SLC5A8, a sodium transporter, is a tumor suppressor gene silenced by methylation in human colon aberrant crypt foci and cancers

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We identify a gene, *SLC5A8*, and show it is a candidate tumor suppressor gene whose silencing by aberrant methylation is a common and early event in human colon neoplasia. Aberrant DNA methylation has been implicated as a component of an epigenetic mechanism that silences genes in human cancers. Using restriction landmark genome scanning, we performed a global search to identify genes that would be aberrantly methylated at high frequency in human colon cancer. From among 1,231 genomic *Not*I sites assayed, site 3D41 was identified as methylated in 11 of 12 colon cancers profiled. Site 3D41 mapped to exon 1 of *SLC5A8*, a transcript that we assembled. In normal colon mucosa we found that *SLC5A8* exon 1 is unmethylated and *SLC5A8* transcript is expressed. In contrast, *SLC5A8* exon 1 proved to be aberrantly methylated in 59% of primary colon cancers and 52% of colon cancer cell lines. *SLC5A8* exon 1 methylated cells were uniformly silenced for SLC5A8 expression, but reactivated expression on treatment with a demethylating drug, 5-azacytidine. Transfection of *SLC5A8* suppressed colony growth in each of three SLC5A8-deficient cell lines, but showed no suppressive effect in any of three SLC5A8-proficient cell lines. *SLC5A8* exon 1 methylation is an early event, detectable in colon adenomas, and in even earlier microscopic colonic aberrant crypt foci. Structural homology and functional testing demonstrated that SLC5A8 is a member of the family of sodium solute symporters, which are now added as a class of candidate colon cancer suppressor genes.

colon cancer

Cytosine methylation within CpG dinucleotides is a recognized epigenetic DNA modification that in normal human tissues is excluded from CpG-rich "islands" that mark the promoters of certain genes (1–3). Global hypomethylation accompanied by aberrant focal CpG island hypermethylation has emerged as one of the signature alterations evidenced by the cancer genome (1–4). Moreover, silencing of gene expression as marked by aberrant methylation of CpG island promoter regions has emerged as a mechanism for the inactivation of tumor suppressor genes that provides an alternative to either mutation or allelic loss (1, 2, 5, 6). Additionally, aberrant methylation of defined genomic sequences can serve as a potentially useful diagnostic marker for detection of human cancers (7, 8).

Restriction landmark genome scanning (RLGS) provides a global analysis of methylation events in a cancer cell by providing a two-dimensional display of the methylation status of genomic *Not*I sites (9). To identify tumor suppressor genes and/or identify genes targeted for methylation in human colon cancer, we carried out RLGS analysis of 12 colon cancer cell lines. This analysis lead to the identification of a transcript, *SLC5A8*, whose aberrant methylation and transcriptional silencing was found to be a common and early event in human colon cancers, and that was found to encode a sodium symporter whose restoration can markedly suppress colony-forming ability of colon cells in which endogenous *SLC5A8* has been inactivated.

Materials and Methods

Sequences. Human *SLC5A8* mRNA and gene sequence GenBank accession numbers as deposited by our group are AF536216 and AF536217. The *SLC5A8* murine homolog can be found under accession number BC017691. Contemporaneously with our entry, *SLC5A8* mRNA sequence was also independently deposited under accession number AY081220 (10).

RLGS. RLGS was performed as described (9).

Amplification and Sequencing of *SLC5A8*. PCR and sequencing primers are provided in *Supporting Materials and Methods*, which is published as supporting information on the PNAS web site, www.pnas.org.

Cell Culture and Clonogenic Assays. Vaco cell lines were cultured as described (6). FET and RKO were gifts of M. Brattain (Roswell Cancer Institute, Buffalo, NY). For colony-forming assays (11), colon cancer cells were transfected with either a SLC5A8 expression vector or a control empty vector, and the number of stable colonies arising after selection in G418 was respectively counted.

Serum DNA Purification. Serum DNA from patients were purified as described (7).

5-Azacytidine Treatment. As described (6), cells were treated for 24 h on day 2 and day 5 with 5-azacytidine (Sigma) at 1.5 μg/ml. The medium was changed 24 h after addition of the 5-azacytidine (i.e., on days 3 and 6).

***Xenopus* Oocyte Studies.** Fifty nanoliters of water (control) or RNA solution (50 nl of 0.6 μg/μl or ≈30 ng of *SLC5A8*-cRNA) was injected into stage V/VI *Xenopus* oocytes, which were

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Abbreviations: ACF, aberrant crypt foci; MS, methylation-specific; RLGS, restriction landmark genome scanning.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. AF536216 (human *SLC5A8* mRNA) and AF536217 (gene sequence)].

