MgCl₂, 0.5 diithiothreitol, and 1 phenylmethylsulfonyl fluoride (PMSF)] and incubated for 10 min on ice followed by centrifugation as above. Cells were resuspended in two packed cell volumes of buffer A, lysed by Dounce homogenization, and then centrifuged (400 g for 20 min at 4°C). The nuclei pellet was resuspended in 3 ml of cold buffer B (20 mM HEPES (pH 7.9), 10% glycerol, 0.42 M NaCl, 1.5 mM MgCl₂, 0.5 mM diithiothreitol, and 2 mM EDTA, and 1 mM PMSF) followed by Dounce homogenization and stirred gently for 30 min at 4°C. After centrifugation (100,000 g for 20 min), the supernatant was precipitated with ammonium sulfate (final concentration 0.33 g/ml) and then centrifuged (25,000 g for 20 min). The pellet was resuspended in buffer C (20 mM HEPES (pH 7.9), 10% glycerol, 0.5 mM dithiothreitol, 0.2 mM EDTA, and 1 mM PMSF) dialyzed against buffer C for 12–18 h at 4°C, and stored at −80°C.

Nuclear extracts were incubated with a radiolabeled GATA region binding site probe for gel shift assay. The

Wild Type 5′ ATCTCTAGATATAACCCGATTTAATA 3′

Mut-A 5′ TACCGAGATATAACCCGATTTAATA 3′

Mut-B 5′ TATCCCTAGCTAACCCGATTTAATA 3′

Mut-C 5′ TATCCCTAGCTAACCCGATTTAATA 3′

Mut-D 5′ TATCCCTAGCTAACCCGATTTAATA 3′

Fig. 1. Sequence of wild-type and mutated GATA region cis element of the rat lactase gene promoter used in this study. The sequence of the wild-type GATA region cis element of the rat lactase promoter is shown at top. Double-stranded mutations corresponding to bases in boldface were incorporated into synthetic oligonucleotides for gel shift analysis (see Fig. 3, MutA, -B, -C, and -D) and into site-directed mutagenesis reporter constructs (see Figs. 5 and 6, pMutA, pMutB, pMutC, and pMutD). The GATA protein consensus DNA binding site is underlined.
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GATA region probe consisted of a double-stranded 25-nt oligonucleotide (5'-TATCCTAGATAACCCAGTTAAATA-3') from -96 to -73 relative to the start site of transcription of the rat lactase gene (21). The Sp1 binding site probe consisted of a double-stranded 28-nt oligonucleotide (5'-GATCGGGGGGGGCGGGGCGAGTAC-3'). The sequence of the mutant oligonucleotides (Mut-A, Mut-B, Mut-C, and Mut-D) is shown in Fig. 1. The Klno fragment-labeled probes were generated by annealing single-stranded oligonucleotides to yield a four-base 5' overhang on each end followed by fill-in incorporation with nucleotide triphosphates, including [32P]dTTP (3,000 Ci/mM). Unincorporated nucleotides were removed by purification with a Bio-Spin P30 column (Bio-Rad). Nuclear extract (15 μg) was incubated in the presence of 40,000 cpm labeled probe (10^6 cpm/ng) with 0.5 μg poly dI:dC with or without 100-fold excess unlabeled probe in 5 mM HEPEs (pH 7.9), 10% glycerol, 25 mM KCl, 0.05 mM EDTA, and 0.125 mM FMSP for 20 min at room temperature. DNA-protein complexes were resolved on a 6% Tris-glycine polyacrylamide gel that was then dried and exposed to X-ray film for autoradiograph detection. Gel supershifts were carried out with the Caco-2 or QT6 nuclei extracts and the gel shift method detailed above with the exception that the reaction mixture was incubated for 30 min at room temperature in the presence of 1 μl of a polyclonal GATA-4 or GATA-6 antibody (Santa Cruz Biotechnology).

