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Distinct Functions Are Implicated for the GATA-4, -5, and -6 Transcription Factors in the Regulation of Intestine Epithelial Cell Differentiation

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Based on conserved expression patterns, three members of the GATA family of transcriptional regulatory proteins, GATA-4, -5, and -6, are thought to be involved in the regulation of cardiogenesis and gut development. Functions for these factors are known in the heart, but relatively little is understood regarding their possible roles in the regulation of gut-specific gene expression. In this study, we analyze the expression and function of GATA-4, -5, and -6 using three separate but complementary vertebrate systems, and the results support a function for these proteins in regulating the terminal-differentiation program of intestinal epithelial cells. We show that xGATA-4, -5, and -6 can stimulate directly activity of the promoter for the intestinal fatty acid-binding protein (xIFABP) gene, which is a marker for differentiated enterocytes. This is the first direct demonstration of a target for GATA factors in the vertebrate intestinal epithelium. Transactivation by xGATA-4, -5, and -6 is mediated at least in part by a defined proximal IFABP promoter element. The expression patterns for cGATA-4, -5, and -6 are markedly distinct along the proximal-distal villus axis. Transcript levels for cGATA-4 increase along the axis toward the villus tip; likewise, cGATA-5 transcripts are largely restricted to the distal tip containing differentiated cells. In contrast, the pattern of cGATA-6 transcripts is complementary to cGATA-5, with highest levels detected in the region of proliferating progenitor cells. Undifferentiated and proliferating human HT-29 cells express hGATA-6 but not hGATA-4 or hGATA-5. Upon stimulation to differentiate, the transcript levels for hGATA-5 increase, and this occurs prior to increased transcription of the terminal differentiation marker intestinal alkaline phosphatase. At the same time, hGATA-6 steady-state transcript levels decline appreciably. All of the data are consistent with evolutionarily conserved but distinct roles for these factors in regulating the differentiation program of intestinal epithelium. Based on this data, we suggest that GATA-6 might function primarily within the proliferating progenitor population, while GATA-4 and GATA-5 function during differentiation to activate terminal-differentiation genes including IFABP.

The intestinal epithelium provides an excellent model system for investigating molecular mechanisms regulating cell lineage establishment, stem cell proliferation, morphogenesis, and the specialization of cell function during terminal differentiation (see references 9 and 16 for reviews). In all vertebrates, the embryonic intestinal lumen is lined by an endoderm-derived epithelial sheet, a monolayer consisting of four principal cell types that are renewed from a proliferating stem cell population. Lineage tracing experiments (7, 39) demonstrated that the four cell types are derived from a small population of multipotential stem cells present near the villus base (the crypt). The differentiating cells migrate from the crypt toward the villus tip, where they eventually die and are extruded into the lumen. Within the epithelium, absorptive enterocytes are the predominant cell type, and they form a highly organized apical brush border. In addition, goblet cells secrete mucus through exported granules, and enteroendocrine cells secrete various hormones and growth factors. The fourth cell type, the lysozyme-producing Paneth cell, differentiates during migration toward the base of the crypt. Although cell position along the polar crypt-tip axis is clearly an important determinant, the mechanisms that regulate the decision to differentiate (and the choice of pathway) are unknown.

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Regulation of gut-specific differentiation programs is likely to involve lineage-restricted transcription factors that control expression of terminal-differentiation genes. Experiments using transgenic mice and cell culture transfection have analyzed the promoters of gut-specific genes in order to identify regulatory elements that mediate lineage, temporal, and spatial control (see reference 52 for a review). In a few cases, potential transcription factors have been identified that can interact with defined regulatory elements. For example, the region including nucleotides −103 to +28 of the murine intestinal fatty acid-binding protein (IFABP) gene directs proper lineage-specific expression; a repeated and conserved element in this region binds two members of the steroid hormone receptor superfamily, HNF-4 and ARP-1 (38). Other transcription factors implicated in the regulation of differentiated epithelia include HNF-1 (5), HNF-3 (8), COUP-TF (31), and Cdx-2 (48). Genetic studies with *Caenorhabditis elegans* and *Drosophila* identified several additional genes that are likely to have conserved functions in vertebrate gut development (reviewed in reference 44).

GATA factors comprise a small family of transcriptional regulatory proteins defined by a highly conserved DNA-binding domain that interacts specifically with DNA *cis* elements containing a consensus WGATAR or related sequence. Six distinct vertebrate GATA factors have been characterized, grouped into two subfamilies based on structural and expression comparisons. The GATA-1, -2, and -3 genes each function

in the hematopoietic system (35). Genetic studies defined a unique role for each in regulating the differentiation state of specific blood lineages (43). The GATA-4, -5, and -6 genes are expressed with overlapping patterns in the developing cardiovascular system and in endoderm-derived tissues including the liver, lungs, pancreas, and gut (12). Several candidate target genes for GATA-4, -5, and -6 have been identified in cardiomyocytes, and data from several experiments indicate a functional role in regulating cardiac differentiation (17, 19, 24). Disruption of GATA-4 function during embryogenesis demonstrated an additional role regulating early morphogenesis critical for normal heart development, although the relevant downstream target genes are not known (26, 32). In addition, GATA-4 is required for the development of visceral endoderm in embryonic stem cell culture (46). In contrast, the function of the GATA-4, -5, and -6 genes in the differentiating gut is not known and is largely untested.

A possible role for GATA factors in regulating gut-specific gene expression was proposed based on the expression of GATA-4 and GATA-5 in the embryonic gut and the adult intestinal epithelium (2, 25, 27). Likewise, GATA-4 and GATA-6 are expressed in gastric epithelium (51), and a few likely target genes for GATA factors in the differentiated stomach have been proposed. A sequence capable of binding GATA factors is present in the promoters of the human and rat α- and β-subunit genes encoding the H^+/K^+-ATPase (30, 33); this proton pump is expressed in stomach parietal cells that also express GATA-4 and GATA-6 (GATA-5 was not analyzed). Similarly, a GATA-binding site is present in the promoter of the rat histidine decarboxylase gene that is expressed specifically in gastric endocrine cells containing abundant GATA-6 transcripts (10). However, these and other putative binding sites have not yet been shown to be functional regulatory elements. Furthermore, candidate targets for GATA factors in intestinal epithelium have not been identified. To address the function and conservation of GATA factors in the gut, we studied the activity of GATA-4, -5, and -6 on a putative downstream target promoter, and the expression patterns for the GATA-4, -5, and -6 genes in chick intestine, and in a human model cell culture system. The data support a hypothesis that these genes have distinct functions in progenitor and differentiated cell compartments of the intestinal epithelium. We identify the gene encoding *Xenopus* IFABP as a downstream target for GATA factors in differentiating intestinal enterocytes. Therefore, GATA factors are likely to have distinct functions in the regulation of the IFABP and other epithelium-specific genes.

MATERIALS AND METHODS

In situ hybridization. Intestines were dissected from adult *Xenopus* females or 1-month-old chicks, washed thoroughly in phosphate-buffered saline (PBS), and fixed in a solution of 4% formaldehyde–2 mM EDTA–PBS at room temperature for 3 h. Pretreatment and hybridization were carried out essentially as described elsewhere (20) with some modifications. Small pieces of intestine were treated with proteinase K for 30 min at room temperature (100 μg/ml for *Xenopus*, 50 μg/ml for chicks). Color development solutions included 10% polyvinyl alcohol (4). The antisense RNA probes were generated by in vitro transcription using bacteriophage polymerases. The cDNA templates used to generate the probes were described previously and shown to be specific in Northern blotting experiments (24, 27). Hybridization solutions contained 0.5 or 0.2 μg/ml of digoxigenin-labeled probes for *Xenopus* and chick samples, respectively. Following development of the alkaline phosphatase reaction, the embryos were refixed in 4% formaldehyde–0.1% glutaraldehyde, dehydrated, embedded in paraplast, and sectioned (5 μm).