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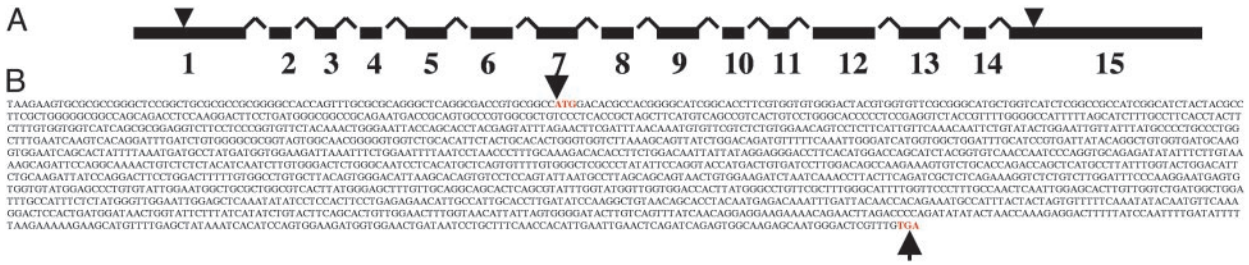

Fig. 1. Identification of *SLC5A8*. (*A*) The genomic structure of the *SLC5A8* gene. Black boxes represent exons, and arrows represent the start and stop codons. (*B*) The nucleotide sequence of the *SLC5A8* coding region.

studied 3–6 days after injection. Oocyte expression of SLC5A8 was verified by immunolocalization of the V5-tag , and oocyte electrophysiology experiments were carried out as described (12) and as detailed in *Supporting Materials and Methods*.

Sodium Bisulfite Treatment, Methylation-Specific (MS)-PCR, and Real-Time MS-PCR. Sodium bisulfite treatment to convert unmethylated cytosine to thymidine and MS-PCR were performed as described (7). Real-time MS-PCR was performed by using *MYOD1* sequences as an internal reference, as described in ref. 8. Primers and PCR conditions are provided in *Supporting Materials and Methods*.

Statistical Methods. Association of *SLC5A8* methylation with sex was analyzed by using Fisher's two-tailed exact test. Association of *SLC5A8* methylation status with tumor site or stage was analyzed by using Pearson's χ^2 statistics. Comparisons of age distributions based on *SLC5A8* methylation were done with Wilcoxon nonparametric tests. Comparisons of colony counts after transfection with different vectors were done by Student's *t* test and linear models.

Aberrant Crypt Foci (ACF). ACF (13–15) were isolated from grossly normal human colonic mucosa according to the method of Bird *et al.* (16) and as described in *Supporting Materials and Methods*. The control for each ACF was a similar number of microscopically normal crypts teased from the same mucosa.

Results

Identification of the *SLC5A8* Gene. Methylation events in genomic DNA from 12 colon cancer cell lines were profiled by restriction landmark genomic scanning. Of 1,231 unselected CpG islands visualized, spot 3D41 was detected as absent and presumptively methylated in 11 of the 12 colon cancer cell lines. A 510-bp genomic fragment surrounding the 3D41 *Not*I site was cloned and shown to correspond to genomic sequence on human chromosome 12q22–23. RNA from normal human colon mucosa was used for connection RT-PCR that linked together over 10 EST sequences mapping to this genomic region. New sequence was generated both by sequencing of these RT-PCR amplified products, as well as by sequencing image clones corresponding to these ESTs (Fig. 7, which is published as supporting information on the PNAS web site). This established that the 3D41 *Not*I site was included within a new transcript encoded by a gene (Fig. 1*B*). This gene, located on chromosome 12q22–23, is comprised of 15 exons, with the *Not*I site from RLGS located in exon1 (Fig. 1*A*). The newly identified transcript includes an in-frame TAA stop codon 5′ to the presumptive ATG start codon, which additionally is embedded within a GCCATGG sequence that conforms to the standard for a good Kozak sequence. BLAST alignment of the predicted protein product of this transcript showed the most closely related proteins to be the human sodium iodide symporter SLC5A5 (46% homology) and the human sodium-dependent multivitamin transporter SLC5A6 (43% homology), both of which belong to the solute carrier 5 family (SLC5) of sodium coupled transporters (Fig. 8, which is published as supporting information on the PNAS web site). Moreover, analysis of the predicted protein by the TMHMM prediction program (www.cbs.dtu.dk/services/TMHMM) identified 13 transmembrane fragments, consistent with structural features of the sodium iodide symporter. Thus, structurally this transcript encodes a member of the SLC5 sodium solute symporter family (SSF) family, and the Human Genome Organization (HUGO) assigned the encoded protein the name SLC5A8. A mouse protein of unknown function shows 77% identity to SLC5A8 and is likely the mouse homolog of the human protein (Fig. 8). RT-PCR confirmed that *SLC5A8* transcript was expressed by normal colon mucosa, as well as by kidney, lung, esophagus, small bowel, stomach, thyroid, and uterus, with the greatest expression seen in kidney (data not shown).