Transient transfection assays. Caco-2 cells or QT6 cells were cultured in DMEM with 10% fetal bovine serum. Forty-eight to sixty hours before transfection, the cells were split and 55-mm dishes were seeded with 2 × 10^5 cells for each reporter construct. A DNA transfection mixture was prepared consisting of 0.6 μg of the construct, 0.5 μg of pRL-CMV (Promega) as an internal control, and pBluescript K5 nicked to adjust to 3.5 μg of total DNA. The individual DNA mixtures were transfected into cells (50–80% confluent) with lipofectamine reagent (BRL) according to the protocol of the manufacturer. For cotransfection experiments, 0.3 pmol of X. tropicalis GATA cDNAs were cloned downstream of the firefly luciferase promoter (GMP) in pCDNA3, which was transfected along with 0.6 pmol of the luciferase reporter constructs. Cells were harvested 48 h after transfection (70–90% confluent), and luciferase activity was measured by the Dual-Luciferase reporter assay system (Promega) as described by the manufacturer, in a Monolight 3010 luminometer. Transfection with promoterless pGL3-Basic was allowed for simultaneous expression and measurement of both reporter enzymes. The lactase promoter-reporter activity correlated with the effect of the promoter sequence or GATA expression, and the activity of the cotransfected pRL-CMV provided an internal control. Experimental lactase promoter-reporter activities were normalized to the activity of the pRL-CMV internal control and expressed as relative luciferase activity (means ± SD, n = 3), thereby minimizing experimental variability caused by differences in cell viability or transfection efficiency.

RNA analysis by RT-PCR. Total RNA was extracted from proliferating Caco-2 cells using Tri reagent (Molecular Research Center) according to the protocol of the manufacturer. RNA concentrations were determined by optical densitometry at 260 nm, and the absence of RNA degradation was confirmed by agarose gel electrophoresis. For RT-PCR, cDNA was initially synthesized from 1.0 μg of Caco-2 cell total RNA using avian myeloblastosis virus RT and the Advantage RT-for-PCR kit (Clontech) according to the protocol of the supplier and brought to a final volume of 100 μL. PCR reactions were then carried out with synthetic oligonucleotide primers corresponding to exon sequences for the human GATA-4 (Ref. 30; GenBank accession no. D78280), GATA-6 (Ref. 26; accession no. NM005257), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (internal standard; Ref. 4; accession no. J04038) genes: GATA-4F 5'-CATCAGACGAGCTTGCGC-3' and GATA-4R 5'-TGACTGTCGGCAAGACCCAG-3' (218-bp product); GATA-6F 5'-CCATGCACTTCCAACC-3' and GATA-6R 5'-AGGGACAGGAGCAGTGACTTCGGG-3' (213-bp product); and GAPDH-F 5'-GGGTCATCATCTCTGCCCCTCTG-3' and GAPDH-R 5'-CCATCACAGTCTTCTGTGGTGGG-3' (208-bp product). To prevent nonspecific amplification hot-start PCR reactions (50 μl) were carried out with 5 μl of the reverse-transcribed cDNA, 0.4 μM primers, 0.20 mM dNTPs, 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl2, and 2.5 units of Taq polymerase (GIBCO BRL) preincubated with 1.28 with TaqStart Antibody (Clontech). Control amplifications confirmed that the reactions were entirely dependent on the RT reaction. Amplification conditions were 94°C for 45 s, 55°C for 45 s, and 72°C for 5 s performed for 15–40 cycles to optimize for PCR products in the linear range of exponential amplification. Simultaneous PCR reactions were performed with first-strand cDNA generated from RNA of different human tissues (MTC; Clontech). PCR products were analyzed after electrophoresis on 2% agarose gels using a Molecular Analyst densitometer (Bio-Rad) and sequenced to confirm their identity.