Isolation of the xIFABP genomic clone, characterization of the genomic structure, and mapping of the transcriptional start site. A lambda genomic library of *Xenopus laevis* DNA was screened with the xIFABP cDNA probe, as described elsewhere (36, 42). Positive clones were isolated following secondary and tertiary screening. The intron-exon boundaries were determined by direct sequencing of the inserts in purified lambda DNA using end-labeled exon-specific primers (37). To determine intron sizes, a lambda genomic clone encompassing the entire coding region and the 3′ untranslated region was analyzed in detail. Restriction analysis showed that the introns were small enough to be amplified by PCR. Therefore, 1 μg of the lambda DNA was used as a template in a 25-μl PCR mixture using two primers (16 to 20 bp; 0.18 μg each) located in neighboring exons. The amplified product therefore contains a defined sequence derived from each of the two exons plus the intervening sequences. The products were analyzed by agarose gel electrophoresis to determine the sizes of the introns, by using known molecular size standards.

For primer extension experiments, 20 μg of total RNA isolated from stage 62 tadpoles (metamorphic climax) or stage 66 frogs (postmetamorphic) was annealed with 1 ng of a [γ-^{32}P]ATP-end-labeled primer in 10 μl of annealing buffer (20 mM Tris [pH 8.3], 0.4 M KCl) by incubating the mixture sequentially at 65°C for 10 min, 55°C for 25 min, and finally, 45°C for 10 min. Following annealing, the reaction mixture was supplemented with 4 μl of 10× reverse transcription buffer (0.5 M Tris [pH 8.3], 60 mM $MgCl_2$), 1 μl of a deoxynucleoside triphosphate mix (25 mM each nucleotide), 0.1 μl of an RNase inhibitor (Gibco/BRL), 1 μl of dithiothreitol (0.1 M), 1 μl of actinomycin D (1 mg/ml; Sigma), 0.2 μl of superscript II reverse transcriptase (Gibco/BRL), and 23 μl of distilled water. The reaction mixture was incubated at 42°C for 1.5 h. Extension products were ethanol precipitated and analyzed by denaturing polyacrylamide electrophoresis, alongside sequencing ladders generated with the same primer and a genomic clone template containing the promoter region. The sequence of the primer is 5′ CCA TAA CTT CCA TGA ATT T (+116 to +98, relative to the transcription start site).

Transient transfection and transactivation assays. The xIFABP promoter was isolated by PCR using the genomic clone as a template. The 7-kb *Xba*I fragment was first subcloned into the pBluescript SK− vector. The two primers used for PCR were T7 (forward, complementary to the T7 promoter in the cloning vector, upstream of the *Xba*I site) and TE356 (reverse, complementary to sequences from +34 to +50 of the xIFABP gene, relative to the transcriptional start site, and including an *Xba*I restriction site). The PCR product was digested with *Xba*I, and the 1,024-bp fragment was purified and subcloned into the *Nhe*I site of the pGL3-Basic luciferase reporter plasmid (Promega). The insert orientation was determined, and the entire sequence was confirmed. The Δ233 mutant promoter (deleting several upstream potential GATA-binding sites) was also generated by PCR, using as primers FABP10 (forward, complementary to nucleotides −233 to −217) and TE356. The product was subcloned into pGL3-Basic as described above. The pm reporter, containing a mutation of the proximal GATA-binding site, was generated by site-directed mutagenesis (QuickChange kit; Stratagene), using the full-length (wild-type [WT]) xIFABP promoter as a template, according to the manufacturer's instructions. The mutagenic primers (TE452 and TE453) introduced by this procedure a 2-bp change within the core of the GATA consensus (GA to CT). The Δ233m reporter was generated similarly, except that the Δ233 reporter was used as a template for mutagenesis. Each of the mutant promoter constructs was confirmed by manual sequencing.

For transactivation assays, the quail fibroblast cell line QT6 was transfected essentially as described elsewhere (14). The evening before transfection, 35-mm dishes were seeded with 2×10^5 cells. Each transfection mixture contained 3 μg of luciferase reporter plasmid, 1 μg of a cytomegalovirus-regulated expression plasmid (either control pCDNA3 vector [Invitrogen] or pCDNA3 containing a full-length xGATA-4, -5, or -6 cDNA), 0.25 μg of the pCH110 β-galactosidase expression plasmid (Pharmacia) as an internal control, and 10 μg of Lipofectamine (Gibco/BRL). Cells were harvested 48 h following transfection, and luciferase activity was measured by using the luciferase assay system (Promega) as described by the manufacturer, in a Turner TD-20e luminometer. The luciferase activity was normalized to β-galactosidase and expressed relative to reporter activity in the control (pCDNA3) transfections. Each transfection was performed in duplicate, and the data in Fig. 4 were averaged from at least four independent experiments. Preliminary experiments were performed to ensure that under these transfection conditions the assay results are linear with respect to reporter input and that the expression of GATA factors is not limiting.

In some cases, transfected cells were harvested and used to prepare nuclear extracts for gel mobility shift experiments, as described elsewhere (56). The probe was an end-labeled double-stranded oligomer generated by hybridizing TE492 and TE493, containing the sequence of the xIFABP promoter from −58 to −31 (indicated in Fig. 2c), including the proximal consensus GATA-binding site. In competition experiments, unlabeled double-stranded oligomer DNA was added (50-fold molar excess). The competitor DNA either was the same as the probe (TE492/TE493), generated with the TE452/TE453 mutant oligomers, or contained the sequence of the GATA-binding site derived from the chick α^D-globin promoter (TE72/TE73 [14]).

The sequences of the primers used are as follows: TE356, 5′ GCG TCT AGA TGA TTG GTG GAG AGA (*Xba*I site underlined); FABP10, 5′ ATA TGC CCT TCC TAA TG (−233 to −217); TE452, 5′ GGA GAT CCC TGT ACA CTT ATG GGG AGA C (mutation underlined); TE453, 5′ GTC TCC CCA TAA GTG TAC AGG GAT CTC C (mutation underlined); TE492, 5′ GGA GAT CCC TGT ACA GAT ATG GGG AGA C (GATA consensus underlined); TE493, 5′ GTC TCC CCA TAT CTG TAC AGG GAT CTC C (GATA consensus underlined).

Cell culture and reverse transcription-PCR (RT-PCR). The human cell lines CaCo-2, HT-29, and SW1417 were obtained from the American Type Culture Collection and maintained according to the supplier's instructions. For HT-29 cells, sodium bicarbonate was added to the medium (0.35 g/liter). Media were changed every day, and cells were passaged to avoid confluence. To induce differentiation, sodium butyrate was added (5 mM) after the cultures reached 80% confluence. Cells were harvested at specific time points up to 48 h (medium was changed at 24 h). In control cultures, PBS was added in place of sodium butyrate and the cells were cultured for the same times (usually an additional 48 h).

Cells were harvested as described elsewhere (14), and total RNA was prepared by using the SNAP RNA isolation kit (Invitrogen) as instructed by the manufacturer. For each sample, cDNA was prepared in a 50-μl RT reaction mixture with 1.25 μg of total RNA, random hexanucleotide primers, and Moloney murine leukemia virus reverse transcriptase. Two microliters of this RT reaction mixture was used directly for semiquantitative PCR, essentially as described elsewhere (57). Preliminary experiments confirmed that the reactions were entirely dependent on the RT reaction and that product accumulates linearly with respect to input RNA and cycle number. In some experiments, reaction mixtures contained trace [α-^{32}P]dCTP; following gel electrophoresis in nondenaturing polyacrylamide gels, the products were analyzed by autoradiography. Reaction conditions were as follows: S14, 1.25 mM $MgCl_2$ and 20 cycles; hGATA-4, 1.0 mM $MgCl_2$ and 28 cycles; hGATA-5, 0.8 mM $MgCl_2$ and 28 cycles; hGATA-6, 1.25 mM $MgCl_2$ and 28 cycles; intestinal alkaline phosphatase (IAP), 0.7 mM $MgCl_2$ and 22 cycles. The following primers were used for PCR: TE442 (5′ GGC AGA CCG AGA TGA ATC CTC A) and TE443 (5′ CAG GTC CAG GGG TCT TGG TCC) for S14, TE444 (5′ AAC GGA AGC CCA AGA ACC TG) and TE445 (5′ TTG TTC TCA GAT CCT TCG GTG C) for hGATA-4, TE454 (5′ GAA CAG CCT GGA ACA GAC CA) and TE451 (5′ TCC CTC ACC AGC CTT CTT GC) for hGATA-5, TE446 (5′ AGG CCA TTT GGT ACA CAT CTC TG) and TE447 (5′ TAA TGT AAA CCA ACC TGC CTG TG) for hGATA6, and TE464 (5′- GCC AGA CAG CGC AGC CAC AGC) and TE465 (5′ TGC ACC AGG TTC TTC CCG TCC AG) for IAP.