***SLC5A8* Is Frequently Silenced and Methylated in Colon Cancer Cell Lines.** RT-PCR was used to further characterize SLC5A8 expression in normal colon mucosa compared with a collection of 31 colon cancer cell lines. Whereas the *SLC5A8* transcript was well expressed in normal colon, it proved absent in 23 of the 31 colon cancer cell lines (Fig. 2*A*). The methylation of *SLC5A8* exon 1 detected by RLGS suggested the hypothesis that aberrant methylation might be the mechanism for silencing of SLC5A8 expression. Consistent with this hypothesis, treatment of SLC5A8-silenced cell lines with the demethylating agent 5-azacytidine reactivated SLC5A8 expression in six of eight colon cancer cell lines tested (Fig. 2*B*; data not shown). Sequencing of the *SLC5A8* transcript in the eight colon cancer cell lines in which it was expressed showed only wild-type sequence with no mutations. Thus, methylation, but not mutation, appeared to be the putative mechanism for inactivating SLC5A8 in colon cancer.

To identify target sequences for aberrant *SLC5A8* methylation in colon cancer, we investigated a dense CpG island (G+C, 70%; CG/GC, 0.9) located in *SLC5A8* exon 1 and surrounding the 3D41 *Not*I site. This region covered 573 bp and included 62 CpG dinucleotides (Fig. 9*A*, which is published as supporting information on the PNAS web site). In contrast, the region immediately 5′ of exon 1 showed only a 46% G+C content. We used sodium bisulfite treatment of genomic DNA to convert unmethylated cytosines to uracil, while leaving methylated cytosines unchanged (17). Sequencing of PCR amplified bisulfite converted *SLC5A8* exon 1 genomic DNA was then used to determine the methylation status of each of the 62 target cytosines within the CpG island domain. Comparing the findings in nine SLC5A8-silenced cell lines versus those in three SLC5A8-expressing cell lines and six samples of SLC5A8 expressing normal colon mucosa defined a 182-bp subregion. In the nine SLC5A8-silenced cell lines this subregion demonstrated uniform methylation of all CpG cytosines, whereas these cytosines were uniformly unmethylated in the three SLC5A8-expressing cell

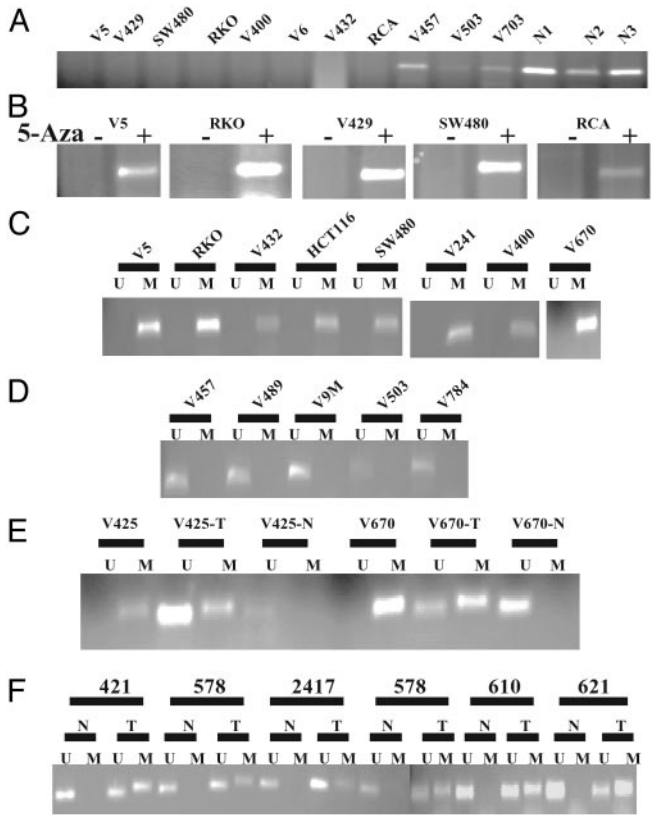

Fig. 2. SLC5A8 expression. (*A*) RT-PCR analysis demonstrating *SLC5A8* transcript expression in three normal colon mucosa samples (N1, N2, and N3) but the absence of *SLC5A8* transcript in most colon cancer cell lines (remaining samples). (*B*) RT-PCR analysis demonstrating reactivation of SLC5A8 expression in cell lines treated with 5-azacytidine (+) compared with untreated controls (−). (*C*) MS-PCR assay for methylated (M) or unmethylated (U) *SLC5A8* exon 1 sequences detects exclusively methylated templates in SLC5A8-silenced cell lines. (*D*) MS-PCR detects only unmethylated *SLC5A8* templates in SLC5A8-expressing cell lines. (*E*) MS-PCR detection of methylated *SLC5A8* templates in colon cancer tumors (T) antecedent to *SLC5A8*-methylated cell lines (V425 and V670). Matched normal colon tissue (N) shows only unmethylated templates. Unmethylated templates in tumor tissue presumptively arise from contaminating nonmalignant cells. (*F*) MS-PCR analysis of colon cancer tumors (T) and matched normal colon tissues (N). Methyl-specific bands are seen in each of the tumor samples but none of the normal controls.