RESULTS

Regulatory regions of lactase gene 5'-flanking DNA mapped by deletional analysis of reporter constructs. Promoter activity was previously mapped to within −200 bp upstream from the transcription start site of the rat lactase gene (6). To identify regions of the lactase gene capable of mediating regulation of gene transcription, deletion fragments of this promoter region were cloned upstream of the firefly luciferase cDNA in the reporter plasmid pGL3-Basic and transfected into Caco-2 cells. The −100, −73, and −58 bp lactase reporter constructs, shown in Fig. 2A, were transiently transfected into Caco-2 cells, a human adenocarcinoma-derived cell line that mimics a small intestinal enterocyte phenotype with respect to expression of several digestive hydrolases including lactase and sucrase-isomaltase. Caco-2 cell extracts were assayed for relative luciferase activity 48 h after transfection. The −100 bp flanking region reporter construct directed maximal transcriptional activity with 12-fold higher relative luciferase expression compared with the promoterless pGL3-Basic. The transcriptional activity driven by the −73 and −58 bp promoter fragments was significantly reduced compared with the −100 bp fragment construct. A positive cis element maps to the region between −73 and −100 bp of the lactase gene promoter. To determine whether the activity mediated by the lactase promoter fragment is cell type specific, the nonintestinal quail fibroblast QT6 cell line was similarly transfected with the lactase promoter-reporter constructs. QT6 cells possess no lactase activity, and, as expected, there was no increase in
relative luciferase activity for any of the reporter constructs compared with pGL3-Basic (Fig. 2B).

Specific nuclear protein(s) from Caco-2 cells interacts with a GATA region element. Inspection of the sequence for the -73 to -100 bp positive cis element region of the lactase promoter reveals a consensus binding site for the GATA family of zinc finger proteins. To identify interactions between this GATA region cis element and nuclear proteins in Caco-2 cells, we used the electrophoretic mobility shift assay (EMSA) or the gel shift assay. The wild-type GATA region oligonucleotide was radiolabeled, incubated in the presence of Caco-2 cell nuclear extract, and then migrated through a 6% nondenaturing acrylamide gel. The autoradiograph in Fig. 3 reveals the position of the rapidly migrating unbound probe at the base of the gel and a DNA-protein complex of slower mobility that is formed after incubation with Caco-2 nuclear extract. The DNA-protein complex is not competed away by 100-fold excess unrelated unlabeled SP1 oligonucleotide but is competed for by 100-fold excess unlabeled wild-type GATA region oligonucleotide. A slightly faster migrating nonspecific band is not displaced with either competitor. The nuclear protein bound to the GATA region probe in Caco-2 cells therefore represents a specific DNA-protein interaction.

To further define specificity of binding, we assayed the abilities of four mutant oligonucleotides to compete for nuclear protein binding to the probe. The mutant oligonucleotides differ from the wild-type GATA region element at clusters of three base pairs that span the length of the cis element and are shown in Fig. 1. Mutant oligonucleotides Mut-A, Mut-C, and Mut-D provided in 100-fold excess were able to compete for protein binding, whereas Mut-B was not (Fig. 3). In synthesizing Mut-B, the wild-type sequence of GATA at nucleotides -90 to -87 was replaced with CGGT. The inability of the Mut-B oligonucleotide to compete for binding suggests that the GATA sequence is essential for nuclear protein binding. The GATA sequence comprises the consensus binding site for the GATA family of zinc finger proteins. A similar interaction between Caco-2 cell nuclear proteins and an upstream GATA cis element of the human lactase promoter was reported by Fitzgerald et al. (7). The factors interacting with that cis element were not identified. We therefore proceeded to determine the identity of the specific Caco-2 nuclear proteins interacting with the GATA region element of the rat lactase promoter.

GATA family proteins interact with the lactase promoter element. Members of the GATA family of transcription factors (GATA-4, -5, and -6) are expressed in intestine and are capable of binding to the GATA cis element in the IFABP promoter (9). To determine whether GATA family proteins in Caco-2 cells bind to the lactase gene-positive cis element identified above, we incubated the radiolabeled GATA region probe and nuclear extract from Caco-2 cells in the absence or presence of a polyclonal GATA-4 and GATA-6 antibody
from proliferating Caco-2 cells was reverse-transcribed and then PCR-amplified with GATA-4 and GATA-6 gene-specific primers. A single PCR product of the exact predicted size was amplified for both the GATA-4 and GATA-6 primer sets in Caco-2 cells. There was no detectable amplification of the RT reaction performed in the absence of RT as a control for genomic DNA contamination. In addition, GATA-4 and GATA-6 mRNA was detected in adult human small intestine and liver. GATA-6 mRNA was also detected in human spleen. Expression of the GAPDH mRNA was detected as a positive control in all of the samples. GATA-4 mRNA was undetectable in control HeLa cells (data not shown).