Northern blotting experiments were performed as described elsewhere (13) by using total RNA isolated from control or butyrate-induced cultures of HT-29 cells. For gel mobility shift experiments, nuclear extracts were prepared and DNA-binding assays were performed as described above. The antiserum used in gel mobility shift experiments was from rabbits immunized with three synthetic peptides consistent with the hGATA-6 coding sequence. These peptides include sequences from highly conserved regions of GATA factors and therefore may contain antibodies to GATA-4 and/or GATA-5 in addition to GATA-6.

RESULTS

The xIFABP promoter is likely a direct target for transcriptional activation by GATA factors in the intestinal epithelium. The cDNA encoding *Xenopus* IFABP was isolated by a differential hybridization screen used to identify targets of thyroid hormone-mediated intestinal remodeling (40). The gene product is intestine specific and provides a marker for absorptive enterocytes of the epithelium (23), a pattern of tissue-specific expression that is conserved in mammalian systems (18, 50). Our previous data indicated that the xGATA-4, -5, and -6 genes are expressed in frog gut and that xGATA-5 transcripts are localized within the stomach and intestine to cells of the epithelium (24, 25). Therefore, we considered whether IFABP might be a direct downstream target for regulation by GATA factors in the gut. However, the expression patterns for xGATA factors were reported previously for adult gut (24, 25), while xIFABP was studied in tadpoles (23). We first confirmed the expression of xIFABP in intestinal epithelium of adult frogs, using a whole-mount in situ hybridization assay. Isolated adult frog intestine was fixed and permeabilized prior to incubation with a digoxigenin-labeled antisense RNA probe synthesized in vitro from the xIFABP cDNA template. Following washing, and detection of the hybridized probe using an alkaline phosphatase-conjugated antibody specific to digoxigenin, the tissue was embedded and sectioned. As shown in Fig. 1a, the xIFABP gene is transcribed in the gut and transcripts are localized to the differentiating epithelium along the distal tip of the villus. No signal is detected in the crypt region or in underlying smooth-muscle layers. The xIFABP transcripts are first detected by this methodology at about 50% of the distance from the crypt zone, and the levels increase along the axis to the villus tip (Fig. 1b). No signal is present in control experiments using the sense strand of the xIFABP cDNA as a probe (Fig. 1c). This experiment confirms that transcription of the xIFABP gene is restricted throughout development to differentiated intestinal epithelium and is therefore a potential downstream target for GATA factors in the gut.

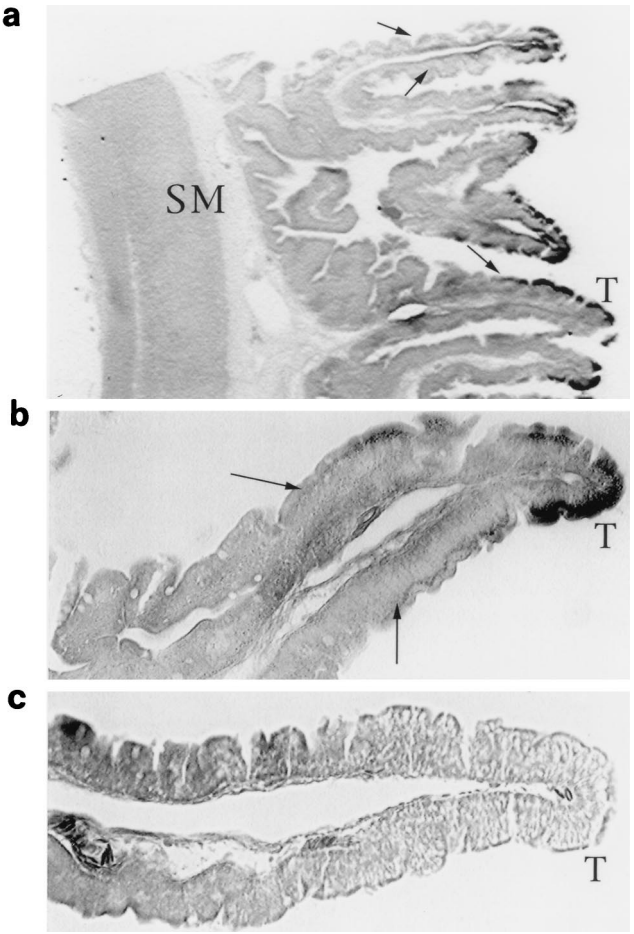


FIG. 1. The xIFABP gene is transcribed in adult *Xenopus* gut specifically in the differentiating epithelium. *Xenopus* intestine was analyzed by whole-mount in situ hybridization. Sections of processed tissue are shown. (a) Villus architecture, with smooth-muscle (SM) layers on the left and the villus proximal-distal axis running left to right toward the tip (T). The dark strain indicates the pattern of xIFABP transcripts following development of the alkaline phosphatase reaction to detect the hybridized antisense xIFABP RNA probe. (b) Higher-magnification view of a villus section. Arrows in both panels indicate the positions along the villus axis that transcripts are first detected in the epithelium. The signal increases in intensity along the proximal-distal axis and is strongest at the distal tip (T). (c) Section from similarly processed tissue that was hybridized with a control sense-strand RNA probe.

In order to identify upstream regulatory sequences controlling the tissue-specific expression of the xIFABP gene, a genomic clone was isolated by screening a bacteriophage lambda library with the xIFABP cDNA insert as the probe. A 7-kb *Xba*I fragment containing sequences for the entire coding region was isolated and used to characterize the structure of the xIFABP gene. The gene is encoded by four exons interrupted by three introns of approximately 250, 1,800, and 620 bp (Fig. 2a). The intron positions are entirely conserved in *Xenopus* (Fig. 2b), mouse (18), rat, and humans (50). The genomic clone includes approximately 1 kb of sequence upstream of the ATG initiation codon, and the primary structure of this entire

