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GATA-4 and *GATA-5* Transcription Factor Genes and Potential Downstream Antitumor Target Genes Are Epigenetically Silenced in Colorectal and Gastric Cancer

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The GATA family of transcription factors participates in gastrointestinal (GI) development. Increases in *GATA-4* and *-5* expression occur in differentiation and *GATA-6* expression in proliferation in embryonic and adult settings. We now show that in colorectal cancer (CRC) and gastric cancer promoter hypermethylation and transcriptional silencing are frequent for *GATA-4* and *-5* but are never seen for *GATA-6*. Potential antitumor target genes upregulated by GATA-4 and -5, the *trefoil factors*, *inhibin*α, and *disabled-2* (*Dab2*) are also silenced, in GI cancers, with associated methylation of the promoters. Drug or genetically induced demethylation simultaneously leads to expression, in CRC cells, of all of the *GATA-4*, *-5*, and downstream genes. Expression of exogenous *GATA-5* overrides methylation at the downstream promoters to activate the target genes. Selection for silencing of both upstream transcription factors and their target genes in GI cancers could indicate that epigenetic silencing of the involved genes provides a summated contribution to tumor progression.

GATA factors are a family of transcription regulatory proteins containing two conserved zinc finger DNA-binding domains recognizing the sequence WGATAR (28, 39). GATA-1, -2, and -3 are important in the development and differentiation of the hematopoietic cell lineage (26). GATA-4, -5, and -6 guide development and differentiation in endoderm-derived organs (24), including the induction of the differentiation of embryonic stem cells (11), specification of proper gut embryogenesis, and guidance of epithelial cell differentiation in the adult (14, 22, 29, 31). *GATA-4*, *-5*, and *-6* have been implicated in cancer development. In this regard, *GATA-6* might be predicted to have oncogenic effects since it is predominantly expressed in proliferating progenitor cells (14, 22, 29, 31). In contrast, *GATA-4* and *-5* would be more likely to behave as tumor suppressor genes since increased expression levels correlate with terminal differentiation in intestinal epithelium (14) and terminal differentiation induced in colorectal cancer (CRC) cells by sodium butyrate (14, 20). *GATA-6* expression decreases in these latter settings, and *GATA-6* may function through a repressive effect on *GATA-4* (14). Diminished *GATA-4* and/or *GATA-5* expression has been reported in serous ovarian cancers (23) and gastric cancer (GC) (3), and the chromosome regions for *GATA-4* (8p23.1-p22) (23) and *GATA-5* (20q13.2-q13.3) (32), are frequent targets of deletion in cancer (13, 18). Importantly, GATA proteins bind the promoters of, and have been suggested as transcriptional activators for, a number of proposed antitumor genes, as discussed in detail below.

Despite growing evidence linking loss of *GATA-4* and *-5* and downstream target functions to cancer development, mutations in these genes have not been frequently found. We now show a high incidence for epigenetic silencing of *GATA-4* and *-5* in both human CRC and GC. Surprisingly, a series of proposed downstream GATA target antitumor genes are also silenced with associated epigenetic silencing marks at their promoters. Both the upstream and the downstream genes are simultaneously reactivated by drug and genetic demethylating strategies. Overexpression of *GATA-5* alone can also activate the target genes. We suggest that a hierarchy of related gene silencing events may cooperate to drive the progression of individual tumors.

MATERIALS AND METHODS

Cell lines and tissue samples. We studied 6 CRC cell lines (RKO, HCT116, DLD-1, HT29, LoVo, and SW480), 1 GC cell line (AZ521), and 45 primary CRC and 27 primary GC samples. All primary normal and neoplastic tissues studied were collected under clinical research guidelines at all participating institutions.

Drug treatment of cells and RNA extraction. The CRC and AZ521 cell lines were grown in Dulbecco modified Eagle medium, minimal essential medium, or McCoy's supplemented with 10% fetal bovine serum, penicillin, and streptomycin. For demethylation studies, cells were treated daily with 5 μM 5-aza-2′deoxycytidine (DAC; Sigma) for 48 h (41). We also treated AZ521 and HCT116 cells with a histone deacetylase inhibitor, TSA (Wako), alone and in a combi-

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nation of DAC plus TSA (6, 41). Total RNA was isolated by using the Trizol reagent (Invitrogen).

RT-PCR procedures. For reverse transcription-PCR (RT-PCR), 2 μg of total RNA was reverse transcribed by using the Superscript kit (Invitrogen), and we amplified all genes with multiple cycle numbers (28 to 35 cycles) to obtain semiquantitative differences in their expression levels. *GATA-4*, *-5*, and *-6* primer pairs were those previously described (3), and the primer sequences and RT-PCR conditions for all other genes are available upon request.

Methylation analyses. DNA extraction, bisulfite treatment, DNA sequencing (Johns Hopkins University School of Medicine Biosynthesis and Sequencing Facility, Department of Biological Chemistry), and methylation-specific PCR (MSP) were performed as previously described (7, 17), and the primer sequences utilized for all genes are available upon request.

Recombinant adenovirus generation and infection procedure. Full-length *GATA-5* was amplified from human GC cDNA according to GenBank sequences (NM080473 and AL499627) and subcloned into a pAdTrack-CMV shuttle plasmid (16). The virus titer was determined by plaque assay in low-passage 293 cells, and infection was performed at doses of 0.4 PFU/cell in HCT116 cells, 8 PFU/cell in RKO cells, and 4 PFU/cell in AZ521 cells to give at least 70% green fluorescent protein-reactive cells with minimal to no cytotoxicity.

Immunoblotting. For examination of GATA-5 protein expression, adenovirus-infected cells were harvested after 48 or 72 h, lysed in sample buffer (LB broth, dithiothreitol, and benzenesulfonyl fluoride), and Western blotting was performed on 5 μg of cell lysate with a goat GATA-5 polyclonal antibody (1:200 dilution; Santa Cruz Biotechnology). For examination of trefoil factor 1 (TFF1/p52) expression, we performed Western blotting with 40 μl of cell culture medium with a mouse anti-pS2 peptide (1:150 dilution; Zymed Laboratories).

RESULTS

Frequent epigenetic silencing of *GATA-4* and *GATA-5* in CRC and GC. As shown by semiquantitative RT-PCR, *GATA-1*, *-2*, and *-3* are expressed in lymphocytes but not in normal colon (Fig. 1A). *GATA-4*, *-5*, and *-6* are all expressed in normal colon, whereas only *GATA-6* is also expressed in lymphocytes (Fig. 1A and Table 1). *GATA-1* is not expressed in any of the cancer cell lines, whereas *GATA-6* is expressed in each. Interestingly, *GATA-2* is expressed in all of the cell lines except RKO CRC cells, and *GATA-3* is absent from RKO and LoVo CRC cells (Fig. 1A). Most strikingly, four of six CRC cell lines and the GC line do not express *GATA-4*; all but LoVo CRC cells lack *GATA-5*, whereas five of seven lines lack both *GATA-4* and *-5* (Fig. 1A and Table 1).

