Cooperative interactions among intestinal GATA factors in activating the rat liver fatty acid binding protein gene

Joyce K. Divine, Lora J. Staloch, Hanna Haveri, Christopher W. Rowley, Markku Heikinheim, and Theodore C. Simon. Cooperative interactions among intestinal GATA factors in activating the rat liver fatty acid binding protein gene. Am J Physiol Gastrointest Liver Physiol 291: G297–G306, 2006. First published April 6, 2006; doi:10.1152/ajpgi.00422.2003.—GATA-4, GATA-5, and GATA-6 are endodermal zinc-finger transcription factors that activate numerous enterocyte-specific genes. GATA-4 and GATA-6 but not GATA-5 are present in adult murine small intestinal enterocytes, and we now report the simultaneous presence of all three GATA factors in murine small intestinal enterocytes before weaning age. An immunohistochemical survey detected enterocyte-specific GATA-4 and GATA-6 at birth and 1 wk of age and GATA-5 at 1 wk but not birth. Interactions among GATA factors were explored utilizing a transgene constructed from the proximal promoter of the rat liver fatty acid binding protein gene (Fabp1). GATA-4 and GATA-5 but not GATA-6 activate the Fabp1 transgene through a cognate binding site at −128. A dose-response assay revealed a maximum in transgene activation by both factors, where additional factor did not further increase transgene activity. However, at saturated levels of GATA-4, additional transgene activation was achieved by adding GATA-5 expression construct, and vice versa. Similar cooperativity occurred with GATA-5 and GATA-6. Identical interactions were observed with a target transgene consisting of a single GATA site upstream of a minimal promoter. Furthermore, GATA-4 and GATA-5 or GATA-4 and GATA-6 bound to each other in solution. These results are consistent with tethering of one GATA factor to the Fabp1 promoter through interaction with a second GATA factor to produce increased target gene activation. Cooperative target gene activation was specific to an intestinal cell line and may represent a mechanism by which genes are activated in the small intestinal epithelium during the period before weaning.

fabp1; GATA-4; GATA-5; GATA-6; suckling-weaning transition

The six members of the GATA transcription factor family share conserved zinc-finger DNA-binding domains that recognize the same DNA sequence, WGATAR (34). GATA factors bind to DNA as monomers, and only one GATA factor occupies the cognate DNA binding site at a time (21). GATA-1, GATA-2, and GATA-3 are found in hematopoietic cells, whereas GATA-4, GATA-5, and GATA-6 are found in endodermic and mesodermic tissues, including the intestine. Numerous genes expressed in the intestinal epithelium are activated in cultured intestinal epithelial cell lines by GATA-4 (4, 12, 16, 20, 45), GATA-5 (12, 16, 20, 27, 30), and GATA-6 (1, 16, 18, 20). We previously performed an immunohistochemical survey for GATA factors in the adult intestine and found that GATA-4 and GATA-6 are expressed in adult murine small intestinal enterocytes, with GATA-6 also present in crypt epithelial cells (12). We did not detect GATA-5 in the adult murine intestine (12), although the Gata5 transcript has been detected in the intestine of both late gestation and adult mice (14, 36).

The presence in a single cell of multiple GATA factors that recognize the same DNA binding site raises the possibility that interactions between GATA factors may occur in transactivating target genes. Although all GATA factors recognize a WGATAR sequence with high affinity, some specificity in gene activation has been observed. GATA-4 is utilized preferentially over GATA-6 in the regulation of myosin α-chain and β-chain genes (7). This specificity has been attributed to preferential binding of individual GATA factors to a particular site, perhaps through interaction with sequences flanking the core GATA binding sequence (3, 7, 49). Interactions with cofactors or other transcription factors that bind nearby a GATA site may also mediate preferential binding or activity of individual GATA factors (4, 10, 12, 13, 30). GATA-4 is subject to phosphorylation (8, 31, 35, 46) or acetylation (26), which can increase DNA binding. Intestinal extracts showed only GATA-4 but not GATA-6 binding to sequences containing GATA binding sites despite the presence of both factors in this tissue, suggesting regulation of binding (4, 14). Another possible means of interaction between GATA factors present in a single cell is direct binding between factors. GATA-1 is capable of a homotypic interaction that results in increased target gene transcription (50) and is required for gene regulation in vivo (38). All GATA factors contain two highly conserved zinc fingers that mediate DNA binding. The homotypic GATA-1 interaction is achieved by binding of the more COOH-terminal zinc finger with the more NH2-terminal finger, allowing formation of multimeric complexes in the absence of DNA (9, 33, 50), dependent on six lysine residues (38). The highly conserved nature of the zinc-finger domains among all the GATA factors suggests that heterotypic interactions may also occur. Binding of GATA-4 to GATA-6 bound to the myosin heavy-chain promoter has been described (7). In this case, only GATA-6 bound the DNA, but GATA-4 was tethered to the promoter through interaction with GATA-6, allowing GATA-4 to contribute to gene activation. These results suggest that factor interactions may contribute to target gene regulation.