lines and six normal colon mucosa samples (Fig. 9*B*). Primers for assay of this subregion by MS-PCR were designed, such that after bisulfite conversion amplification products would selectively be derived from either methylated or unmethylated genomic templates (17). MS-PCR assay of 31 total colon cancer cell lines demonstrated that *SLC5A8* exon 1 methylation was present in 16 cases (52%), and in each of these methylated cell lines no *SLC5A8* transcript was detectable (Fig. 2*C*). In contrast, in each of the eight SLC5A8-expressing cell lines MS-PCR assayed exon 1 as unmethylated (Fig. 2*D*). In seven remaining instances, SLC5A8 expression was absent, but aberrant methylation was not identified as the reason. Moreover, in the case of two of the *SLC5A8*-methylated cell lines (V425 and V670), DNA from antecedent tumor and matched patient normal tissue was also available. In each of these cases, MS-PCR confirmed that *SLC5A8* methylation was present in the primary tumor tissues, but was absent in the matched normal tissues (Fig. 2*E*). Thus the *SLC5A8* methylation and silencing detected in colon cancer cell lines reflects somatic aberrations present in primary colon cancer tissues. We note that the finding of gene silencing associated with aberrant methylation in a first exon region

Table 1. *SLC5A8* methylation in colon tumors and matched normal mucosa

	Normal tissue	
Tumor tissue	Methylated	Unmethylated
Methylated	3 (5%)	35 (54%)
Unmethylated	0 (0%)	26 (41%)

Shown is the characterization of 64 pairs of colon cancer tumors and matched normal colon tissues assayed for methylation of *SLC5A8* exon 1 by MS-PCR. Indicated are the numbers (and percentages) of tissue pairs with each of the four possible methylation phenotypes.

corresponding to 5′ untranslated sequences has existing precedent at other loci (2, 18). In previous studies, our group has noted that in colon cancers aberrant methylation of hMLH1 and HLTF commonly silences both maternal and paternal alleles in the same tumor (6, 11). Consistent with this mechanism, testing of microsatellite markers D12S1041 and D12S1727, which flank *SLC5A8*, showed the presence of two distinguishable parental *SLC5A8* chromosomal regions in 10 of 10 colon cancer cell lines that showed the presence of only methylated *SLC5A8* exon 1.

***SLC5A8* Methylation Is Commonly Present in Primary Colon Cancers and in Colon Adenomas.** To further establish the frequency of *SLC5A8* exon 1 methylation in primary colon cancer tumors, we analyzed by MS-PCR an additional 64 pairs of primary colon cancer tumor tissues, as well as their accompanying matched normal colon tissues. *SLC5A8* methylation was detected in 38 of 64 (59%) primary colon cancers (Fig. 2*F*, Table 1). In 35 of 38 cases (92%) in which colon tumors showed *SLC5A8* methylation, this methylation was not detected in the same individuals' normal colon tissues. *SLC5A8* exon 1 methylation thus substantially arose in these individuals' cancers as part of and during the neoplastic process. In three cases in which *SLC5A8* methylation was detected in both an individuals' cancerous and normal colon tissues, these findings likely indicate either the presence of some cancer cells within the grossly normal resected tissue or the possibility that the cancer arose from a field of *SLC5A8*-methylated cells. The rarity of detecting *SLC5A8* methylation in normal colon tissues is highlighted by noting that no *SLC5A8* methylation was detected in any of the 26 normal colon tissues in which the accompanying colon cancer was also unmethylated (Table 1), and moreover, that no *SLC5A8* methylation was detected in any of 12 additional normal colon tissues from resections done for non-cancer diagnoses (data not shown).

Among all primary cancers and cell lines analyzed, the finding of *SLC5A8* methylation in colon cancer tumors and cell lines was not significantly correlated with either sex ($P = 0.39$) or age ($P = 0.52$), with a median age of 69 in persons with *SLC5A8*-methylated cancers versus 67 in those with *SLC5A8*-unmethylated cancers. Moreover, the distribution by tumor stage (Dukes' stage B, C, and D primary tumor or metastatic cancer deposit) was not significantly different between *SLC5A8*-methylated and nonmethylated colon cancers ($P = 0.77$; Table 2, which is published as supporting information on the PNAS web site). *SLC5A8*-methylated and -unmethylated cancers also showed no significant difference with respect to site of origin in the rectum, left colon, or right colon ($P = 0.47$; Table 3, which is published as supporting information on the PNAS web site).