We used the demethylating agent DAC to initially study the epigenetic status of *GATA-4*, *-5*, and *-6* in each of the cell lines (Fig. 1A and Table 1). Each basally silent GATA gene, except *GATA-1*, which is not expressed in normal colon, is reexpressed by this treatment. Furthermore, the silenced *GATA-4* and *-5* genes had characteristics of hypermethylated tumor suppressor genes (41), (6), since treatment with the histone deacetylation inhibitor TSA alone fails to reactivate these genes but is synergistic with a low dose of DAC in doing so (Fig. 1B). Finally, expression of both *GATA-4* and *-5* is restored in HCT116 CRC DKO cells (Fig. 1C) in which two key DNA methyltransferase genes, *DNMT1* and *DNMT3b*, have been biallelically disrupted with resultant virtual abolition of DNA methyltransferase activity (34), and there is very minor expression of *GATA-4* in HCT116 cells in which *DNMT1* alone (35) is knocked out (Fig. 1C).

***GATA-4* and *-5* have aberrant promoter CpG island methylation in cultured and primary CRC and GC.** We studied the promoter methylation status of *GATA-4*, *-5*, and *-6*. Through RT-PCR studies combined with new expressed sequence tag (EST) identification in database searches, we have clarified the 5′ structure of each of the genes and identified CpG islands associated with the most 5′ promoter regions of each (Fig. 2). MSP analyses revealed these islands to be typical in having a nonmethylated status regardless of the gene expression state (4) in normal lymphocytes and normal colon from patients without cancer (Fig. 3A and Table 1). *GATA-4* has a weak methylation signal in 2 normal colon mucosa samples from patients with cancers in which the gene is hypermethylated, as detailed below, but is not methylated in 12 other normal samples from patients with CRC (Fig. 3A and C). Methylation of *GATA-5* is not seen in any normal samples (Fig. 3A and C), and neither gene is methylated in five normal gastric mucosa samples (data not shown).

In contrast to the normal patterns described above, the promoters of *GATA-4* and *-5* were abnormally methylated in cultured gastrointestinal (GI) cancers in which these genes are basally silent, whereas *GATA-6* was not methylated in any of these same cultures (Fig. 1D and Table 1). In the CRC HCT116 cells, the wild-type cells contained only signal for methylated alleles of *GATA-4* and *-5*, whereas only unmethylated alleles were found in the DKO cells (Fig. 1D). In addition, *GATA-4* and *-5* are also frequently hypermethylated in primary tumors, with strong MSP methylation signals in 30 of 45 (66.7%) and 28 of 44 (63.6%) primary CRC tissues, respectively, and both genes are hypermethylated in 24 (53%) of the tumors (see, for example, Fig. 3A and B). Of 27 GC tissues, 9 (33.3%) were found to be *GATA-4* methylation positive, 11 (40.7%) were found to be positive for *GATA-5*, and 7 (26%) had hypermethylation of both genes (Fig. 3D).

In selected samples, we verified MSP results by bisulfite sequencing (Fig. 4). *GATA* expression-negative cultured tumor cells (*GATA-4* [HCT116] and *GATA-5* [RKO]) and methylation-positive primary CRC samples show dense methylation of the promoter CpG islands, but expression-positive cultured tumor cells (*GATA-4* [RKO] and *GATA-5* [NuGC-2]), a *GATA-5*-positive GC line (3), and normal colon samples showed only scattered methylation within the examined regions. Even one of the normal colon samples with a weak MSP methylation signal for *GATA-4* (C1N) showed hypermethylation only in the 5′ and 3′ borders of the promoter CpG island (data not shown). The primary colon cancer, C10, shown to be hypermethylated for the promoter regions of *GATA-4* and *-5* by MSP (Fig. 3A), was densely methylated for most alleles, as determined by bisulfite sequencing (Fig. 4).

***GATA* genes and their candidate downstream targets have independent epigenetic silencing in GI cancers.** We next sought to determine how *GATA* gene silencing might correlate with the expression of candidate downstream genes that have been reported to be upregulated by these transcription factors and some of which are speculated to act as putative antitumor genes. One such group, the TFF genes, are predominantly expressed in gastric (44) and colonic epithelium (40) and encode for secreted proteins that help guide epithelial cells properly during repair of damaged GI epithelium (25) (9). *TFF1* (also known as pS2) is known to be a tumor suppressor gene. Approximately 30% of *TFF1* knockout mice develop gastric adenomas and carcinomas (25), and occasional mutations, allelic deletions, and reduced expression occur in human primary GCs (12, 33). Importantly, *TFF1* and *TFF2* (1) are upregulated by the CRC prevention agents, nonsteroidal anti-inflammatory drugs, indomethacin, and aspirin (2). We found that *TFF-1* to