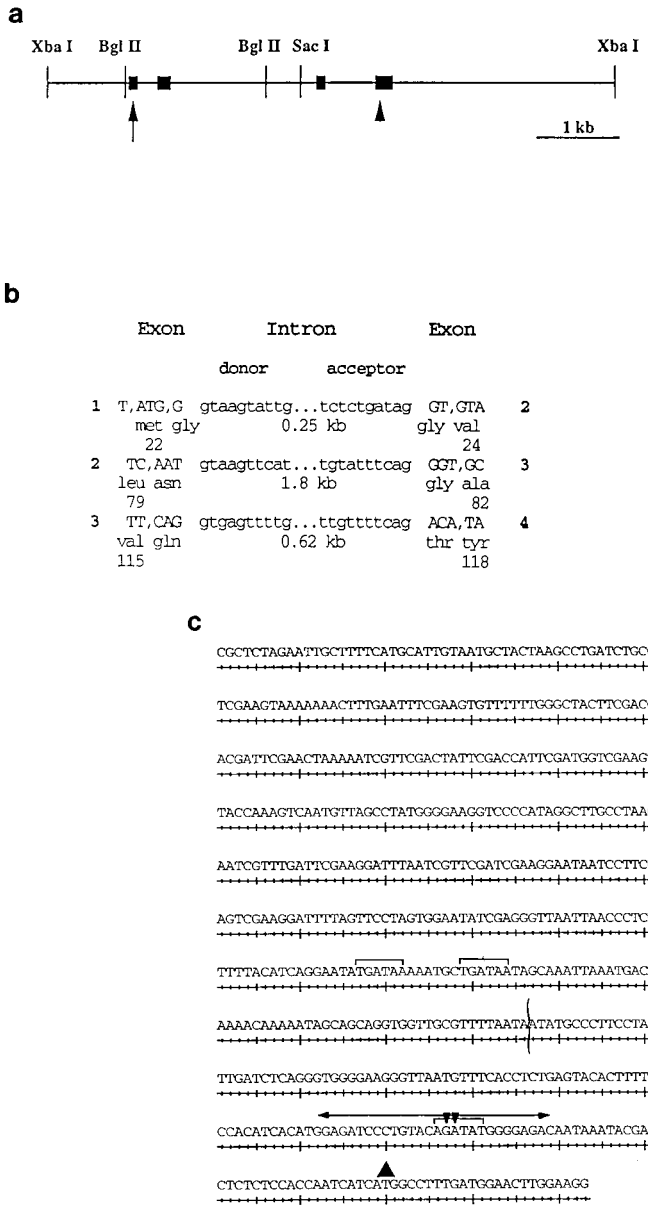


FIG. 2. Structure of the xIFABP gene and sequence of the promoter region. (a) Genomic organization of exons along a 7-kb *Xba*I fragment. The positions of *Bgl*II and *Sac*I restriction sites are also indicated. Solid boxes represent the four exons, and the arrow and arrowhead indicate the positions of the ATG translational initiation codon and stop codon, respectively. The positions of the exons, placement of the introns, and sizes of the introns separating the exons were determined by PCR and direct sequencing. (b) Sequence encompassing the exon/intron boundaries, relative to the xIFABP coding sequence. (c) Sequence of the xIFABP gene from −969 (around the upstream *Xba*I site) to +75, relative to the transcriptional start site (see Fig. 3). Small brackets (three located between −360 and −280 and one located around −42) indicate several sequences consistent with the GATA consensus binding sites. The larger bracket around −290 encompasses a conserved sequence shown in previous experiments to be important for regulating IFABP expression in transgenic mice (45). Note that the deviated GATA site in this sequence (TGATG) is in the reverse orientation. The vertical brace indicates the position at which upstream sequences were deleted in the Δ233 reporter. The long double-headed arrow indicates the sequence of the oligomer probe used in the gel mobility shift experiments (Fig. 5 and 8), and the small arrowheads point to the bases mutated in the pm reporter (Fig. 4) and in the mutant oligomers used in competition experiments (Fig. 5 and 8). The angled arrow indicates the transcriptional start site designated +1 (from the data in Fig. 3), and the solid triangle identifies the ATG translational initiation codon.

region was determined (Fig. 2c). An oligonucleotide primer complementary to nucleotides +49 to +67 (relative to the ATG initiation codon) was used in primer extension experiments to determine the transcriptional start site of the gene. As shown in Fig. 3, a single extension product is detected in RNA isolated from stage 66 adult intestine but not in RNA isolated from stage 62 tadpoles. Stage 62 is the climax of metamorphosis, when the xIFABP gene is not expressed due to intestinal remodeling (23, 41). The position of the single start site maps 49 nucleotides upstream of the translational initiator ATG, consistent with the size of the cDNA clone. PCR products obtained with IFABP-specific primers in a 5′ rapid amplification of cDNA ends procedure were sequenced, and the ends were found to be consistent with the primer extension results (not shown). The transcriptional start site (+1) is indicated on the sequence shown in Fig. 2c.

The promoter region upstream of the xIFABP start site contains potential regulatory elements that might restrict expression of the gene to differentiated intestinal epithelium. The consensus binding site for GATA factors is (A/T) GATA (A/G) (15). Several sequences consistent with this consensus, representing potential binding sites for GATA factors, are present in the promoter region, indicated by brackets over the 6-bp sites in Fig. 2c. A fragment including all of the upstream sequences derived from the genomic clone (down to but not including the ATG initiation codon) was isolated by using specific primers in a PCR, and the resulting product was fused in frame with a luciferase reporter gene. The recombinant reporter gene therefore contains 969 bp of the xIFABP promoter regulating expression of the luciferase coding sequence (the resulting RNA includes the 49-nucleotide xIFABP 5′ untranslated region but contains the ATG initiation codon of the luciferase gene). This reporter plasmid was used to test directly the ability of *Xenopus* GATA factors to transactivate the xIFABP promoter. We used a QT6 fibroblast cell line to express ectopically xGATA-4, -5, and -6 using expression plasmids

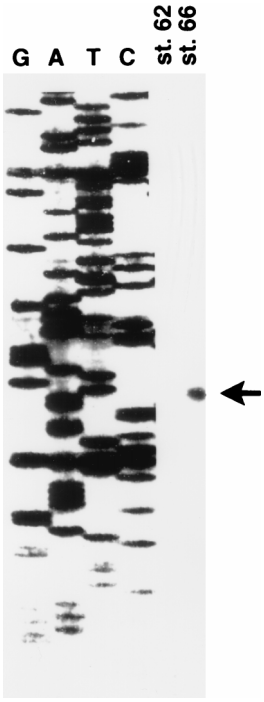


FIG. 3. Primer extension analysis mapping of the xIFABP transcriptional start site. RNA from stage (st.) 62 tadpoles or stage 66 adult frogs was analyzed with a specific labeled primer. The reaction products were analyzed on a denaturing polyacrylamide gel alongside sequencing ladders made with the same primer and the genomic clone as a template. The arrow indicates the position of the single extension product.

regulated by a cytomegalovirus promoter. This system was used previously to analyze the ability of GATA-1 to activate expression of a target globin promoter (14), and to define functional domains of GATA-1 (55, 56), because of the low levels or absence of endogenous GATA factors.

The luciferase reporter regulated by the xIFABP promoter was cotransfected into QT6 cells with the expression vector alone or containing an xGATA factor cDNA, and with a plasmid containing the *lacZ* gene as an internal control for transfection efficiency and lysate recovery. After 2 days in culture, cell lysates were harvested from transfected cells and luciferase activities were measured relative to β-galactosidase enzyme levels. As shown in Fig. 4A, each of the GATA-4, -5, and -6 factors is able to transactivate the luciferase reporter containing the xIFABP promoter, although at different levels. Specifically, GATA-4 and GATA-5 expression activates the promoter between five- and ninefold. GATA-6 is less potent, resulting in only a two- to threefold activation relative to the basal expression level of the xIFABP promoter in the QT6 cells. Expression of the luciferase reporter is entirely dependent on the presence of the xIFABP promoter sequences (not shown). Coexpression of the GATA factors in various combinations did not further augment the activity of the luciferase reporter over that of GATA-4 alone (not shown). Because specific antibodies are not available to quantify ectopic expression levels, it is possible that xGATA-6 binding activity does not accumulate at the same level in the transient transfection system, relative to xGATA-4 and -5. Analysis of binding activities derived from transfected cells is consistent with this possibility (see Fig. 5). Nevertheless, GATA factors known to be expressed in gut epithelium are capable of activating directly the xIFABP promoter in a nonintestinal cell type, in some cases nearly 10-fold.

Specific mutations of the xIFABP promoter were generated and analyzed to test whether specific GATA consensus sites mediate promoter activity in the presence of ectopic GATA factors. The transactivation data for xGATA-4 regarding these mutated reporters is shown in Fig. 4B. Essentially the same results were obtained for xGATA-5 and xGATA-6, although the reporter activities are relatively lower (not shown). Two regions were targeted for analysis. First, several consensus GATA-binding sites are clustered between nucleotides −270 and −350 (Fig. 2c). Of particular note, a putative GATA-binding site centered at −285 lies within a sequence shown previously to be important for regulating lineage-specific expression of the murine gene in transgenic mice (45). The *Xenopus* promoter contains a 12/15 match with the mouse sequence at this position. PCR was used to delete sequences from the promoter upstream of nucleotide −233, relative to the transcriptional start site. Deletion of these sequences, including the potential GATA-binding sites, had minimal effect on transactivation by GATA factors (Fig. 4B, Δ233). A second region containing a consensus GATA-binding site, centered around −42 relative to the start site, was next targeted for mutation. Mutation of this proximal GATA-binding site had a significant effect on the function of ectopic GATA factors (Fig. 4B, pm); the 2-bp mutation in the context of an otherwise wild-type promoter results in an 80% decrease in the level of transactivation. Deletion of the upstream sequences in addition to mutation of the proximal GATA site (Fig. 4B, Δ233m) did not have a significant effect on transactivation, relative to mutation of the proximal site alone. A low level of residual transactivation by GATA factors occurs with the Δ233m reporter. It is not clear if this results from binding at a cryptic GATA element elsewhere in the promoter or plasmid or is due to GATA-dependent activation that is not dependent on specific DNA binding. However, this low-level (twofold) transactivation has been noted in previous transfection experiments (14) and may not be relevant to the normal regulation of the gene by GATA factors.