To determine the timing of onset of SLC5A8 silencing in colon carcinogenesis, we additionally analyzed a group of 29 adenomas for *SLC5A8* exon 1 methylation. *SLC5A8* methylation was detected in 17 of the 29 (59%) adenoma cases. *SLC5A8* methylation thus appears to be an early event that is already established in colon neoplasia by the adenoma stage.

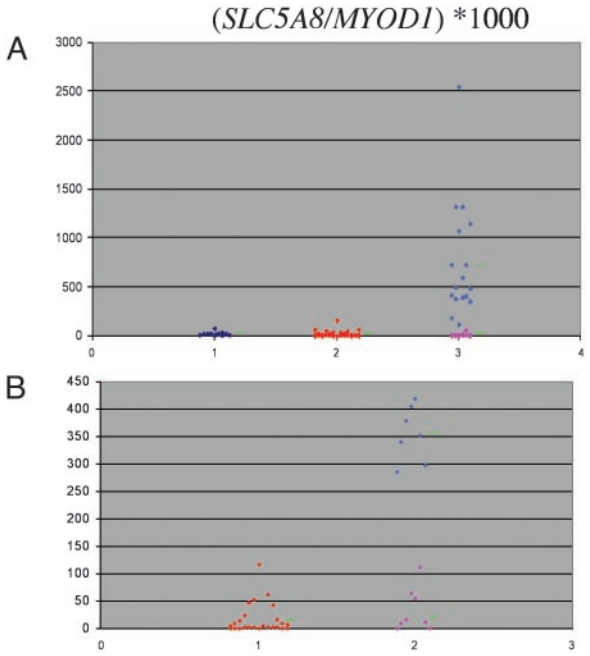

Fig. 3. Real-time MS-PCR analysis of *SLC5A8* methylation. Plotted is 1,000 times the ratio of measured *SLC5A8*-methylated product to the control *MYOD1*-derived product. (*A*) Detection of *SLC5A8* methylation in primary colon cancer tissues. Column 1 displays values for normal colon tissues harvested from non-cancer resections (dark blue diamonds). Column 2 displays values for normal colon tissues harvested from colon cancer resections (red diamonds). Column 3 displays values for colon cancer tissues divided into unmethylated samples falling within the normal tissue range (pink diamonds) and methylated samples showing values greater than the normal tissue range (light blue diamonds). Adjacent green bars indicate population means. (*B*) Real-time MS-PCR analysis of *SLC5A8* methylation in ACF. Column 1 displays values for 24 normal colon tissues harvested from colon resections of 11 individuals (red diamonds). Column 2 displays values for 15 ACF harvested from the same 11 individuals' resections. Pink diamonds indicate unmethylated samples within the normal range, and blue diamonds indicate methylated samples falling within the range previously demonstrated by methylated cancers. Adjacent green bars indicate the mean value for each group.

Quantitative Assay of *SLC5A8* Exon 1 Methylation. To derive a quantitative measure of *SLC5A8* methylation we used a real-time MS-PCR assay the results of which were expressed as 1,000 times the ratio of methylated *SLC5A8* reaction product to a control *MYOD1* reaction product (8). In this assay, 0 methylation was detected in the Vaco9 SLC5A8-expressing colon cancer cell line, and a methylation value of 1,000 was detected in the *SLC5A8*-methylated and silenced RKO colon cancer cell line. As shown in Fig. 3*A*, assay for *SLC5A8* exon 1 methylation in 11 normal colon mucosal samples derived from non-cancer resections yielded only barely detectable methylation values (mean value, 24; range, 4–82) and defined an "unmethylated normal range" of values all <100. Analysis of 29 normal colon samples derived from colon cancer resections gave similarly low values with a mean value of 22 and a single outlier sample (value, 159) falling outside the range defined by the non-cancer-derived normal tissues. This observation essentially replicated our previous observation of rare faint methylation events detected in some cancer-associated normal tissue. In contrast, analysis of colon cancer samples clearly distinguished two populations of tumors. Twelve cancers were deemed unmethylated, because they showed methylation values well within the population normal range (mean value, 12; range, 0–58; Fig. 3*A*) and hence were indistinguishable from unmethylated normal tissues. In contrast, 17 cancers with methylation values greater than the normal range comprised a distinct "methylated" group of cancers that was characterized by a mean methylation value of 747 and a range of 121–2,549 (Fig. 3*A*). The mean methylated colon cancer thus displayed 75% the level of methylation measured in a pure cell line population of methylated RKO cells. The heterogeneity in measured methylation values among the methylated colon cancers may in part derive from differences among the tumors in levels of contaminating and infiltrating non-cancer cells. The methylated and unmethylated cancer populations defined by real-time MS-PCR corresponded to the tumors classified as unmethylated and methylated, respectively, in the previous nonquantitated MS-PCR reaction.