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in tissues such as the intestinal epithelium where multiple GATA factors are present. We explored this possibility utilizing the liver fatty acid binding protein gene (Fabp1) as a model.

*Fabp1* is abundantly expressed in small intestinal enterocytes beginning at the time of epithelial cytodifferentiation at embryonic day 17 in rodents (39). Enterocytic *Fabp1* expression remains high throughout the preweaning period and adulthood and is primarily regulated at the transcriptional level (2, 22). We have utilized a transgene constructed from rat *Fabp1* nucleotides −596 to +21 to study enterocytic gene regulation (11). A transgene constructed of *Fabp1* nucleotides −596 to +21 relative to the start site of transcription is active in all small intestinal epithelial cells, including enterocytes (41). There are three consensus GATA sites in the proximal promoter of the *Fabp1* transgene in cultured cells through interaction with these sites (11). GATA-4 and GATA-5 activate the *Fabp1* transgene in cultured cells through preferential interaction of the most proximal of the three GATA binding sites present in the promoter (12). The potential interactions between pairs of endodermal GATA factors in activating the *Fabp1* promoter, and GATA factors activate the *Fabp1* transgene in cultured cells through interaction with these sites (11). GATA-4 and GATA-5 activate the *Fabp1* transgene in cultured cells through preferential interaction of the most proximal of the three GATA binding sites present in the promoter (12). The potential interactions between pairs of endodermal GATA factors in activating the *Fabp1* transgene were determined in intestinal Caco-2 cells. We found that GATA-4, GATA-5, and GATA-6 were simultaneously present in the small intestinal epithelium of mice before weaning, that cooperative functional interactions occurred between specific pairs of GATA factors, and that heterotypic and homotypic binding occurred among these factors.

**MATERIALS AND METHODS**

**Immunohistochemistry.** Approval was obtained from the Institutional Animal Care and Use Committee for all experiments involving animals. Immunohistochemistry for murine GATA-4, GATA-5, and GATA-6 was performed as previously described (12). The normal small intestinal tissue samples were harvested from FVB/N mice on postnatal days 1, 7, 12, 15, 18, and 21. Briefly, tissues were fixed in 4% paraformaldehyde and embedded in paraffin before sectioning. Primary antibodies were polyclonal goat anti-mouse GATA-4 IgG, polyclonal goat anti-mouse GATA-5 IgG, and polyclonal rabbit anti-mouse GATA-6 IgG (all from Santa Cruz Biotechnology), used at respective dilutions of 1:200, 1:50, and 1:50. Nonimmune IgG was used as the primary antibody for negative controls. Primary antibodies were visualized with a commercial avidin-biotin immunoperoxidase system (Vectastain Elite ABC Kit, Vector Laboratories) and substrate 3,3′-diaminobenzidine (Sigma). Staining for each sample was repeated at least twice.