Following similar transfections, cell lysates were harvested and nuclear proteins were extracted. These extracts were used in gel mobility shift experiments, as shown in Fig. 5. The labeled probe was a double-stranded oligomer sequence of the xIFABP promoter, centered around the proximal (−42) consensus GATA site. Lysates from cells transfected with GATA-4, -5, and -6 expression plasmids contained binding activity that was specific for the xIFABP promoter sequence, generating a single major complex, not present in cells transfected with the control expression vector (Fig. 5, lanes 1, arrow). This complex was inhibited by an excess of unlabeled oligomer (lanes 2) but not by an oligomer containing the 2-bp mutation present in the pm construct, which changes the GATA consensus binding site (lanes 3). Therefore, the same mutation that decreases the transactivation of the xIFABP promoter also abolishes the ability of ectopic GATA factors to bind to this promoter sequence. The complex is similarly inhibited by unlabeled excess oligomer containing a well-characterized consensus GATA-binding site from the chick α^D-globin promoter (lanes 4). We conclude that GATA factors are able to activate directly the xIFABP promoter, that a specific GATA-binding site around −42 can mediate much of this response, and that this site is therefore likely to be a target for GATA factors during gut epithelium differentiation. Multiple GATA-binding sites (in addition to other gut-specific factors) might contribute to full promoter activity, particularly in vivo under conditions of potentially limiting factors. Also, the more

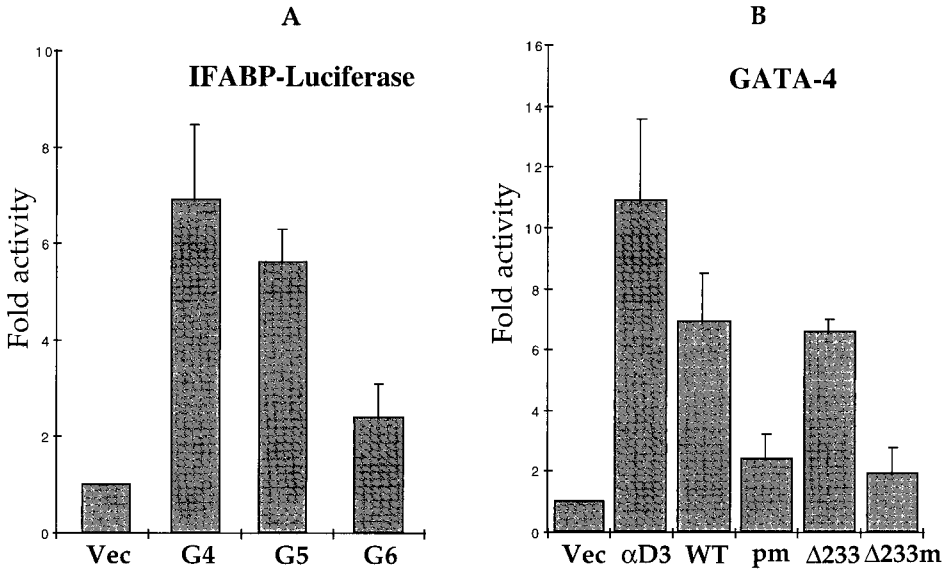


FIG. 4. The xIFABP promoter is a direct target for transactivation by GATA factors. The graphs show the relative activity of a reporter gene regulated by the xIFABP promoter. (A) The basal activity of the full length (−1 to −969) promoter when cotransfected with the control (empty) expression vector was arbitrarily assigned a value of 1 (Vec). The relative activity of this promoter when cotransfected with expression vectors for xGATA-4 (G4), xGATA-5 (G5), or xGATA-6 (G6) is shown. The data were averaged from at least four independent experiments, and error bars indicate the standard deviations. In all cases, the transfection includes saturating amounts of the GATA factor expression vector, determined in preliminary transfections. (B) Similar transfections were performed with the xGATA-4 expression vector but with either the full-length reporter (WT) (presented as 100% activity) or reporters containing mutated promoters as indicated. The activity of a control GATA-dependent promoter (αD3 [14]) is shown for comparison. Note that the majority (but not all) of the reporter activity in the presence of GATA-4 is eliminated when the proximal GATA site is mutated (pm).

distal sites (perhaps including the conserved region around −285) might not be functional in the cell culture system or in the absence of additional cell-specific factors.

GATA-4 and GATA-5 transcript levels correlate with terminal differentiation, while GATA-6 mRNA is the predominant GATA factor message in proliferating progenitor cells. The ability of different GATA factors to function in the transactivation assay raises the question of whether there is any specificity for GATA-4, -5, and -6 regarding regulation of the xIFABP and other terminal-differentiation genes. Therefore, we determined the expression patterns for these genes within the intestine as an indication of potential gene function. For this purpose, given the high degrees of conservation for the sequence and expression of the IFABP gene in vertebrate evolution, we analyzed the expression patterns of GATA factors in chick gut, because the villus crypt-tip axis is particularly distinguished in the chick relative to that in the frog. Intestine was isolated from 1-month-old chicks and fixed prior to incubation with antisense chick GATA-4, -5, and -6 RNA probes in whole-mount in situ hybridization experiments, and the relative transcript patterns for these three genes were compared.

As shown in Fig. 6, the transcript patterns for cGATA-4 and (particularly) cGATA-5 are consistent with a function in regulating terminal differentiation. Transcripts for cGATA-4 (Fig. 6a, upper panel) are detected at a very low level in the crypt cell zone, and levels increase substantially towards the villus tip. Transcripts are also detected below the crypt in the region where Paneth cells reside. The transcripts encoding cGATA-5 are first detected about 50% along the villus axis, with highest levels toward the distal end (Fig. 6a, middle panel). Note the similarity of this pattern with the xIFABP pattern shown in Fig. 1. The transcripts for cGATA-4 and cGATA-5 are not generally detected at the extreme tip of the villus (seen only in certain correctly oriented sections [T in Fig. 6a]); these cells do express high levels of alkaline phosphatase activity (specific to differentiated epithelial cells [not shown]) but are in the process of dying prior to being extruded into the lumen. In contrast to cGATA-4 and cGATA-5, the cGATA-6 gene is regulated very differently along the crypt-tip axis (Fig. 6a, lower panel). The cGATA-6 gene is not expressed in the differentiated epithelial cells near the tip of the villus. Instead, relatively high transcript levels are present in the proximal region of the villus associated with proliferating cells. As shown in Fig. 6b, the cGATA-6 pattern is essentially complementary to the cGATA-5 pattern, along the villus epithelium. Therefore, cGATA-6 is the predominant GATA factor expressed in the less differentiated, proliferating progenitor cell population, while cGATA-4 and cGATA-5 are expressed abundantly in the differentiated epithelium.