**GATA-4, -5, and -6 can activate lactase gene transcription.** To determine whether the intestinal GATA proteins are capable of either activating or repressing lactase gene transcription, Caco-2 and QT6 cells were cotransfected with wild-type or mutated promoter-reporter constructs and with the expression constructs for GATA-4, -5, and -6. In cloning the constructs, *Xenopus* GATA cDNA inserts were ligated downstream of a recombinant CMV promoter in the expression vector pCDNA3. Cotransfection with each of the GATA proteins results in a three- to fourfold transcriptional activation of the wild-type lactase promoter-reporter construct pgLac (Fig. 6A, compare pxGATA-4, -5, and -6 vs. pCDNA3). Gao et al. (9) reported a similar activation of the *Xenopus* IFABP gene promoter using the identical GATA-4/5/6 expression constructs. To determine whether GATA factor overexpression was capable of activating the lactase promoter in nonintestinal cells, we assayed QT6 cells cotransfected with the GATA expression constructs. Overexpression of the GATA proteins in the nonintestinal QT6 cells resulted in comparable transactivation of the lactase promoter-reporter construct (Fig. 6B).

**Fig. 4.** Gel supershift with GATA region probe and anti-GATA-4 or -6 or preimmune serum. Gel retardation was carried out with nuclear extract (10 μg) from Caco-2 cells and 32P-radiolabeled GATA region probe. The reaction mixture was incubated for 30 min at room temperature alone (−) or with 1.0 μl of polyclonal GATA-4 or GATA-6 antibody or preimmune serum.

GATA-5 binding could not be assayed because human-reactive GATA-5-specific antibody was not available. The complexes were analyzed by electrophoresis on a nondenaturing gel and analyzed for specific supershift of the previously identified complex. Both the GATA-4 and GATA-6 antibodies recognized the nuclear protein bound to the GATA region probe, resulting in a complex of reduced gel mobility or a supershift (Fig. 4). There is residual bound DNA-protein complex that is not supershifted by the GATA-4 or GATA-6 antibody in each reaction, consistent with the presence of both proteins interacting with the GATA region probe. Although GATA-5 binding could not be assayed directly, residual complex remained even in reactions incubated in the presence of both GATA-4 and -6 antibodies (Fig. 4). As expected, no supershift was observed for the binding reaction carried out in the presence of nonimmune serum (Fig. 4).

**GATA-4 and GATA-6 mRNA is present in proliferating Caco-2 cells.** To confirm GATA-4 and GATA-6 gene expression in Caco-2 cells, the presence of GATA-specific mRNA was assayed (Fig. 5). Total RNA isolated

**Fig. 5.** GATA-4 and GATA-6 mRNA detection in Caco-2 cells. One microgram of total RNA extracted from proliferating Caco-2 cells was reverse-transcribed (+) or not (−) and PCR-amplified with GATA-4-, GATA-6-, lactase-, or glyceraldehyde-3-phosphate dehydrogenase (GAPDH)-specific primers (left). Similar PCR reactions were performed with RT cDNA from adult human thymus, intestine, liver, and spleen tissue (right). A single PCR-amplified product of the exact predicted size was visualized for each primer set after ethidium bromide staining of a 2% agarose gel. The negative gel images are shown.

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and they differ from the wild-type promoter at the base pairs corresponding to the respective Mut-A, -B, -C, and -D oligonucleotides (see Fig. 1). Cotransfection of the pMutA and pMutC reporter constructs with the GATA-4 expression constructs results in a 2.5-fold or greater transcriptional activation, comparable to that of the wild-type promoter construct (Fig. 7A). However, cotransfection of the pMutB reporter construct with the GATA-4 expression construct results in a significant disruption of GATA factor activation. Cotransfection of nonintestinal QT6 cells with pMutA and pMutC and the GATA-4 construct results in a similar lactase promoter transactivation (Fig. 7B). Again, cotransfection with the pMutB reporter construct results in a disruption of GATA-4 transactivation in QT6 cells. The GATA consensus binding sequence WGATAR is mutated in the pMutB construct. As predicted from the results of the gel shift analysis, the GATA proteins cannot bind to the mutant lactase promoter in pMutB and therefore cannot activate transcription. GATA protein activation of the pMutD construct was also disrupted, suggesting that bases mutated downstream of the GATA element may be involved in lactase transcriptional activation. In QT6 cells, GATA protein activation of the pMutC construct was significantly greater than that of the wild-type promoter construct. The mutated MutC sequence may disrupt a repressive element or create an enhancing site capable of interacting with QT6 cell nuclear factors.