**Plasmids.** The construction of the *Fabp1*- transgene and GATA-expression plasmids has been described (11), as has construction of mammalian expression plasmids pTS186 (GATA-4), pTS218 (GATA-5), and pTS473 (GATA-6) luciferase plasmids. All experiments were repeated at least twice. Solution binding assays were performed as described with modifications (11). GST-GATA fusion proteins were produced in BL21 (DE3) Escherichia coli, purified with glutathione resin, and dialyzed against a buffer containing 50 mM Tris (pH = 7.5), 150 mM NaCl, 100 μM ZnCl₂, 0.3% Nonidet P-40, and 1 mM dithiothreitol. 35S-labeled target proteins were synthesized from mammalian expression plasmids pTS186 (GATA-4), pTS218 (GATA-5), and pTS236 (GATA-6) using the TNT T7 coupled reticulocyte lysate kit (Promega). Binding assays were conducted by mixing 4 μg of GST or GST-GATA fusion protein with 20 μl of each target synthesis reaction in a total volume of 400 μl binding buffer (20 mM...
RESULTS

GATA-4, GATA-5, and GATA-6 are all present in enterocytes of the mice before weaning. We previously reported that GATA-4 and GATA-6 are present in the adult murine enterocyte but did not detect the presence of immunoreactive GATA-5. Since Gata5 mRNA was reported to be present at high levels in intestinal epithelial cells of mice in late gestation (36), we utilized immunohistochemistry to determine the presence of GATA-5 and other GATA factors in newborn and 1-wk-old murine small intestines (Fig. 1). GATA-4 and GATA-6 immunoreactivity were detected in small intestinal epithelial cells on the villus and in the intervillus regions of newborn mice and mice at 1 wk of age. Staining for both GATA-4 and GATA-6 was more intense in intervillus and lower villus cells. The cytoplasmic staining observed with the GATA-6 antibody is most likely nonspecific, with similar cytoplasmic staining observed in the cytoplasm of brain cells that lack GATA-6 expression (12). However, most cytoplasmic GATA-6 expression was evident in cells with nuclear staining and not evident in cells without nuclear staining. GATA-6 has been identified in the cytoplasmic compartment as well as in the nucleus of ovarian tumor cells (6), and it is possible that the cytoplasmic staining may be specific in some cells. GATA-5 was not detected in newborn animals but was easily detected in upper and midvillus cells at 1 wk of age. GATA-5 immunostaining was also readily detected in the same cellular distribution in mice of age 12 and 15 days but was greatly decreased in mice of 18 or 21 days of age (data not shown). Thus villus enterocytes of mice before weaning contain GATA-4, GATA-5, and GATA-6, whereas GATA-5 expression is lost during the suckling-weaning transition.

Fabp1 transgene activation by GATA-4 or GATA-5 is saturable in Caco-2 cells. We have utilized a transgene constructed from nucleotides −596 to +21 of Fabp1 to study GATA factor transcriptional regulation (12). Four consensus GATA binding sites are present in Fabp1 nucleotides −596 to +21 (Fig. 2). The most proximal consensus site at −128 overlaps with an imperfect site at −130, which might be utilized if the −128 site was mutagenized to ablate binding. The Fabp1 transgene was activated by GATA-4 and GATA-5 but not GATA-6 in intestinal Caco-2 cells and other cells in transient transfection assays (12). Moreover, transgene activation by GATA-5 and GATA-6 was mediated almost entirely through the most proximal site at −128 (12). We then determined the effect of different amounts of GATA expression plasmids on Fabp1 transgene activation in transient transfection assays in Caco-2 cells (Fig. 3A). The Fabp1 transgene was active in Caco-2 cells without addition of exogenous transcription factors, and this level of activity was set to 1.0 for comparison with activity in the presence of added transcription factors. Addition of 0.25 μg GATA-4 or GATA-5 expression plasmid to the assay resulted in an increase in Fabp1 transgene activity in Caco-2 cells to almost three times that of the transgene in the absence of exogenous transcription factors. Inclusion of additional GATA-4 or GATA-5 expression plasmid in the transfection mixture produced greater Fabp1 transgene activation up to 2.0 μg of added plasmid. Addition of more than 2.0 μg of GATA factor expression plasmid did not result in greater transgene activation, indicating that Fabp1 activation by GATA-4 or GATA-5 is saturable in Caco-2 cells. Addition of any amount of GATA-6 expression plasmid did not result in a significant increase of Fabp1 transgene activity.