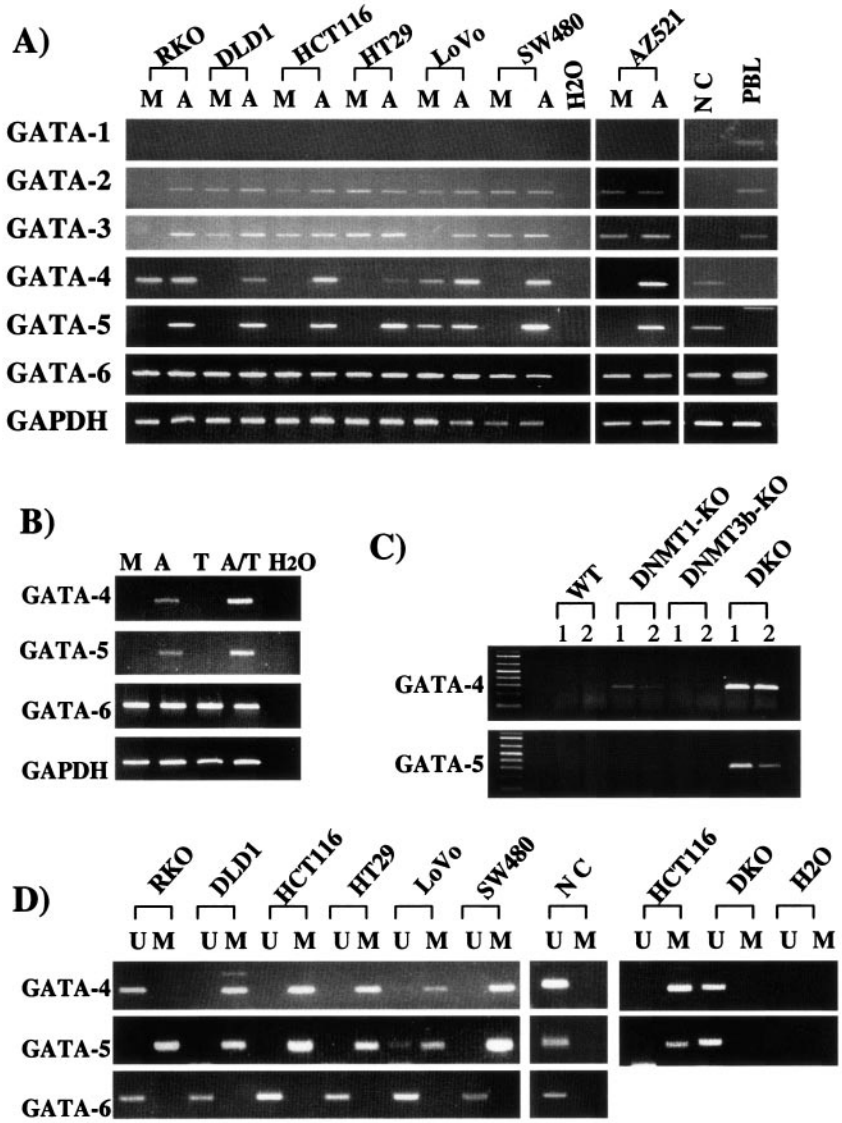

FIG. 1. *GATA-4*, *-5*, and *-6* expression in GI cancer cell lines. (A) *GATA-4*, *-5*, and *-6* expression levels were examined by RT-PCR in seven cancer cell lines (CRC lines RKO, DLD1, HCT116, HT29, LoVo, and SW480 and GC cell line AZ521) with (lanes A) and without (lanes M [mock]) treatment with DAC and in normal colonic mucosa (lane NC) and peripheral blood lymphocytes (lane PBL). *GAPDH* expression is used as an internal loading control for the RT-PCR, and H_2O indicates no RNA added. (B) GC cell line AZ521 was treated with low-dose DAC alone (lane A), TSA (lane T), a combination of these two drugs (lane A/T), or mock treatment (lane M), and examined by RT-PCR as described in panel A. (C) *GATA-4* and *GATA-5* expression was examined as described in panel A in wild-type (WT) HCT116 colon cancer cells and two clones each of these cells in which both alleles of DNA methyltransferases 1 (*DNMT1*-KO) and 3b (*DNMT3b*-KO) or both DNA methyltransferases (DKO) were knocked out (21). (D) MSP analysis of the promoter CpG islands of *GATA-4* and *-5* (primer regions depicted by black arrows [MSP] with an asterisk in Fig. 2) in six CRC lines and normal colon mucosa. PCR products recognizing unmethylated (lanes U) and methylated (lanes M) CpG sites are analyzed in 2.5% agarose gels stained by ethidium bromide. To the right, this MSP analysis is shown for *GATA-4* and *-5* in the HCT116 colon cancer wild-type and the *DNMT1* plus *DNMT3b* knockout cells (DKO) used in panel C.

-3, while not expressed in normal lymphocytes, were all expressed in normal colon but that *TFF1* expression was absent from four, *TFF2* was absent from three, and *TFF3* was absent from three of seven cancer cell lines (Table 1 and Fig. 5A).

Both GATA-1 and -4 are reported to bind to and upregulate the promoter of the *inhibin*α gene, a member of the transforming growth factor β superfamily (10, 21), which induces gonadal sex cord-stromal tumors when disrupted in mice (27). We could not detect expression of *inhibin*α in either normal lymphocytes or colon. However, a serial analysis for gene expression tag and EST (GenBank no. BM987739) have been identified for normal colon mucosa, and this gene was clearly expressed in some of the tumor cell lines (Fig. 5A and Table 1). However, expression of this gene was absent in two of the seven cancer lines studied, and the gene was barely to poorly expressed in two other cancer lines (Fig. 5A and Table 1).

Disabled-2 (*Dab2*) is an important candidate tumor suppressor gene reported to be directly activated by GATA-6 (30).

TABLE 1. Summary of methylation status, expression levels of *GATA* genes, and candidate target genes in cancer cells before and after DAC treatment

Gene	RKO			DLD1			HCT116			HT29			LoVo			SW480			AZ521			Normal colon		PBL[c]	
	Mt[a]	Expr[b]		Mt	Expr		Mt	Expr		Mt	Expr		Mt	Expr		Mt	Expr		Mt	Expr		Mt	Expr	Mt	Expr
		M	A		M	A		M	A		M	A		M	A		M	A		M	A				
GATA-4	U	+	+	M	−	+	M	−	+	M	−	+	U/M	+	++	M	−	+	M	−	+	U	+	U	−
GATA-5	M	−	+	M	−	+	M	−	+	M	−	+	U/M	+	++	M	−	+	M	−	+	U	+	U	−
GATA-6	U	+	+	U	+	+	U	+	+	U	+	+	U	+	+	U	+	+	U	+	+	U	+	U	+
TFF1	M	−	+	U	+	++	M	−	+	U	+	++	U	+	++	M	−	+	M	−	+	U/M	+	M	−
TFF2	NA	−	−	ND	+	+	M	−	+	M	+	+	ND	+	+	ND	+	+	NA	−	−	ND	+	ND	−
TFF3	ND	−	+	ND	+	+	M	−	+	M	+	+	ND	+	++	ND	+	+	ND	−	+	ND	+	ND	−
*inhibin*α	U	+	+	M	−	+	M	−	+	U/M	−	+/−	U/M	+	++	U	+	++	U	+	++	U	−	U	−
Dab2	M	−	+	U	+	+	U	+	+	U	+	+	U	+	+	U	+	+	U	+	+	U	+	U	+

[a] Mt, methylation status. Abbreviations: U, unmethylated; M, methylated; NA; genomic sequences were not dected by either MSP and bisulfite sequencing; ND; not done.
[b] Expr, expression. Abbreviations: M, mock treatment; A, DAC treatment. Key: −, undetectable level; +, detectable level; ++, increased expression level from the "+" basal level. Analyses were done by RT-PCR.
[c] PBL, peripheral blood lymphocytes.

Studies of this activation, however, have been confusing because a more recent study (37) suggested that the true promoter region and exon 1 for this gene are at least 14 kb farther upstream from the promoter region described in the earlier report. Our findings, presented below, clarify the role of this new region even further and link it to expression of the gene. *Dab2* is not expressed at a very high frequency in breast and ovarian cancers, and its absence has been correlated to the ability of epithelial cancer cells to grow independently of the basement membrane (38). We found that *Dab2* is expressed in normal colon and lymphocytes. However, using both RT-PCR primers for the internal coding region and primers linking the newly reported upstream first exon to this coding area, we found that expression was absent in one of the CRC lines (i.e., RKO cells) tested here (Table 1 and Fig. 5A).

In considering the pattern for basal expression of all of the candidate genes for GATA regulation (Table 1), line RKO lacks five of the six genes, line HCT116 lacks four, and line AZ521 lacks three. However, despite evidence that GATA factors can upregulate the expression for all of the downstream genes studied, the simple absence of *GATA-4* or *GATA-5* expression does not seem to account for the loss of downstream gene expression. For example, cell lines HCT116, DLD1, HT29, SW480, and AZ521 all demonstrated a decrease in *GATA-4* and *-5* expression. However, *TFF1* was not expressed in HCT116, SW480, and AZ251 but was expressed in DLD1 and HT29 (Table 1). *TFF2* was not expressed in HCT116 and AZ251 but was expressed in DLD1, HT29, and SW480. Similar discrepancies were apparent for other genes (Table 1).

Importantly, however, silencing of all of the downstream genes, despite this lack of coordination between expression of upstream and downstream genes, appeared to be, as for *GATA-4* and *-5*, under epigenetic regulation in many of the GI cancer cell lines. When the cells were treated with DAC, each downstream gene is activated in virtually every cell line in which the gene lacks basal expression, and in many instances (*TFF1*, *TFF3*, and *inhibin*α) a low basal expression was further increased (Fig. 5A and Table 1). Two exceptions occurred in cell lines RKO and AZ521, wherein *TFF2* was not reexpressed after DAC treatment (Fig. 5A and Table 1). As described below, the promoter region for *TFF2* appeared to be either homozygously mutated and/or deleted from the genome of these above two cell lines. In the CRC cell line HCT116, in which the *TFF2* promoter is present, the gene was readily activated by DAC (Fig. 5A and Table 1). Also, all four silent genes in wild-type HCT116 cells, *TFF1* to *-3* and *inhibin*α, were reexpressed in the HCT116-DKO cells (Fig. 5A).