GATA factor binding to DNA correlates with functional promoter activation. To correlate binding of the GATA factors to the lactase cis element DNA with the functional promoter activation data, we performed gel...
shift and supershift analysis with nuclear extract isolated from QT6 cells transfected with the GATA-4/5/6 expression constructs as described above. A GATA-specific complex, a slower migrating complex A, a faster migrating prominent complex B, and a nonspecific complex are detected in QT6 cells cotransfected with the GATA-4, -5, and -6 constructs (Fig. 5A). The relative migration of the GATA-specific complex is slightly different for each factor, with the GATA-6 reactions consistently resulting in a less abundant complex. The GATA-specific complex is not detected in the QT6 cells cotransfected with the empty pcDNA3 expression vectors. The GATA-specific complex is competed by 100-fold excess unlabelled probe but not by the MutB probe, in which the GATA sequence is mutated. In addition, the GATA-4-specific complex is supershifted with the GATA-4 antibody (Fig. 8B). The faint GATA-6-specific complex is similarly supershifted with GATA-6 antibody (data not shown), and the GATA-5-specific complex could not be assayed because human-reactive GATA-5 antibody was not available. These results indicate that the lactase promoter construct is inactive in QT6 cells (Fig. 2B) but that overexpression of GATA factors can result in factor binding to the lactase GATA region cis element and can function to stimulate promoter activity.

DISCUSSION

The GATA-4, -5, and -6 subfamily of zinc finger proteins are expressed in overlapping patterns in the developing heart and endoderm-derived organs of the gastrointestinal tract including the stomach, intestine, liver, and pancreas (1, 5, 16, 19, 20). GATA binding sites have been identified in transcriptional regulatory elements of several cardiac-specific target genes. However, few intestinal target genes have been identified for the GATA-4/5/6 factor subfamily. Fitzgerald et al. (7) demonstrated that GATA-6 can activate the human lactase promoter in cell culture. Subsequently, the gene encoding IFABP was identified by Gao et al. (9) as an in vitro target for the GATA-4/5/6 factors in intestinal cell culture. Both genes are expressed in absorptive enterocytes. To identify regions of the intestinal lactase promoter involved in regulating lactase transcription, we have characterized the promoter activity of various lactase reporter-promoter constructs. Fragments of the 5’-flanking region linked to the luciferase reporter gene and transfected into Caco-2 cells were assayed for transcriptional activity. A −100 bp promoter-reporter construct possessed maximal activity whereas the −73 bp deletion construct was essentially inactive compared with the promoterless pGL3-Basic vector (Fig. 2A). As expected, the promoter constructs were inactive when transfected into nonintestinal QT6 cells, which do not express the lactase gene (Fig. 2B).

Close inspection of the sequence in this positive element mapped between nucleotides −73 and −100 of the lactase promoter revealed a consensus binding sequence for the GATA family of zinc finger transcription factors. Fitzgerald et al. (7) mapped a positive GATA cis element to a similar region in the human lactase gene. The 28-bp GATA region sequence is highly conserved in the rat, pig, and human lactase genes, providing support for an important functional role in regulating intestinal lactase transcription.