Fig. 1. The presence of GATA-4, GATA-5, and GATA-6 in sections of the proximal small intestine of newborn and 1-wk-old mice was defined utilizing immunohistochemistry. Primary antibodies were visualized with brown peroxidase staining on sections counterstained with hematoxylin. The cytoplasmic staining observed in sections incubated with the GATA-6 antibody is nonspecific.
Renilla luciferase with a flexible 17-amino-acid linker. The GATA-4 and GATA-5 but not GATA-6 fusion proteins activated the Fabp1 transgene but with less activity than the native factors (22.9% for GATA-4 and 80.8% for GATA-5 at the 1 μg level). Steady-state fusion protein levels increased proportionally with added dosage of expression plasmid as determined by Renilla luciferase activity (Fig. 3B), suggesting that the plateaus of transgene activation and lack of GATA-6 activity are not the result of limiting protein synthesis. The total amount of plasmid DNA added to each well of cells in each transfection experiment was kept constant by addition of pSG5 plasmid, which lacks any eukaryotic protein expression.

GATA factors exhibit cooperative activation of the Fabp1 transgene in Caco-2 cells. GATA-4 and GATA-5 but not GATA-6 activate the Fabp1 transgene in Caco-2 cells. Since GATA-4, GATA-5, and GATA-6 are present in overlapping cell populations in the intestinal epithelium, we explored potential interactions among GATA factors in Fabp1 transgene activation in Caco-2 cells (Fig. 4). Fabp1 transgene activation by pairs of GATA factors was compared with activation by individual factors. Since only one GATA factor can bind to each GATA site (21), activation by a pair of GATA factors together would be predicted to be between the activations of the two factors individually. This effect was observed with GATA-4 and GATA-6 (Fig. 3A), where Fabp1 transgene activation by the two factors in combination lies between the values obtained with each factor individually. However, a different result was obtained with the combination of GATA-4 and GATA-5 or GATA-5 and GATA-6. These combinations of endodermal GATA factors resulted in a significantly greater transgene activation than predicted by competitive interaction, although the interaction of GATA-5 and GATA-6 could be due to the greater amount of GATA-5 present or a greater binding affinity of GATA-5 than GATA-6. These factor interactions were mediated through the three Fabp1 GATA binding sites, since a transgene with all these sites mutagenized was not activated by any individual GATA factor or combination of GATA factors (gray bars).

The cooperative activation observed with pairs of GATA factors could be due to GATA factor binding at more than one GATA site in the Fabp1 promoter, although we found that individual GATA factors activated the Fabp1 transgene only through the most proximal site (12). This possibility was tested with a transgene constructed from an Fabp1 promoter sequence with the GATA sites at −228 and −556 inactivated by mutagenesis (Fig. 2). Figure 4, D–F, shows transfections performed with this transgene but otherwise identical to those shown in Fig. 4, A–C. Despite the presence of only a single functional GATA site, cooperative Fabp1 transgene activation was observed with GATA-4 and GATA-5 or GATA-5 and GATA-6, although interaction between GATA-5 and GATA-6 might be due to increased quantity or greater DNA binding affinity of GATA-5 than GATA-6. The gray bars in Fig. 4, D–F, are transfections with an Fabp1 transgene with all GATA sites mutagenized, confirming that activation by individual or paired GATA factors is dependent on the intact GATA binding site. The transfections shown in Fig. 4, A–F, were conducted with saturating amounts of GATA factor expression plasmid, as verified by the comparison with additional wells of cells that received higher amounts of expression plasmid during the same experiment (Fig. 4G).