Detection of *SLC5A8* Methylation in ACF. The finding of *SLC5A8* methylation in colon adenomas prompted us to consider that *SLC5A8* methylation might be an early event in human colon neoplasia. The earliest morphologically identifiable colon neoplasias putatively are ACF (13). These microscopic morphologically aberrant multicrypt structures are recognizable in unembedded colon under low power magnification. Moreover, a subset of ACF lesions demonstrate both histologic dysplasia and mutations of the *APC* tumor suppressor gene (14, 15), suggesting that at least some ACF have the potential to progress to colon adenomas and cancers. To assess a possible role of *SLC5A8* methylation in ACF development, 15 ACF, composed of from 17–155 crypts (48 ± 36 crypts, mean ± SD), were dissected from 11 different patients' colons bearing either cancer or adenomas. From these same 11 cases, 24 similarly sized tissue samples were dissected from mucosal regions that appeared normal under low-power magnification. Real-time MS-PCR analysis of *SLC5A8* methylation in the 24 control normal samples gave results similar to those obtained in previous normal mucosal samples, with a mean *SLC5A8* methylation value of 12, and with only one of these 24 new samples (methylation value of 117) falling just outside of the previously determined normal limit of 100 (Fig. 3*B*). In contrast, analysis of DNA from the ACF revealed two distinct populations: 8 of 15 ACF falling within the normal range (mean, 34; range, 0–113) and 7 of 15 ACF samples demonstrating *SLC5A8* values well within the range of methylated cancers (mean, 355; range, 287–420; Fig. 3*B*). In contrast, none of these 15 ACF demonstrated aberrant methylation of *hMLH1*, which thus likely arises later in colon carcinogenesis. These findings suggest that *SLC5A8* methylation is indeed an early aberration that precedes adenoma formation and is detectable in ACF. This finding also further strengthens the model that suggests a subset of ACF are likely to progress to more advanced colonic neoplasms.

***SLC5A8* Methylation as a Serologic Marker of Colon Cancer.** *SLC5A8* methylation was detected in 59% of our primary colon samples. In these same samples we had previously noted a 44% frequency of methylation of *HLTF*, a SWI/SNF family gene (11), and also found a 44% frequency of methylation of *p16* (Fig. 10, which is published as supporting information on the PNAS web site; ref. 19). These data suggest that *SLC5A8* methylation might be a high-quality marker of colon cancer presence. In this regard, we and others have shown that aberrantly methylated genomic DNA from specific loci can be detected in the serum of some cancer patients (7). Accordingly, we characterized the level of *SLC5A8* methylation in ethanol-precipitable DNA prepared from the serum of colon cancer patients (7). *SLC5A8* methylation was totally undetectable with a measured value of 0 in DNA extracted from each of 13 serum samples from individuals with colon cancers in which *SLC5A8* assayed as unmethylated (Fig. 4). In contrast, *SLC5A8* methylation was detectable in serum DNA from 4 of 10 patients in which the underlying colon cancer assayed as *SLC5A8*-methylated (Fig. 4). A positive signal for *MYOD1* verified the presence of input DNA into each of these

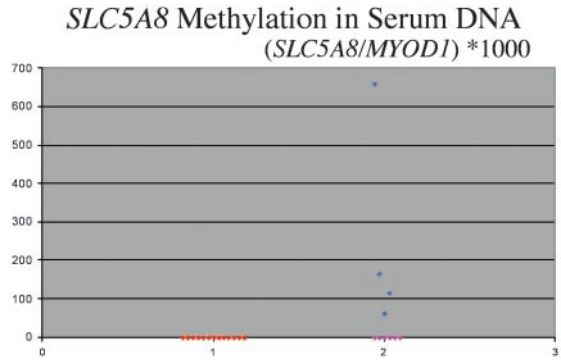

Fig. 4. Real-time MS-PCR analysis of *SLC5A8* methylation in DNA precipitated from the serum of colon cancer patients. Plotted is 1,000 times the ratio of measured *SLC5A8*-methylated product to control *MYOD1*-derived product. Column 1 displays an absence of detectable *SLC5A8* methylation in serum of 13 individuals whose colon cancer tumors assayed as unmethylated by MS-PCR (red diamonds). Column 2 displays values of *SLC5A8* methylation in the serum of 10 individuals whose colon cancer tumors assayed as methylated by MS-PCR. Pink diamonds indicate six sera without detectable methylation, and blue diamonds indicate four sera in which *SLC5A8* methylation was detectable.

assays. Although serologic assays for methylated DNA as a marker of cancer are clearly in the early stages of investigation, we note that a panel of methylated genes that included *SLC5A8*, *HLTF*, *p16*, and *hMLH1* provided greater sensitivity than any single locus alone for detecting an aberrant methylation event in our set of 64 primary colon cancers (Fig. 10).