What might account for the independent epigenetic regulation of upstream *GATA* and downstream target genes? We found that, as for *GATA-4* and *-5*, the expression of the downstream genes was associated, for the most part, with methylation of their promoter regions, although several different types of methylation patterns were involved. Basal silencing in cancer cells of the candidate *GATA* target genes, *inhibin*α and *Dab2*, appeared to involve classic tumor specific hypermethylation of promoter CpG islands. We found that the initial region defined as the promoter for *inhibin*α (Fig. 5B) was CpG poor, contained only two GATA-binding motifs (36), and was methylated in normal and tumor cell lines regardless of expression status (data not shown). However, we found a typical CpG island located ca. 700 bp upstream that contained multiple additional GATA-binding sites (Fig. 5B). The methylation status of this CpG island correlated exactly with the expression status being unmethylated in normal colon or lymphocytes, unmethylated or only partially methylated in cancer lines that basally express *inhibin* α, and fully methylated in the three lines in which the gene is basally silent (Table 1 and Fig. 5B). The upstream promoter region and untranslated exon 1 of *Dab2* (37) has an associated CpG island, which in current databases lies 30 kb from exon 2 (Fig. 5C). By MSP analysis, this island was determined to be unmethylated in normal colon and lymphocytes and in the six cell lines that express the gene but fully methylated in RKO cells in which this gene is basally silent and reactivated after DAC treatment (Table 1 and Fig. 5C).

The epigenetic silencing of the TFF genes appears to be much different than for all of the above genes. No CpG islands are found between these three genes, which cluster together in order within 45 kb on chromosome 21q22.3, nor between *TFF1* and a separate gene located 5 kb upstream (Fig. 6A). The CpG-poor promoters of these genes are thus more typical for

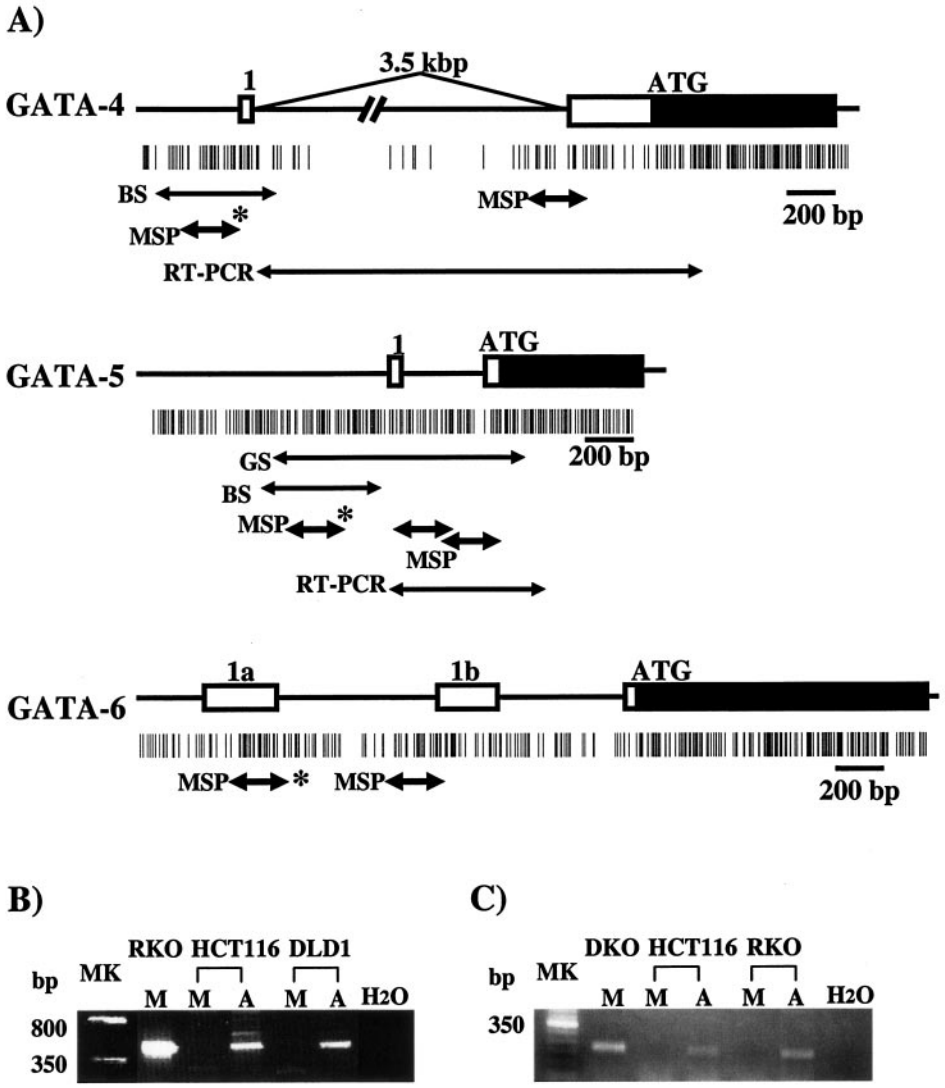

FIG. 2. Schematic representation of 5′ regions of *GATA-4*, *-5*, and *-6* and MSP analyses of promoter methylation status. (A) Schematic representation of the genes. The *GATA-4* diagram includes a new exon 1, located 3.5 kb upstream of the previously designated exon 1 (identified from EST BG718444), genomic sequences containing this EST (AC090790 and AC069185), and a confirmatory PCR approach showing the EST to be contained in the single transcript amplified for this gene (see panel B). The newly reported GATA5 cDNA (no. NM080473) includes one 5′-untranslated exon (41 bp). This newly identified exon 1 is located 387 bp upstream of exon 2 that contains the translation start site in the genomic *GATA-5* sequence (no. AL499627). The data for the genomic structure of *GATA-6* (A and B) was obtained from the newly reported sequence of this gene (GenBank no. AC009669), which reveals two 5′-untranslated exons (1a and 1b). Boxes indicate exons, including coding (black) and noncording (white) regions. Vertical bars show CpG sites. Black arrows below the CpG sites indicate the regions analyzed by MSP, genomic sequencing (GS), and bisulfite sequencing (BS) in the present study. The regions analyzed by MSP for which methylation status corresponded to *GATA-4*, *-5*, and *-6* expression are indicated by an asterisk. (B) RT-PCR analysis of 5′-untranslated region of *GATA-4* by using the primer set (see panel A, RT-PCR) in CRC lines. (C) RT-PCR analysis of 5′-untranslated region of GATA5 with the primers designated as in panel A. Lanes (B and C): MK, 50-bp ladder marker; M, mock treatment of cells; A, treatment with DAC; H_2O, no RNA added.

those of tissue-specific genes, which can normally be differentially methylated in correlation to gene expression status (4). Indeed, for each of the cell lines and normal tissues in which *TFF1* was basally silent, only methylated alleles for this gene were detected by MSP, whereas the promoter is only partially methylated in normal colon and unmethylated in the three cell lines in which *TFF1* was basally expressed (Fig. 6B and Table 1). The promoter regions for *TFF2* and *TFF3* were examined by bisulfite sequencing. As noted above, no promoter region sequences could be amplified for *TFF2* in lines RKO and AZ521, wherein this gene was not basally expressed or reactivated by the demethylating maneuvers. Otherwise, both genes were methylated in cell lines in which the genes were basally expressed or silent (Fig. 6C and D). Thus, these were the only two downstream genes in our study for which promoter methylation did not correlate with expression status.