Induction of terminal differentiation leads to an early increase in GATA-5 mRNA levels and a concomitant decrease in levels of transcripts encoding GATA-6. To provide further evidence that different GATA factors have distinct functions in intestine epithelial cell development, we analyzed the expression of GATA factors during in vitro cell differentiation. Therefore, we studied human adenocarcinoma-derived cell lines that are characterized as inducible models for gut cell differentiation. The sequences for the human GATA-4 (22, 54) and GATA-6 (49) genes were described previously. These sequences, and a partial sequence deposited in the GenBank database for human GATA-5, were used to design RT-PCR primers to measure in a semiquantitative assay transcripts for human GATA factors in RNA isolated from various cell lines. In preliminary experiments, RNA was isolated from growing and subconfluent (uninduced) CaCo-2, HT-29, or SW1417 adenocarcinoma-derived cells. As shown in Fig. 7a, CaCo-2 cells contain transcripts for each of the GATA-4, -5, and -6 genes. The PCR analysis detects very low levels of hGATA-4 and hGATA-5 mRNA in uninduced cells and relatively higher levels of transcripts for hGATA-6. Transcripts for hGATA-4 were

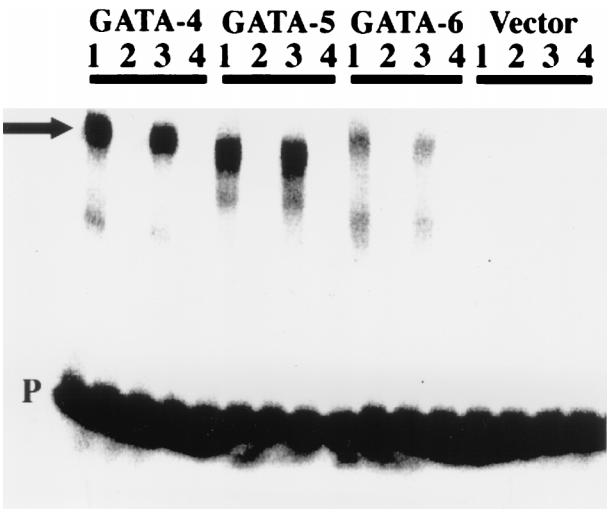


FIG. 5. GATA factors bind specifically to the xIFABP proximal promoter element. Gel mobility shift assays were performed by using as probe an oligomer containing sequences from −58 to −31 (Fig. 2c), including the proximal consensus GATA-binding site. Extracts were derived from cells transfected with the expression vector alone or the xGATA-4, xGATA-5, or xGATA-6 expression vector, as indicated. In addition to the probe and extract, incubation mixtures contained no competitor (lanes 1), competitor containing the WT promoter sequence (TE492/TE493) (lanes 2), competitor containing the pm mutated sequence that alters the GA of the consensus GATA-binding site (TE452/TE453) (lanes 3), or competitor containing a well-characterized GATA-binding site from the chicken α^D-globin promoter TE72/TE73 (lanes 4). The position of the major specific complex (arrow) and the position of the labeled probe (P) are indicated. The GATA-6 reactions consistently generate a less-abundant complex; it is not known if this is due to lower expression levels or binding affinity.

not detected in the undifferentiated HT-29 or SW1417 cells. The SW1417 cells express relatively abundant levels of hGATA-5 mRNA, while HT-29 cells contain only hGATA-6 transcripts. These results are consistent with previous studies that indicate that uninduced HT-29 cells represent a relatively early or crypt-like cell type (58); based on our hypothesis (see below), the SW1417 cells may therefore represent a more differentiated epithelial cell.

We used the HT-29 cells to analyze changes over time in GATA factor transcript levels during sodium butyrate-induced differentiation. When undifferentiated HT-29 cells are grown to near confluence and then treated with sodium butyrate, they undergo differentiation and eventually develop a well-organized brush border morphology (not shown). At various times following induction, cells were harvested and RNA was prepared and analyzed for levels of GATA factor transcripts, relative to those encoding the terminal differentiation marker IAP and a control mRNA encoding the S14 ribosomal protein (28). Transcript levels were measured by a semiquantitative RT-PCR assay, under conditions that ensured a linear accumulation of signal with respect to input RNA and cycle number. As shown in Fig. 7b, sodium butyrate induction leads to significant changes in GATA factor transcript levels, prior to changes in expression of terminal-differentiation markers. In particular, the hGATA-5 transcript levels are increased substantially by 2 h. Transcript levels continue to rise relative to the control S14 transcript levels during the 48-h induction period. The IAP levels also rise dramatically as the cells initiate differentiation, but these changes are not significant until 24 h after induction. In marked contrast, GATA-6 transcript levels decline appreciably between 1 and 24 h, before eventually recovering to the initial levels by 48 h. Although the significance of the recovery of GATA-6 transcript levels is not clear, it is confirmed by Northern blotting experiments (Fig. 7c) and probably indicates a proliferating subset of cells that escaped butyrate-induced differentiation. The hGATA-4 mRNA was not detected in HT-29 cells. The timing of IAP transcript accumulation is consistent with that described previously in Northern blotting experiments (3, 21). The rapid accumulation of GATA-5 indicates that it might function relatively early in the differentiation program induced by sodium butyrate.

Nuclear extracts were prepared from uninduced or induced HT-29 cells; gel mobility shift analysis demonstrates that HT-29 cells express GATA-binding factors that interact specifically with a DNA probe containing the IFABP proximal GATA element (Fig. 8a). Most interestingly, the abundance of this specific binding activity increases during the 48-h induction period (compare lanes 1 in Fig. 8a) following an initial decrease at 24 h (Fig. 8b). We prepared polyclonal antibodies by injecting rabbits with peptides derived from the predicted human GATA-6 protein sequence. These antibodies interfere specifically with binding of the GATA activity to the IFABP element-containing probe (Fig. 8b, lanes 3). Because the human GATA-5 cDNA is not available, we cannot test if the antibodies cross-react with the GATA-5 protein, but this is likely, considering that at least some of the antigen is derived from highly conserved regions. We conclude that GATA-binding activity is present in uninduced HT-29 cells and that this is probably predominantly GATA-6 (based on the RNA studies). GATA-6 transcript levels and total GATA-binding activities decline during earlier stages of butyrate-induced differentiation (Fig. 8b, 24 h, lane 1). By 48 h, after GATA-5 transcript levels are increased, there is a corresponding increase in GATA-binding activity, although this might also include reaccumulating levels of GATA-6.

DISCUSSION

Our results provide strong evidence for evolutionarily conserved but distinct functions for different GATA factors in the regulation of intestinal epithelial differentiation. We used three different vertebrate developmental systems that provide distinct but complementary information on the function and regulation of GATA-4, -5, and -6 during intestinal epithelium development. In each system we find evidence correlating the expression of GATA factors with changes in the differentiation state of gut epithelium. First, each of the xGATA-4, -5, and -6 factors, when expressed ectopically, can activate the promoter for the terminal-differentiation marker xIFABP in transient transfection experiments. This appears to be a direct function of binding to GATA consensus binding sites in the promoter. In particular, mutation of a proximal GATA consensus site affects significantly the ability of expressed GATA factors to activate the xIFABP promoter. This proximal GATA-binding site is approximately 10 bp upstream of a potential TATA box; a similar arrangement of regulatory elements is present in the chick α-globin gene that is activated in similar experiments by ectopic GATA-1 expression (14). Ectopically expressed xGATA-6 is less active on the xIFABP promoter relative to xGATA-4 or xGATA-5. Although we cannot rule out that this is due to differences in protein accumulation, stability, or binding affinity, this might indicate that GATA-4 and/or GATA-5 are primary regulators of IFABP transcription. The activation of the xIFABP promoter by xGATA-4, -5, and -6 in the cell culture system is relatively modest (no more than 10-fold), indicating that additional cell-specific factors are likely required for regulation in vivo. Second, the expression patterns in chick intestine are consistent with a model in which GATA-4 and/or GATA-5 regulate the expression of terminal-differen-