***SLC5A8* Encodes a Functional Sodium Transporter.** Sequence homology suggested that *SLC5A8* encodes a member of the sodium solute symporter family, a family of transporters that utilizes the energetically favorable transport of sodium into cells to cotransport a second coupled substrate (20). To determine whether SLC5A8 indeed functions as a Na^+-coupled solute transporter, we expressed V5 epitope-tagged SLC5A8 protein in *Xenopus laevis* oocytes by injecting cRNA. Immunofluorescence using an anti-V5 antibody demonstrated intense staining of the oocyte plasma membrane in cRNA-injected cells, but not in water-injected controls (Fig. 5*A*). Using microelectrodes, we monitored mock-injected and *SLC5A8* cRNA-injected oocytes, and in a constant perfusion chamber simultaneously measured membrane potential (V_m) and intracellular Na^+ activity (aNa_i) (21). Control oocytes demonstrated a resting V_m of -49.6 ± 1.4 mV ($n = 4$) and an aNa_i of 3.4 ± 0.2 mM. *SLC5A8*-injected oocytes maintained a similar membrane potential ($V_m = -56.7 \pm 4.6$ mV; $n = 6$), but compared with control showed a significantly elevated aNa_i (12.3 ± 1.4 mM; $n = 6$), indicating Na^+ uptake by these SLC5A8-expressing oocytes (Fig. 5*B*). Thus far, our results do not indicate whether this Na^+ uptake is substrate-coupled or is a "Na^+ leak" similar to that typically observed among of all of the previously characterized members of the SLC5 family (22–24). However, our findings provide direct functional data that *SLC5A8* encodes a sodium transporter and is therefore highly likely to be a bona fide member of the SLC5 family of sodium solute symporters.

SLC5A8 Suppression of Colon Cancer Colony Formation. The high frequency of *SLC5A8* methylation observed in colon cancer suggested that inactivation of this gene might confer a selective advantage. To assay for such an advantage, we examined the effect of *SLC5A8* transfection in three colon cancer cell lines (V400, RKO, and FET) in which the endogenous *SLC5A8* gene was methylated and silenced, as compared with three colon cancer cell lines (V457, V9M, and V364) in which the endogenous *SLC5A8* gene remained unmethylated and expressed. Reconstitution of SLC5A8 expression in *SLC5A8*-methylated cells suppressed colony-forming ability by at least 75% in each of the three lines tested ($P < 0.01$; Fig. 6*B*). In contrast, transfection of *SLC5A8* did not show significant colony suppression in the any of the three cell lines that already expressed an endogenous *SLC5A8* allele (Fig. 6*A*; $P < 0.01$ for the difference in effect of *SLC5A8* transfection in *SLC5A8*-methylated versus -unmethylated cell lines). Transient transfection showed that both *SLC5A8*-methylated and -unmethylated cells were able to express comparable levels of exogenous SLC5A8, as determined by Western analysis for a V5 epitope tag attached to the *SLC5A8* cDNA. These findings suggest that *SLC5A8* methylation and silencing confers a specific growth advantage in the subset of colon cancers in which this locus is inactivated. Consistent with this interpretation, we found that four of five of the rare SLC5A8-expressing clones that grew out after transfection of the *SLC5A8*-methylated V400 colon cancer cell lines were markedly suppressed in their ability to form xenograft tumors in athymic mice (Fig. 11, which is published as supporting information on the PNAS web site).

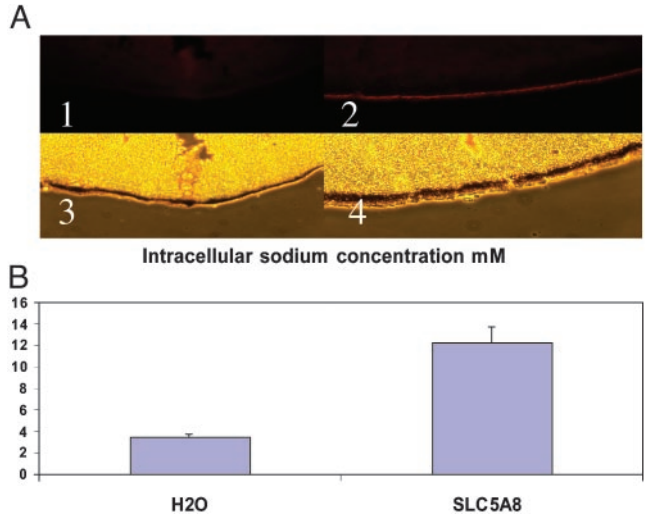

Fig. 5. Analysis of SLC5A8 function in *Xenopus* oocytes. (*A*) Immunofluorescent visualization of the V5 epitope tag in mock-injected oocytes (*1*) or in oocytes injected with cRNA for V5-tagged SLC5A8 (*2*). The corresponding phase photomicrographs are shown in *3* and *4*. (*B*) Intracellular sodium concentration [aNa_i] (mM) as measured by a sodium electrode in control water-injected and *SLC5A8* cRNA-injected oocytes.