***GATA-5* overexpression partially overrides epigenetic silencing of downstream target genes.** The epigenetic profile derived above suggests a complicated scenario in GI cancers wherein there is a potentially redundant epigenetic silencing of

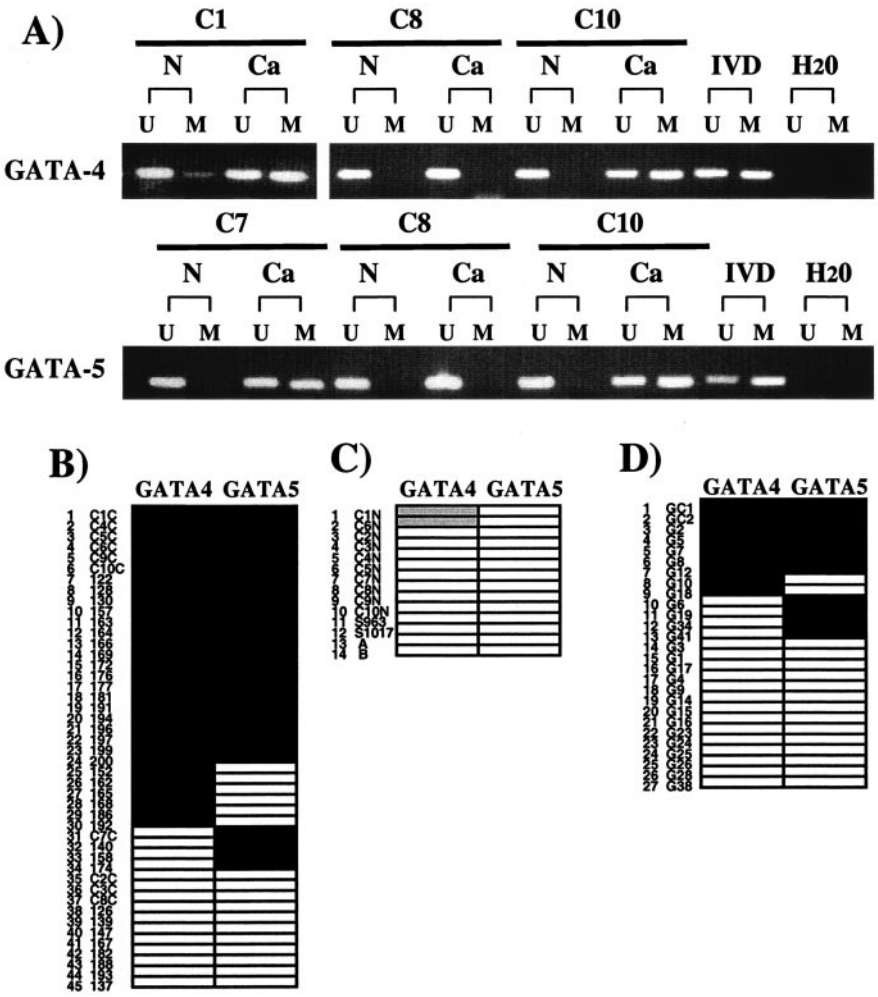

FIG. 3. Methylation analysis of *GATA-4* and *-5* in noncultured normal and neoplastic GI samples. (A) Examples of MSP analyses (carried out as described for Fig. 1D) of *GATA-4* and *-5* in noncultured colon cancer tissues (Ca) and corresponding normal mucosae (N). Lanes: U, unmethylated alleles; M, methylated alleles; IVD, in vitro-methylated control; H_2O, no DNA added. (B) Summary of the analyses for *GATA-4* and *-5* methylation in 45 primary CRCs. Each number in the vertical column represents a single tumor. Key: black, detection of methylated alleles; white, detection of unmethylated alleles only. (C) MSP analyses for normal colon mucosa samples from patients without ($n = 2$) or with ($n = 12$) cancer. Shaded boxes indicate weak detection of methylated alleles in two patients with *GATA-4* simultaneously hypermethylated in cancer. (D) Summary for 27 primary GCs.

both upstream transcription factors and genes that have been implicated as potential downstream targets for activation by these factors. We thus questioned whether the changes at the downstream genes might be insufficient to completely prevent their activation by GATA genes. Using an adenovirus system (16), we transiently expressed exogenous GATA-5 protein (Fig. 7A and B) in HCT116, RKO, and AZ521 cells, in which this gene is basally silent. This resulted in reactivation, at the transcript level (Fig. 7C and Table 1) of *inhibin*α and *TFF1* in all cell lines in which these genes are basally silent. When examined at the protein level, the GATA-5 overexpression resulted in expression of the secreted TFF1 protein (15) in the media of the cell cultures (Fig. 7D). *TFF2* is not activated in RKO and AZ521 lines, in which the promoter is mutated or deleted, as noted previously, but is reexpressed in HCT116 cells, in which the GATA-binding sites for this gene are present (Fig. 7C). *Dab2* is also reactivated at the transcript level in the one cell line, RKO, in which it is silent (Fig. 7C). Only one of the downstream genes, *TFF3*, is not reactivated in the lines in which the gene is basally silenced (Fig. 7C) and thus may not be a target for GATA-5.

In each case described above in which gene reactivation by GATA-5 occurred, the involved genes remain fully methylated (for examples for *TFF2*, see Fig. 6C and D; for examples for *inhibin*α and *TFF1*, see Fig. 7E) even though our analyses indicated robust activation of the genes in short-term studies.

DISCUSSION

Despite growing evidence linking loss of function for GATA transcription factor genes and several of their candidate downstream target genes to cancer development, mutations in such genes have not generally been found. Since epigenetic gene silencing, as well as mutations, can account for tumor suppres-