GATA factors exhibit cooperative activation of the synthetic minimal promoter transgene in Caco-2 cells. Cooperative activation of the Fabp1 transgene by GATA-4 and GATA-5 or GATA-5 and GATA-6 might be due to interactions with other factors present endogenously in Caco-2 cells and that bind to the Fabp1 promoter. For example, we have shown that GATA-4, GATA-5, and GATA-6 functionally and physically interact with HNF-1α to activate the Fabp1 transgene, and that Fabp1 transgene activation by GATA family factors is reduced in Caco-2 cells when an HNF-1 binding site at −95 is mutagenized (12). We found that activation of the Fabp1 transgene with a mutagenized HNF-1 binding site by both GATA-4 and GATA-5 was decreased compared with the native transgene, but the same relative degree of cooperation was observed between the two factors (data not shown). To determine potential effects of transcription factors besides HNF-1 family factors, we assessed cooperativity between GATA factors utilizing a transgene (47) consisting of a single GATA site
to that of GATA-5 alone, indicating cooperativity or a greater DNA binding affinity and/or greater amount of GATA-5 synthesized (Fig. 4C). Each transfection contained 2 μg of GATA expression plasmid, and factor mixtures contained 2 μg of both factors. Figure 4D compares minimal promoter transgene activation by 2 μg and 4 μg of each GATA factor to demonstrate that the transfections shown in Fig. 4, A–C, were conducted under conditions where activation by each GATA factor was saturated. These results were essentially identical to those obtained with the Fabp1 transgene and show that certain combinations of two GATA factors can interact at a single site in Caco-2 cells to produce greater transcriptional activation than either factor alone.

GATA factor cooperativity is observed in Caco-2 cells but not HeLa cells. The results shown in Fig. 5 demonstrate that cooperativity between pairs of endodermal GATA factors in Caco-2 cells was not dependent on the specific promoter utilized. We next determined whether cooperativity was cell-type dependent by performing transfections with the Fabp1 transgene in a nonintestinal cell line. HeLa cells were chosen since they do not contain detectable amounts of GATA factors (44). No significant cooperativity was observed between any pair of GATA factors in activating the Fabp1 transgene HeLa cells (Fig. 6). These results indicate a cell type-specific functional interaction between the pairs of GATA factors.

Homotypic and heterotypic binding among GATA-4, GATA-5, and GATA-6. When enough GATA-4 was present to saturate Fabp1 transgene expression, addition of GATA-5 resulted in additional activation and vice versa. One possible mechanism mediating this cooperativity would be direct binding of the two GATA factors to each other. Only one GATA factor occupies the DNA GATA site at a time, but the second GATA factor could bind to the first GATA factor and not directly to the DNA. This mechanism was reported for the cooperative activation of GATA-4 and GATA-6 in activating genes expressed in cardiomyocytes (7). We utilized an in vitro binding assay to determine whether GATA-4 or GATA-5 could interact with GATA-4, GATA-5, or GATA-6 in solution (Fig. 7). We found that GATA-5 bound in solution to both GATA-4 and GATA-5 and that GATA-4 and GATA-6 could bind to each other as previously reported (7). In addition, we found GATA-5 capable of homotypic binding, allowing the possibility of multimeric complexes. These results are consistent with involvement of GATA-GATA factor interactions in GATA-factor cooperativity in Caco-2 cells, although the functional interactions must also be mediated by cell-specific proteins since they occur only in the intestinal cell line.

**DISCUSSION**

We detected immunoreactive GATA-4, GATA-5, and GATA-6 in villus enterocytes of the murine small intestine before weaning age. We had previously found that immunoreactive GATA-5 was not detectable in the adult murine intestinal epithelium (12), and now did not detect GATA-5 immunoreactivity in newborn intestinal epithelial cells or in mice of ages 18 and 21 days. That GATA-5 was induced during the period after birth and before weaning suggests a role in regulating genes that are active during this period and reduced at the suckling-weaning transition. Others have detected the Gata5 transcript in both the late-gestational (36) and adult
small intestine (14, 36), in contrast to our failure to detect GATA-5 protein in the newborn or adult small intestine. This discrepancy may reflect a difference in transcript vs. protein expression as characterized for GATA-6 (5), or a difference in assay sensitivity between mRNA detection and immunohistochemistry, or a difference in cellular gene expression in the intestinal epithelium. A detailed study of immunoreactive GATA factors in the adult murine intestinal epithelium found GATA-5 expression was confined to nonenterocytic lineages (15). The immunohistochemical assay performed in this work clearly demonstrates a substantial increase in GATA-5 expression in absorptive enterocytes at 1 wk compared with the newborn or adult animal. A separate study detected higher levels of Gata5 transcript at postnatal day 7 or 14 than in the adult (17). This increase in GATA-5 after birth and before weaning may contribute to activating genes specific for this period. Lactase-phlorizin hydrolase has been utilized as a model for genes most active before weaning but suppressed at the suckling-weaning transition (29). In contrast, sucrase-isomaltase is first detected at 2 wk of age but increases after weaning (29). A transgene constructed from the proximal promoter of sucrase-isomaltase is activated by GATA factors in Caco-2 cells (4, 16, 30). However, a lactase transgene was more sensitive to GATA activation than the sucrase transgene, leading to the proposal that GATA factors play a unique role in intestinal gene expression between birth and weaning (30). Fabp1 is expressed at a high and constant level from birth to weaning, with expression rising somewhat in the adult tissue (22), perhaps due to the large number of transactivating factors that bind to the proximal promoter (11), and reflecting the requirement for lipid metabolism in enterocytes in both suckling and adult animals.