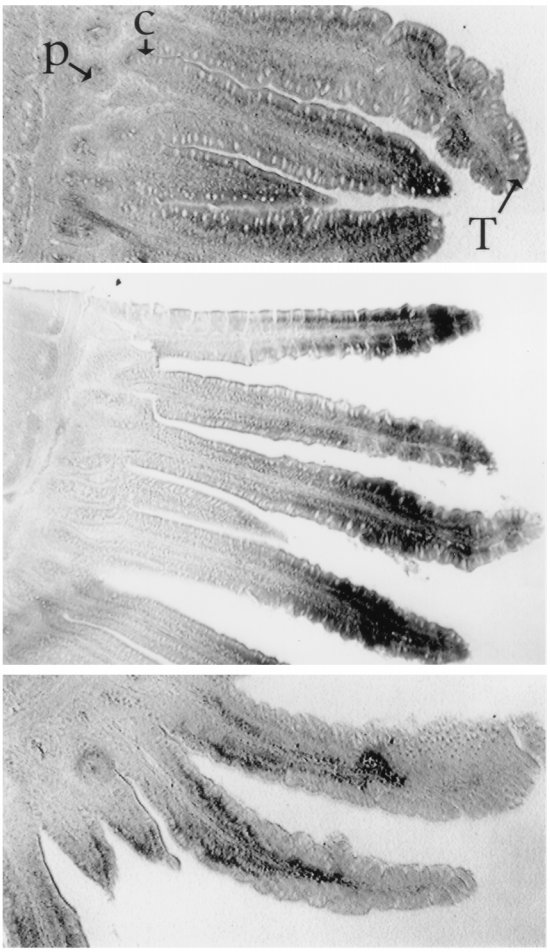
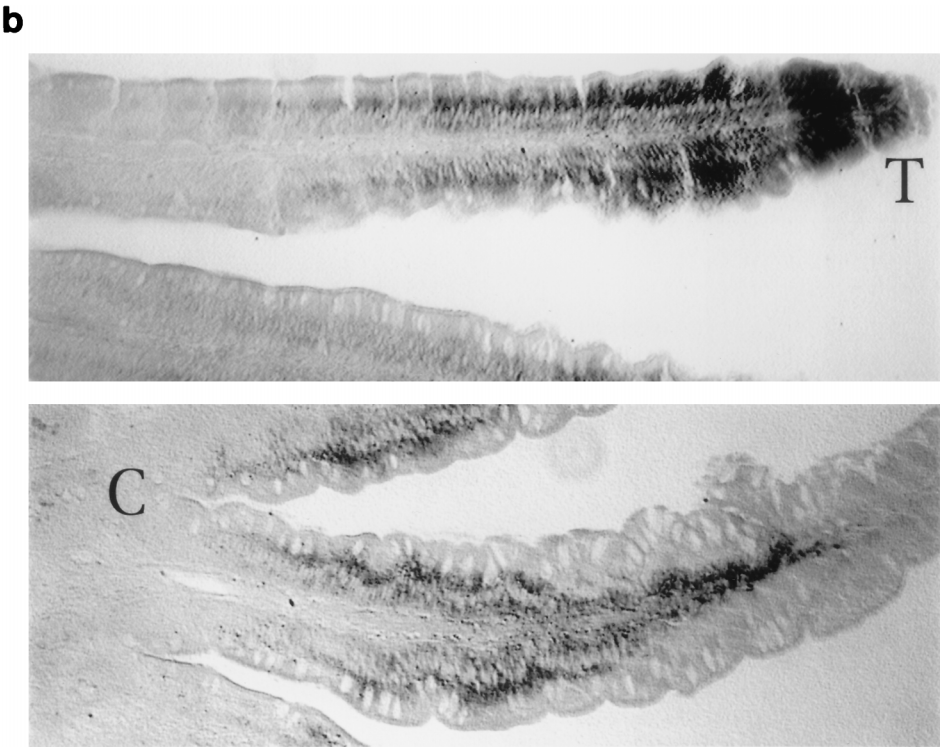


FIG. 6. GATA factors are differentially regulated along the villus axis within intestinal epithelium. (a) Intestine was isolated from 1-month-old chicks and processed by in situ whole-mount hybridization. Sections from tissues incubated with antisense RNA probes for cGATA-4 (upper panel), cGATA-5 (middle panel), or cGATA-6 (lower panel) are shown. The dark signal indicates the pattern of GATA factor transcripts, as in Fig. 1. The relative positions of the crypt (c) progenitor cells, the Paneth (p) cells, and the distal-tip (T) cells are indicated. Note that GATA-4 transcripts are distributed from the crypt to the tip, with increasing levels accumulating toward the distal tip. In contrast, GATA-5 transcripts are highly localized within differentiating cells of the tip, and GATA-6 transcript levels are highest in the less differentiated region located closer to the crypt zone. (b) Higher-magnification views of sections derived from tissue hybridized to probes for cGATA-5 (upper panel) or cGATA-6 (lower panel). Note that the transcript patterns are essentially complementary.



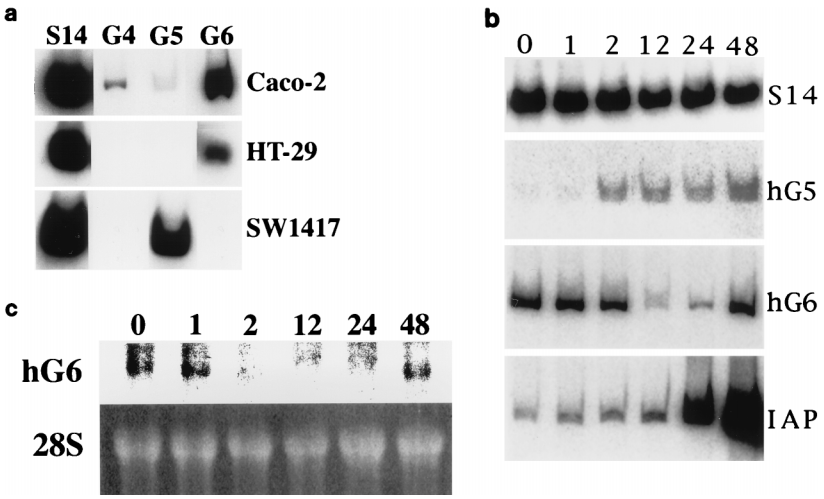


FIG. 7. Changes in GATA factor transcript levels correlate with induction of terminal differentiation in human gut epithelium cell lines. (a) RT-PCR analysis was used to measure the relative levels of RNA encoding human GATA-4, -5, and -6 (G4, G5, and G6, respectively) in samples derived from uninduced and proliferating CaCo-2, HT-29, or SW1417 cells. The S14 gene encodes a relatively abundant rRNA protein message that does not change significantly in different samples and is used as a positive control for the RT reactions. PCR products were labeled by including trace radiolabeled nucleotides in the reaction. The products were detected after gel electrophoresis by autoradiography. (b) HT-29 cells were either uninduced (lane 0) or induced with 5 mM sodium butyrate, and RNA was harvested at various times (in hours) to measure relative transcript levels by semiquantitative RT-PCR analysis. A representative autoradiograph following gel electrophoresis of the PCR products is shown. The IAP gene is a terminal-differentiation marker that is induced to high levels by 48 h. The hGATA-5 gene is an early target for activation by sodium butyrate, while the hGATA-6 transcript levels decline initially during differentiation before recovering at later time points. Similar kinetics were consistently noted in multiple experiments. (c) The same RNA as that used for the RT-PCR analysis in panel b was analyzed for hGATA-6 mRNA in a Northern blotting experiment. Total RNA was electrophoresed, blotted, and hybridized to an hGATA-6 cDNA probe. As shown in the upper panel, the RNA levels decrease prior to reaccumulating by 48 h, confirming the RT-PCR results. The lower panel shows the ethidium bromide-stained 28S rRNA, demonstrating equal RNA loading for each lane.

tiation genes including IFABP and IAP. Both GATA-4 and GATA-5 are transcribed at highest levels in a zone of epithelial cells apical of the progenitor crypt cells. The patterns are consistent with a "gradient" of increasing GATA-4 and -5 expression as the cells migrate from the crypt to the villus tip, where they are fully differentiated and, after a finite period of time, eventually die by apoptosis. In contrast, GATA-6 levels are relatively high in the proliferating and less differentiated cell population and transcript abundance decreases in the differentiating cells. As a consequence, this gene is relatively abundant in the progenitor cell population, suggesting a role in proliferation or early differentiation. Third, in the human HT-29 cell line, induction of differentiation leads to rapid changes in steady-state mRNA levels that are distinct for different GATA factors: GATA-5 transcript levels increase, while GATA-6 levels decline (but eventually recover), prior to activation of the terminal-differentiation marker IAP. There is, after 48 h of induction, a corresponding increase in GATA-binding activity that can interact specifically with the xIFABP proximal promoter element.