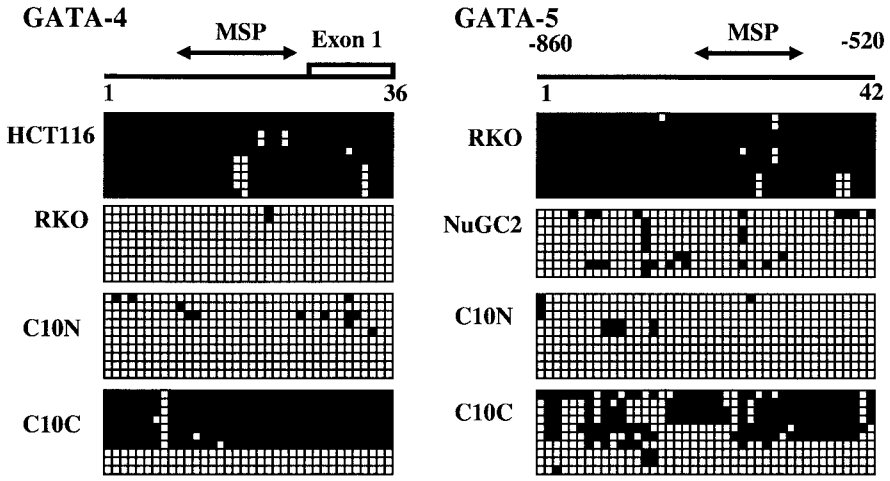

FIG. 4. Sodium bisulfite DNA sequencing of *GATA-4* and *-5* in colorectal (RKO and HCT116) and gastric (NuGC2) cancer cell lines and in various noncultured GI tissue samples. Each horizontal row of squares represents analysis, in a single clone of bisulfite-treated DNA, of 36 (*GATA-4*) or 42 (*GATA-5*) CpG sites contained in the region shown. Solid and open squares represent methylated and unmethylated CpG sites, respectively. *GATA* expression-negative cell lines (*GATA-4* in HCT116 and *GATA-5* in RKO) show densely methylated clones, but expression-positive cells (*GATA-4* in RKO and *GATA-5* in NuGC2 GC cells) have predominantly unmethylated clones. A primary colon cancer (case C10C) has predominantly methylated clones of *GATA-4* and *-5*, and normal colon mucosa from the same patient (C10N) has unmethylated clones.

sor gene loss in cancer (19), our present data suggest that *GATA-4*, *GATA -5*, *inhibin*α, *Dab2*, and *TFF* genes could play tumor suppressor gene roles in CRC and GC. In addition to growing evidence that loss of function for the *GATA-4* and *-5* genes could be important for cancer, *inhibin*α and *TFF1* both induce tumors when knocked out in mice (25, 27), although this is true for the former gene only in the setting of the testes. Interestingly, with respect to the *TFF* genes, we have also now found homozygous genetic disruption of *TFF2* sequences in two cell lines, as well as epigenetic silencing of this gene, findings that also attest to the potential importance of its antitumor effects.

The potential of our findings for the *GATA-4*, *-5*, and *-6* genes to be important in GI tumor development are particularly compelling. Many cancers involve various degrees of failure to complete cell differentiation and therefore manifest a phenotype of maturation arrest. Our patterns of silencing of the GATA genes would foster this situation. GATA-4, -5, and -6 are known to play distinct roles in embryonic GI development and also appear to do so for differentiation of mature GI epithelium (14, 20, 24). Loss of the differentiation stimuli of GATA-4 and -5, with concomitant retention of the proliferative stimulus of GATA-6, would predictably impede differentiation and could thus play a distinct role in the progression of cancers with this gene expression profile.

An intriguing aspect of our findings concerns why tumors might select for simultaneous epigenetic silencing of both upstream activating transcription factors and multiple downstream candidate target antitumor genes. Multiple possible explanations exist. First, it is certainly possible that, in the GI tract, a tight physiologic linkage between the transcription factors and the candidate downstream genes we have studied does not exist and, therefore, the epigenetic events we highlight here might also not be linked. Each epigenetic gene silencing event could then arise stochastically, and some may not play any true role in the progression of the GI tumors under study. However, as discussed previously, there is certainly experimental evidence pointing to the possible regulatory significance of GATAs for activating the genes we have studied. Further, the potential for all of the genes to play a role in cancer suggests that any coordination between the transcriptional factors and activation of the downstream genes would have great ramifications for cancer progression. Our current findings, given the growing recognition of the importance of epigenetic gene silencing for cancer, emphasizes the need for continuing exploration of the suggested relationships in detail.

A second possible scenario may explain some aspects of our findings. Some of the gene silencing events we observed could reflect normal states that transiently precede differentiation in proliferating and renewing GI cell epithelial compartments. This might be true especially in light of the methylation data for the *TFF* genes that have no promoter CpG islands. Differential promoter methylation is not unusual in normal tissues for nonhousekeeping genes with CpG-poor rich promoters (4). Thus, the presence or absence of promoter methylation in such genes can accompany their different transcriptional status during differentiation (4). However, promoter CpG islands for most genes, like those found in *GATA-4*, *GATA-5*, *inhibin*α, and *Dab2*, are generally not methylated in normal cells regardless of expression status (4). We could not find evidence, by sensitive techniques, for such methylation in the normal tissues examined. Perhaps expansion of a normally rare population of adult cells, such as stem cells, with the methylation profiles we observed could occur during tumorigenesis. Our data could provide useful markers to explore this possibility in future studies.

We suggest that one final hypothesis is appealing to consider and might well constitute a common paradigm for events that help fuel tumor progression. For the genes we studied, perhaps the most powerful antitumor effect may come from a summation of their epigenetic inactivation during tumor progression. Finding simultaneous mutations for each involved gene in the