The nuclear proteins interacting with the GATA region element were assayed using nuclear extract prepared from Caco-2 cells. The predominant DNA-protein complex seen on gel shift analysis was determined to be a specific interaction that was competed for by excess GATA region probe but not by an unrelated oligonucleotide (Fig. 3). To further define specificity of binding, we assayed for the ability of four mutant GATA region oligonucleotides to compete for nuclear protein binding to the probe. Only Mut-B, in which the wild-type sequence of GATA at nucleotides −90 to −87 was replaced with CGGT, was unable to compete for binding to the wild-type probe. The inability of the Mut-B oligonucleotide to compete for binding suggests that the GATA sequence is essential for nuclear protein binding. The sequences surrounding the GATA region comprise the consensus binding site, WGATAR, for the GATA family of zinc finger transcription factors. Fitzgerald et al. (7) reported a similar interaction between Caco-2 cell nuclear proteins and the upstream GATA cis element of the human lactase promoter. We have identified the proteins interacting with the GATA region element. Specific antibodies against GATA-4 and GATA-6 recognized the nuclear protein bound to the GATA region probe, resulting in a supershifted EMSA complex (Fig. 4). Both GATA-4 and GATA-6 proteins therefore are expressed in Caco-2 cells and appear to interact with the GATA element of the lactase promoter. GATA-5 binding could not be directly assayed because human-reactive GATA-5 antibody was not available. However, we speculate that the residual EMSA complex not supershifted in the presence of both GATA-4 and -6 antibodies may represent GATA-5 binding.

GATA-4/5/6 are each expressed in developing gastrointestinal and gut-derived tissues (5). Fitzgerald et al. (7) demonstrated that GATA-6 RNA is present in Caco-2 cells but were unable to detect GATA-4 protein or RNA in Caco-2 cells by gel supershift or Northern
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A

Cotransfected GATA: 4 6 5
Competitor: WT WT WT WT MutB MutB MutB MutB

GATA-region
Free Probe

B

Serum: - GATA-4 primers

GATA-region
Free Probe

Supershift
GATA transcription factor function has been implicated in the regulation of intestinal epithelial cell differentiation (9). To determine whether GATA family proteins are capable of either activating or repressing lactase gene transcription, Caco-2 cells were transfected with wild-type or mutated promoter-reporter constructs and with GATA-4, -5, and -6 expression constructs. Each of the GATA expression constructs was capable of activating the luciferase reporter driven by the wild-type lactase promoter in Caco-2 cells (Fig. 4). Fitzgerald et al. (7) initially reported a similar construct and with GATA binding element (Fig. 4) and that both mRNA species are expressed in Caco-2 cells (Fig. 5). The discrepancies may be attributable to differences in GATA-specific antibody affinities and the enhanced sensitivity of RT-PCR for mRNA detection. Variations or shifts in the phenotypic expression pattern of Caco-2 cells cultured by different investigators over time may also account for the discrepancies. In addition, as expected (26), mRNA for both GATA-4 and GATA-6 was detected in both liver and small intestine, but only GATA-6 mRNA was detected in spleen, and no expression was detected in thymus (Fig. 5).

GATA-4 and GATA-5 proteins, in addition to GATA-6, are capable of activating lactase promoter activity in intestinal cells. In addition, there was transactivation of the lactase promoter-reporter construct by the GATA-4/5/6 expression constructs in cotransfected QT6 fibroblasts (Fig. 6A). Fitzgerald et al. (7) demonstrated that GATA-6 can stimulate activation of the lactase promoter. We have demonstrated that each of the members of the zinc finger GATA binding protein family expressed in intestine (GATA-4 and GATA-5 in addition to GATA-6) can recognize a GATA consensus binding sequence within a positive cis element of the lactase promoter and can activate transcription. Gao et al. (9) demonstrated that the GATA-4/5/6 proteins are also capable of binding to a GATA cis element in the promoter of the IFABP gene and can activate transcription of the IFABP promoter in enterocyte cell culture. These findings for two intestine-specific genes (lactase and IFABP) support a role for the GATA family of transcription factors in regulating intestinal gene expression. Gao et al. (9) described differences in relative abundance of specific GATA proteins along the small intestine crypt-villus axis and in differentiating intestinal cells in culture. Relative levels of each GATA factor within enterocytes may therefore regulate transcription during cell differentiation. Other gene regulatory nuclear proteins may also interact with the GATA proteins to mediate their ability to activate or repress transcription during enterocyte differentiation. It will be of interest to determine whether the complex interactions between multiple GATA transcription factors function to regulate gut differentiation and development.

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