Discussion

In this study, we have identified a gene, *SLC5A8*, that we demonstrate is a candidate colon cancer suppressor gene. We find that *SLC5A8* encodes a sodium transporter and is a member of the sodium solute symporter family (SLC5). *SLC5A8* is frequently targeted for methylation and silencing in human colon cancer, with aberrant *SLC5A8* exon 1 methylation detected in 52% of colon cancer cell lines and 59% of primary colon cancers. All colon cancer cell lines that showed *SLC5A8* exon 1 methylation were silenced for SLC5A8 expression, and SLC5A8 expression could be restored by treatment with the demethylating agent 5-azacytidine. We therefore conclude that epigenetic gene silencing, which is reflected by aberrant *SLC5A8* methylation, represents the principal mechanism for inactivating this gene in colon cancer. Moreover, our finding that exogenous SLC5A8 specifically suppresses colony-forming activity in colon cells that have inactivated this allele supports the hypothesis that SLC5A8 inactivation confers a selectable advantage in neoplas-

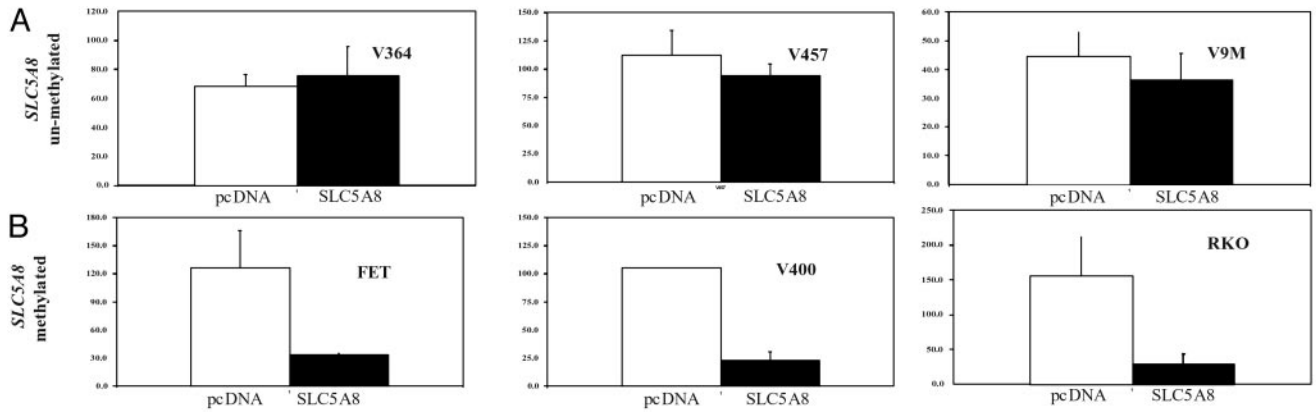

Fig. 6. SLC5A8 suppression of colon cancer colony formation. Shown are the number of G4180-resistant colonies arising from transfection with a SLC5A8 expression vector (SLC5A8) or a control empty expression vector (pcDNA) in *SLC5A8*-unmethylated and -expressing V364, V457, and V9M cells (*A*) as compared with *SLC5A8*-methylated and -deficient FET, V400, and RKO cells (*B*).

tic colon epithelial cells. Colon cells that retain SLC5A8 are insensitive to the introduction of an exogenous allele and presumably bear a mutation elsewhere that renders them tolerant to continued SLC5A8 expression. Also supporting the hypothesis that *SLC5A8* methylation is a pathogenetic event in colon neoplasia is our finding that *SLC5A8* methylation is a highly early event detectable in 47% of ACF, which are the earliest detectable morphologic abnormality of the colon epithelium. *SLC5A8* methylation may also play an etiologic role in malignancies additional to colon cancer. In early studies we note that *SLC5A8* methylation is present in a subset of cancers of the breast and stomach (Table 4, which is published as supporting information on the PNAS web site). Both molecular homology and functional data suggest that SLC5A8 functions as a sodium solute symporter. There are 109 currently known members of the sodium solute symporter family, which functions to cotransport sodium coupled to solutes as diverse as iodine (NIS/SLC5A5), glucose (SGLT1/SLC5A1;SGLT2/SLC5A2), inositol (SMIT/SLC5A3), and water-soluble vitamins (SMVT/SLC5A6) (22–24). Elucidating the putative solute cotransported by SLC5A8 may provide both insight into the mechanism of SLC5A8 growth suppression and leads for potential development of novel agents useful for colon neoplasia prevention and treatment.

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