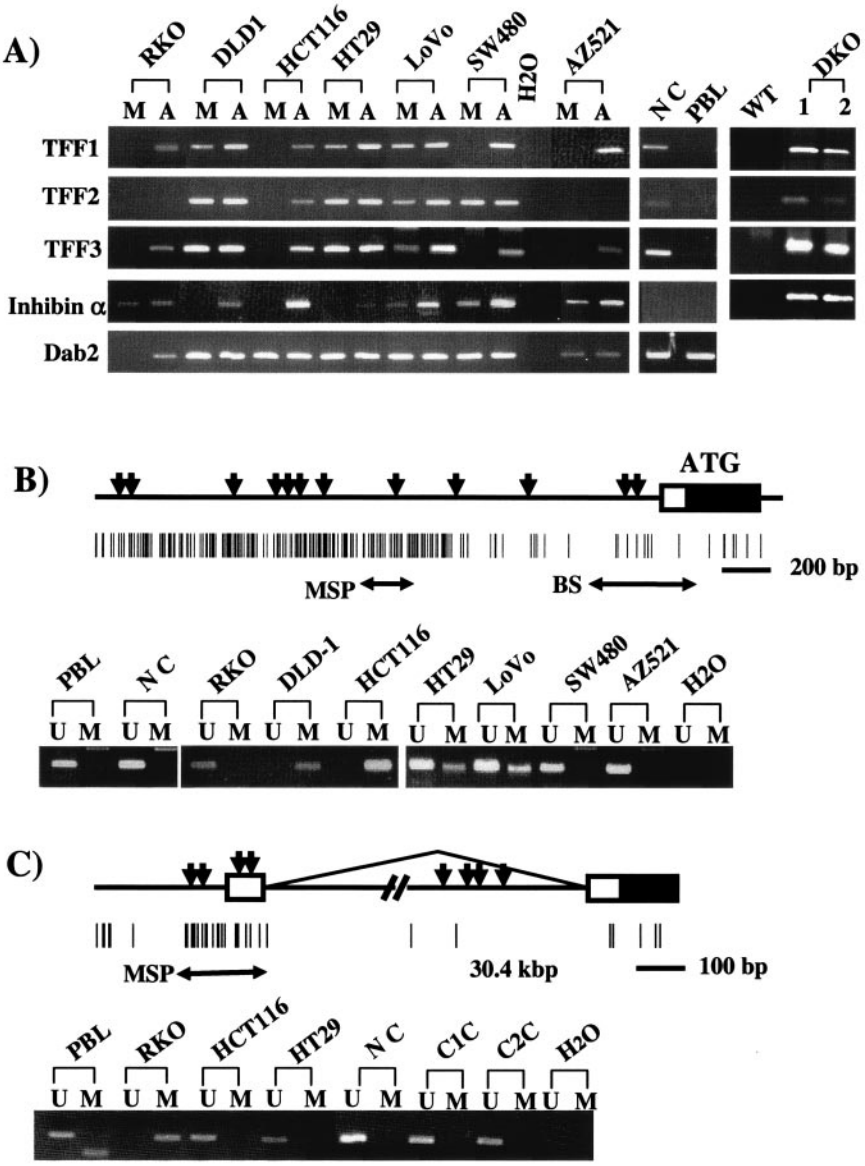

FIG. 5. Expression and methylation status of GATA downstream target genes in cultured colon cancer and GC cells and normal tissues. (A) RT-PCR analyses of the expression of each gene in the same colon cancer and GC cell lines and normal tissues as given for *GATA* analyses in Fig. 1A. Also, to the far right, is shown an analysis of expression, carried out as described above, of the *TFF* genes and *inhibin*α in the HCT116 colon cancer wild-type (WT) and *DNMT1*$^{-/-}$ plus *DNMT3b*$^{-/-}$ cells (DKO) shown in Fig. 1C. Lanes: M, mock treatment of cells; A, treatment with DAC; NC, normal colon; PBL, normal lymphocytes; H_2O, no RNA added. (B) Methylation status of the *inhibin*α gene. A schematic of the 5′ region of the gene is shown above in which the rectangle depicts the first exon, and the blackened area denotes the coding region within this exon. The black triangles represent positions of consensus GATA-binding sites, and the vertical lines each represent a CpG site. The large arrow (BS) denotes a region of bisulfite sequencing for the CpG poor region previously thought to be the only promoter region (see the text), and the smaller arrows (MSP) represent the positions of primers used for the MSP analysis in all of the cancer cell lines of the newly defined CpG island discussed in the text and shown in the lower part of panel B. Lanes: U, unmethylated alleles; M, methylated alleles; PBL, normal lymphocytes; NC, normal colon; H_2O, no DNA added. (C) Methylation status of *Dab2*. A schematic of *Dab2* is shown above in which a 5′ untranslated exon 1 (open box) is located upstream from exon 2 which contains the ATG for start of the coding region (black area within the square for exon 2). Arching line, mRNA splicing which joins exon 1 to exon 2; black triangles, positions of GATA-binding sites; vertical lines, CpG sites and the island around exon 1; arrows at the bottom (MSP), position of MSP primers used to analyze the methylation status of the CpG island as shown in the panel below. The lower part of panel C shows examples of MSP results for the methylation status of the *Dab2* 5′ CpG island. Lanes: U, unmethylated alleles; M, methylated alleles; PBL, normal lymphocytes; NC, normal colon; C1C and C2C, colon cancers.

same tumor might be quite rare. However, individual tumors epigenetically silence multiple genes (8), and this mode of gene inactivation, where protein function is less permanently, and perhaps less completely, disrupted than is the case for mutational events might be an excellent candidate mechanism for inactivating complex antitumor gene networks. Selection of cells might involve any combination of sporadic epigenetic inactivation events, which facilitate evolution of a cancer. For

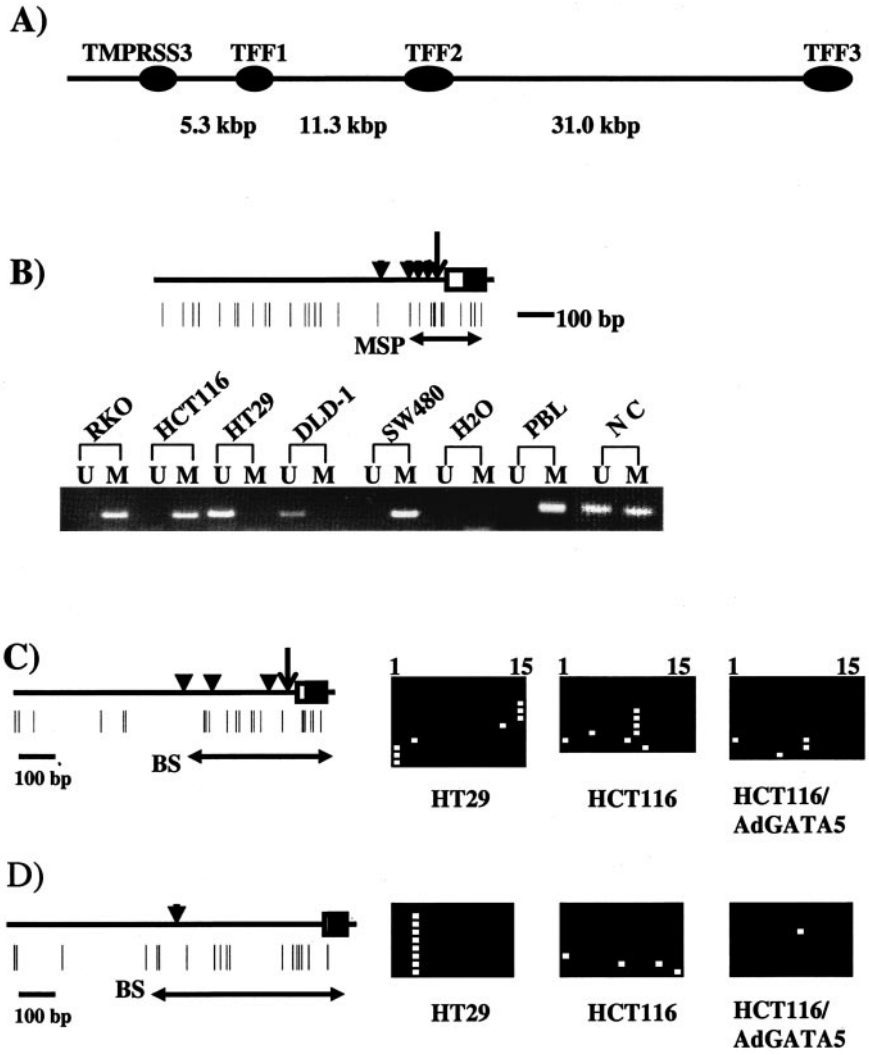

FIG. 6. Methylation status of *TFF1* to *TFF3* in cultured colon cancer cells. (A) Schematic of the alignment of the three *TFF* genes on chromosome 21q22.3. The location of a separate gene, *TMPRSS3*, upstream from *TFF1* is also shown. No CpG islands could be located anywhere along the depicted stretch of genomic sequence. (B) Methylation status of *TFF1*. A schematic of the 5′ region of the gene depicts the transcription start site (large vertical arrow) and exon 1 is shown in the rectangle, with the coding region portion shown in solid black. Vertical black triangles, GATA binding sites; vertical lines, CpG sites. Horizontal arrows (MSP) show the primer sites for the MSP analysis in the panel below for selected cancer cell lines and normal tissues (NC, normal colon; PBL, peripheral blood lymphocytes). (C) Schematic of the 5′ region of *TFF2*. All symbols are exactly as for those in Fig. 6A except that the horizontal arrow (BS) show the area represented in the bisulfite sequencing shown directly beside the schematic. For the sequencing all horizontal squares represent CpG sites in individual sequenced clones (white, unmethylated; black, methylated). The sequencing is shown for (i) HT29 cells in which the gene is expressed, (ii) HCT116 cells in which it is not, and (iii) these same cells which express the gene after adenoviral expression of *GATA-5* (see Fig. 7). (D) Schematic of the 5′ region of *TFF3*. All symbols are as described for the other panels and, again, the horizontal arrow (BS) shows the area represented in the bisulfite sequencing shown directly beside the schematic.