Cooperative functional interactions between pairs of endodermal GATA factors were specific to the intestinal cell line, Caco-2. GATA activation of the target genes in Caco-2 cells became saturated, where adding more GATA factor did not increase target gene activation. Saturation is not due to a maximum production of factor by the expression construct,
since the luciferase fusion proteins were produced in proportion to expression plasmid added and since no saturation is reached in some cell lines (data not shown). In addition, the lack of cooperativity in HeLa cells at saturating levels indicates simple addition of more GATA factors is not sufficient for increased transgene activation. This limit on maximum activity is not inherent to the Fabp1 transgene, which was activated in Caco-2 cells over 100-fold by a transcription factor mixture containing GATA-4 (11), compared with the 3- to 5-fold activation by the individual GATA factors. Endogenous GATA-4 and GATA-6 expression have been detected in Caco-2 cells (16) with higher levels of GATA-6 (18), whereas GATA binding factors are not detected in HeLa cells (25). Target gene activation by a single GATA factor has been shown to parallel DNA binding (34), and only one GATA factor occupies the cognate DNA binding site at a time (21). Equal competition between pairs of factors would be predicted under conditions with saturating amounts of GATA factors, as Fig. 5. GATA factor cooperativity was assessed with a transgene containing a single GATA site upstream of a minimal promoter linked to the human growth hormone (hGH) gene as a reporter (47). Transient transfections in Caco-2 cells were performed with the minimal promoter transgene and GATA factor expression plasmids individually and in pairs as described in Fig. 4. A: transgene activation by GATA-4 ($P < 0.00004$) and the combination of both factors ($P < 0.0001$), but not GATA-6 ($P > 0.01$). B and C: GATA-5 (B) ($P < 0.000001$), GATA-4 and GATA-5 together (B) ($P < 0.000003$), and GATA-5 and GATA-6 together (C) ($P < 0.000003$) also activated. Each transfection contained 2 $\mu$g of GATA expression plasmid, and factor mixtures contained 2 $\mu$g of both factors. Total plasmid in all assays was held constant by addition of pSG5 control vector. D: comparative graph of minimal promoter transgene activation by 2 $\mu$g and 4 $\mu$g of each GATA factor to demonstrate that the transfections shown in A–C were conducted under conditions where activation by each GATA factor was saturated ($P > 0.1$ for all GATAs). Transgene activity was normalized to that of the minimal promoter transgene in the absence of added factors. Bar height is the mean of values obtained with three separate wells of cells with error bars indicating the SD.