An important question regarding the function of GATA factors in regulating differentiation programs is whether coexpressed genes perform similar or distinct functions. The GATA-1, -2, and -3 genes are each expressed in the hematopoietic system and may be coexpressed in certain cell types. However, GATA-2 is implicated in the regulation of proliferating stem cell populations (6, 53), while GATA-1 and GATA-3 are critical for differentiation of specific cell lineages (34, 43). In this context, the genes are regulated differentially within a single lineage with regard to the developmental time frame and presumably activate distinct sets of target genes. By analogy, it is possible that similar specialization of function occurs in the gut epithelial system. In this case, GATA-6 might be important within a proliferating cell population for regulating the transition to a differentiating cell type, while GATA-4 and/or GATA-5 might function later, upon an environmental cue, to activate directly the differentiation program. Further evidence for specific and distinct functions of GATA factors will require additional experiments in which specific expression patterns are altered during development. Different cells within the epithelium might express distinct subsets or levels of GATA factors at various stages of development and differentiation, and this might be relevant to cell type identity.

A highly conserved function for GATA factors in intestinal epithelium is consistent with recent results regarding roles for GATA factors during invertebrate development. At least one GATA factor is involved in intestine-specific gene expression in *C. elegans* (11). McGhee and colleagues identified a cell-specific enhancer of the gut esterase 1 (ges-1) gene located 1,100 bp upstream of the transcriptional start site. A 36-bp region containing a tandem GATA binding site is critical for directing gut-specific transcription and for efficient repression of the gene in the tail or pharynx. The tandem GATA enhancer directs gut-specific expression of a linked reporter gene; the factor that interacts with these GATA sites is apparently distinct from a previously identified *C. elegans* GATA factor (47). A *Drosophila* GATA factor called "serpent" (*srp*) is required for midgut development in addition to hematopoiesis. *srp* can activate the expression of downstream fat body genes (1). It is unlikely that *srp* is involved directly in terminal differentiation because the gene is expressed prior to this and transiently (before the end of germ band extension). However, *srp* might be important for regulation of primordial development, while other GATA factors that are expressed in the midgut (dGATA-c) might regulate terminal differentiation (29). Therefore, distinct functions for GATA factors in regulating different developmental compartments of the gut might be a highly conserved mechanism of regulation.

Understanding the mechanism for GATA factor function in development requires identification of the genes that they regulate. While several potential targets for GATA factors in the gut have been described, our data indicate that the IFABP

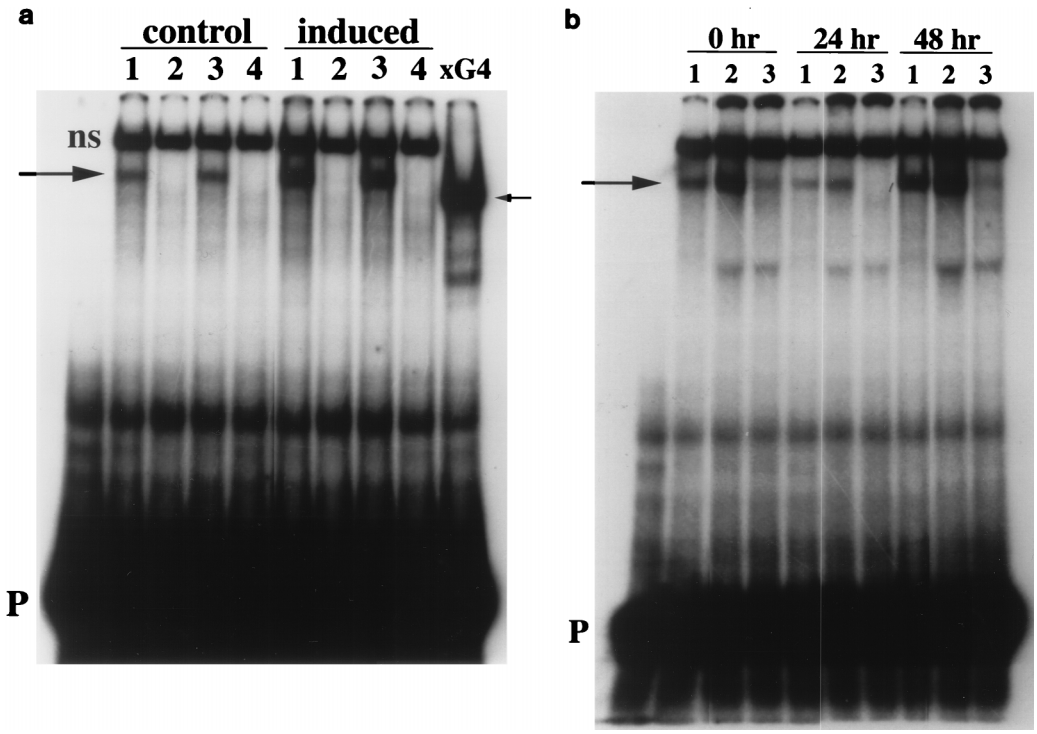


FIG. 8. Nuclear extracts derived from HT-29 cells contain binding activity that interacts specifically with the IFABP proximal GATA element, and the levels of this activity increase following sodium butyrate-induced differentiation. (a) The oligomer probe containing the xIFABP proximal promoter element (TE492/TE493, as in Fig. 5) was end labeled and incubated with nuclear extracts derived from uninduced (control) or induced cells cultured for 48 h in the presence of butyrate. Control cultures were incubated for 48 h in a mock induction, and equal amounts of total protein lysates were used in mobility shift assays. The positions of the free probe (P) and a complex formed with a nonspecific DNA-binding activity (ns) are indicated. The first lane contains the probe incubated in buffer alone. Lanes: 1, no specific competitor DNA; 2, 100-fold-excess unlabeled-probe competitor; 3, 100-fold-excess competitor DNA containing a specific mutation of the GATA *cis* element (TE452/TE453); 4, 100-fold-excess competitor containing the GATA-binding site from the chicken α^D-globin promoter (TE72/TE73). Note that the specific complex (large arrow), but not the nonspecific complex, is increased in abundance severalfold in extracts from induced cells. Lane xG4, extract from QT6 cells transfected with the xGATA-4 expression vector (as in Fig. 5) as a positive control for a specific complex (small arrow). (b) Gel mobility shift experiments were performed as for panel a with extracts derived from uninduced HT-29 cells (0 h) or cells induced for 24 or 48 h. In all cases, cells were cultured for 48 h following addition of butyrate to the 48-h sample; equal amounts of total nuclear proteins were incubated with the probe. Lanes: 1, no additional competitor; 2, 1 μl of preimmune rabbit serum; 3, 1 μl of immune serum derived from rabbits injected with synthetic peptides consistent with several regions of the hGATA-6 sequence. Note that the antibodies specifically inhibit or disrupt formation of the complex (arrow), while preimmune serum actually enhances binding. Although it is not known, the antibody is likely to interfere with binding in all GATA-4, -5, and -6 interactions in this experiment. P, free probe.

gene is a direct target. However, transcriptional regulation in the gut is highly complex, involving numerous changes along both the crypt-villus axis and the proximal-distal axis. In addition, expression is regulated developmentally and is lineage specific within the four distinct subpopulations of epithelial cells. Thus, it will be of interest to determine how GATA factors cooperate with other transcription factors to achieve temporal and spatial regulation of the IFABP gene during development. While our studies provide data implicating GATA factor function in differentiating intestinal enterocytes, others have reported possible functions for these factors in gastric parietal cells (33) and gastric endocrine cells (10). Therefore, the functions of each GATA factor are likely diverse and largely dependent on the relative levels of each GATA factor and the complement of available additional transcription factors within a specific cell type.

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