the downstream gene events, such silencing may only partially blunt transcriptional response to upstream activating factors. Thus, continued expression of GATA factors, and probably also other transcription factors with which these proteins are known to partner (5, 42), could activate downstream genes despite their local promoter methylation. Thus, selection during tumorigenesis for inactivation of one or both of the upstream *GATA-4* and *GATA-5* genes would be additive to the downstream silencing events to ensure the most powerful selection for loss of function of a group of *GATA* regulated antitumor genes.

Whatever the final full biological explanation for our data, our results have a significant translational implication. Genetic changes are not reversible but, as we demonstrated here, multiple epigenetically silenced candidate antitumor genes can be simultaneously reactivated in single tumors. This concept should receive careful attention with respect to cancer therapeutic strategies. Also, epigenetic silencing of important genes can occur early in the progression of cancers (19, 43). Upregulation of several of the genes we have studied, especially the TFF factors, has been suggested as important for CRC chemoprevention approaches. Thus, gene reac-

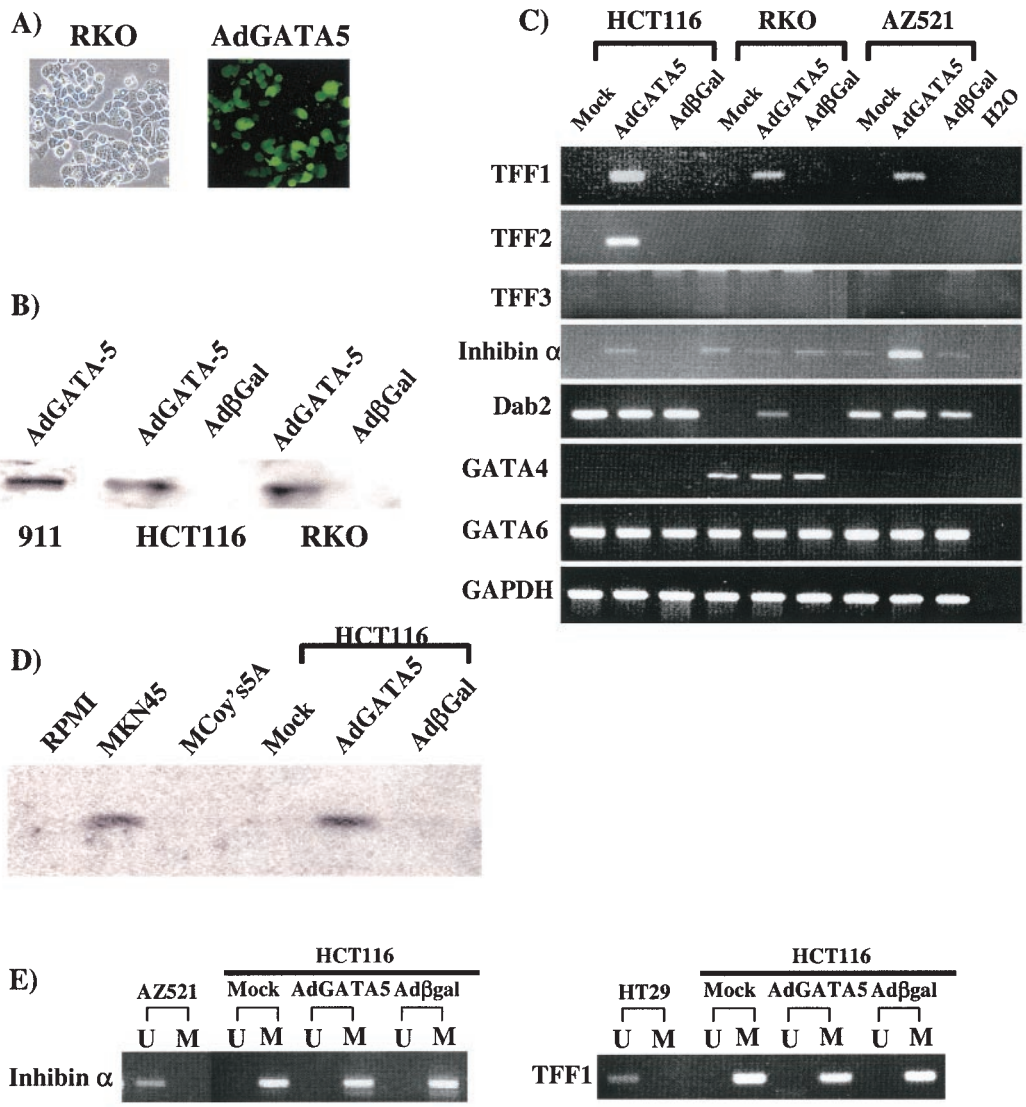

FIG. 7. Overexpression of *GATA-5* in GI cancer cells. (A) Morphological analysis in RKO cells after a *GATA-5* construct was overexpressed by using an adenovirus system (20). The left subpanel shows the phase-contrast morphological appearance, and right subpanel shows green fluorescent protein expression in the same fields. (B) Immunoblotting with anti-GATA-5 antibody in cancer cells. The 911 cell line (16) used to package the viral construct served as a positive control for production of the protein. GATA-5 protein is basally undetectable in HCT116 and RKO colon cancer cells, which exhibit *GATA-5* promoter methylation. Adenovirus (AdGATA-5) overexpression of *GATA-5* results in a strong expression of the expected M_r 45,000 form in both cancer cell lines. The virus expressing the *Escherichia coli* β-galactosidase gene was used as negative control (Adβgal). (C) RT-PCR analyses of expression for GATA-5 target genes. RNA was extracted from adenovirus-infected cells for PCR analyses for expression of each gene. Note that *GATA-5* overexpression induces reexpression of each candidate target gene, except for *TTF3* in all cell lines and *TFF2* in RKO and AZ521 cells, where the gene is homozygously mutated and/or deleted, in each cell line where basal expression is absent. (D) Immunoblotting with anti-TFF1 antibody in culture medium collected after 48 h from a positive control GC cell line MKN45, which has an unmethylated and expressed TFF1 gene, and colon cancer HCT116 cells in which the gene is hypermethylated and silenced. Note the TFF1 protein in the MKN45 cell media but only in the HCT116 media when cells are infected with adeno-GATA-5 but not when cells are infected with adeno-β-Gal. The RPMI culture medium used for MKN45 and the McCoy's 5A medium used for HCT116 cells are shown as additional negative controls. (E) MSP analyses of 5′ region methylation status of *inhibin*α and *TFF1* before and after overexpression of *GATA-5*.

tivation approaches might constitute cancer prevention, as well as therapeutic strategies.

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