Fig. 6. Cooperativity between GATA factors in activating the Fabp1 transgene was assessed in HeLa cells with transient transfections. A–C: transfections with the Fabp1 transgene and expression plasmids for GATA-4, GATA-5, or GATA-6 individually and in pairs. A–C: activation by GATA-4 (A) ($P < 0.000001$), GATA-6 (A) ($P < 0.0001$), GATA-4 and GATA-5 together (B) ($P < 0.000007$), and GATA-5 and GATA-6 together (C) ($P < 0.00007$). GATA-5 produces little transgene activation in HeLa cells ($P < 0.003$). Each transfection contained 2 $\mu$g of GATA expression plasmid, and factor mixtures contained 2 $\mu$g of both factors. Total plasmid in each transfection was held constant by addition of pSG5 control vector. D: comparative graph of Fabp1 transgene activation by 2 and 4 $\mu$g of each GATA factor to demonstrate that the transfections shown in A–C were conducted under conditions where transgene activation by each GATA factor was saturated, with expression at 4 $\mu$g less than 2 $\mu$g for all GATAs ($P < 0.02$ for GATA-4, $P < 0.001$ for GATA-5, and $P < 0.0001$ for GATA-6). Transgene activity was normalized to that of the native Fabp1 promoter transgene in the absence of added factors, and bar heights are the mean of values obtained with three separate wells of cells with error bars indicating the SD.
Fig. 7. 35S-GATA-4, 35S-GATA-5, or 35S-GATA-6 were incubated with either glutathione-S-transferase (GST) or a fusion protein consisting of GST linked to GATA-4 or to GATA-5. Each solution binding experiment is represented by three lanes, indicated by the 35S-labeled GATA factor above the lanes. The third lane of the set contains an aliquot of the 35S-labeled GATA factor run directly on the gel as a control for synthesis and molecular size marker (labeled “input only”). The first lane of each set is a binding assay conducted with GST as bait and is compared with the second lane of each assay where the bait is the GST-GATA fusion protein.

we observed with GATA-4 and GATA-6. However, GATA-4 and GATA-5 or GATA-5 and GATA-6 displayed a much larger degree of activation when combined than predicted by competition for a single site under saturating conditions. An alternative interpretation of the observed activation by GATA-5 and GATA-6 together is that increased synthesis and/or DNA binding of GATA-5 ameliorates any competitive action of GATA-6.

One mechanism for cooperativity between pairs of factors is heterotypic binding, where one factor tethers the other to the DNA, thus allowing both to contribute to gene activation. This type of interaction has been observed in cardiac cells for GATA-4 and GATA-6 (7). Homotypic interactions have also been reported for GATA-1 (9, 50). Large complexes of GATA-1 have been detected in the nucleus of GATA-1-expressing cells, and these same complexes contained GATA-2 or GATA-3 when these factors were also present (14). GATA-1 homotypic binding occurs through interactions at the zinc fingers (33), and the zinc-finger motif has been found to have a general role in protein-protein interactions as well as the DNA-binding function originally described in Ref. 32. GATA-1 homotypic binding is dependent on lysine residues conserved among GATA-1, GATA-2, and GATA-3, but these residues are not present in GATA-4, GATA-5, or GATA-6 (38). We found that GATA-5 but not GATA-4 was capable of homotypic binding, and heterotypic binding could occur between GATA-4 and GATA-5 or GATA-5 and GATA-6, as well as the previously described interaction between GATA-4 and GATA-6. These results are consistent with extensive interactions between GATA factors independent of DNA binding.

The existence of a maximum limit for GATA factor activation suggests that additional proteins besides the GATA factors are involved in target gene activation and that these accessory proteins are limiting. The observed cooperativity also is consistent with a mechanism where each GATA factor acts through an independent accessory factor to activate the target gene. Additional evidence for cell-specific factors critical for GATA factor transactivation of target genes is provided by the observation that GATA family Fabp1 transgene activation differs between cell lines. GATA-4 or GATA-5 but not GATA-6 activates the Fabp1 transgene in Caco-2 cells, whereas GATA-4 or GATA-6 but not GATA-5 activates the Fabp1 transgene in HeLa cells. Cell type-specific activation of a lactase-phlorizin hydrolase transgene by GATA-5 has been reported (30), whereas Caco-2 cells contain GATA-4, GATA-5, and GATA-6, with GATA-6 the predominant form (18). Despite the ubiquitous nature of in vitro binding between the GATA factors, we observed cooperative functional interactions only between GATA-4 and GATA-5 or GATA-5 and GATA-6 in Caco-2 cells. Competitive interactions were observed between GATA-4 and GATA-6 in Caco-2 cells and all pairs tested in HeLa cells. A cooperative interaction between GATA-4 and GATA-6 has been reported in cardiac cells (7). These results suggest that a functional interaction between the factors is dependent on cell-specific factors that may stabilize the interaction in each cell type.

Many proteins are known to bind to the GATA factors, and interacting proteins may contribute to cell specificity in heterotypic binding or family member target gene activation. Fast oxidative-glycolytic (FOG) family factors were identified as widely expressed regulatory cofactors that bound to GATA factors, and FOG-2 is critical for GATA-4 function in vivo (42). Cell specificity of GATA-factor function/factor-factor interaction may also be influenced by the binding of GATA factors to other cell-specific transcription factors. HNF-1α is a transcription factor found in intestine but not heart, and GATA-4, GATA-5, and GATA-6 all interact functionally and physically with HNF-1α to activate the Fabp1 transgene (11). HNF-1α is found in Caco-2 cells (11) but not HeLa cells (30) and may contribute to the cell specificity of the observed GATA interactions. We observed the GATA interactions with a Fabp1 promoter transgene lacking an HNF-1 binding site and with a synthetic promoter with no HNF-1 binding site, but interactions independent of HNF-1α DNA binding may occur. Direct binding of GATA-4, GATA-5, or GATA-6 to other transcription factors found in the intestinal epithelium has also been reported (27, 30, 40, 44, 46). Cell-specific protein modification may also contribute to the regulation of interactions between GATA factors. GATA-4 is phosphorylated, and the phosphorylated form has increased stability (7, 28) and increased binding to C/EBPβ, another transcription factor found in the intestinal epithelium (43).

Heterotypic binding of the GATA factors could in principle tether any GATA factor present in the cell to a competent GATA binding site in any gene through interaction with a different GATA factor. Many cells contain at least two of the nonhematopoietic GATA factors: GATA-4, GATA-5, or GATA-6. The intestinal epithelium contains all three of these factors in unique spatiotemporal patterns. GATA-4 is present in differentiated enterocytes of the newborn, suckling, and
adult mouse small intestine (12, 44). GATA-6 is present in crypt (proliferating) epithelial cells as well as differentiated (villus) enterocytes in the adult and neonate (12). GATA-5 is present in differentiated villus enterocytes of the small intestine through 7–15 days of age, diminishing during the suckling-weaning transition, and absent in the adult small intestinal epithelium (12). The existence of three factors with partially overlapping expression patterns in the intestinal epithelium suggests separate functions for each GATA factor and/or use of multiple factors to achieve expression patterns for a single function that are more complex than possible with a single factor. It is interesting to note that the appearance of GATA-5 in the intestine before weaning allows cooperative interactions in enterocytes with both GATA-4 and GATA-6, which are present throughout the entire postnatal developmental period and thus available for interaction. These interactions may be critical for regulating genes expressed uniquely during this period. The possibility of heterotypic interactions between these factors provides an additional rationale for the presence of multiple factors in a single cell, where target gene activation may be increased by a single GATA site by multiple GATA factors. There is evidence that different GATA factors can activate the same genes in the same cells. Intestinal target genes are activated by more than one GATA factor in cell transfection assays, including Fabp1 (16, 20, 37). Ectopic expression of GATA-4, GATA-5, or GATA-6 in Xenopus laevis oocytes activates the same cardiac-specific genes (23). cGATA-4, cGATA-5, and cGATA-6 are all expressed during chicken cardiac development, and repression of all three genes results in a profound defect in cardiogenesis but not loss of any two GATA factors (24). However, GATA factor activation of target genes is not entirely interchangeable, since Gata6 expression cannot fully rescue the phenotype in endodermal differentiation of Gata6-deficient embryonic stem cells (19). All in all, GATA-4, GATA-5, and GATA-6 may have similar individual as well as cooperative roles in the maturing intestinal epithelium.

ACKNOWLEDGMENTS

We are grateful to David Wilson for provision of the synthetic GATA site transgene and helpful discussions and to Josh and Lilia Risman for technical assistance.

GRANTS

This work was supported in part by National Institute of Diabetes and Digestive and Kidney Diseases Grant DK-56361 (to T. C. Simon), research Grant 1-FY99–606 from the March of Dimes Birth Defects Foundation (to T. C. Simon), the Washington University Diabetes Research Training Center Grant DK-20579 (to T. C. Simon), the Washington University Digestive Diseases Research Core Center Grant DK-52574 (to T. C. Simon), the Finnish Pediatric Foundation (to H. Haveri and M. Heikinheimo), and the Sigrid Juselius Foundation (to M. Heikinheimo).

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