Molecular cloning and promoter analysis of downregulated in adenoma (DRA)

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Alrefai WA, Wen X, Jiang W, Katz JP, Steinbrecher KA, Cohen MB, Williams IR, Dudeja PK, Wu GD. Molecular cloning and promoter analysis of downregulated in adenoma (DRA). Am J Physiol Gastrointest Liver Physiol 293: G923–G934, 2007. First published August 30, 2007; doi:10.1152/ajpgi.00029.2007.—Downregulated in adenoma (DRA), also referred to as SLC26A3, is an intestinal anion transporter essential for intestinal chloride absorption. Mutations in DRA result in congenital chloride diarrhea. DRA expression has been shown to be induced by differentiation and to be modulated by cytokines. However, mechanisms of DRA gene transcription and its tissue-specific targeting have not yet been investigated. In this study, we cloned a 3,765-bp promoter fragment of human DRA gene and characterized its activity in human colonic LS174T and Caco-2 human colon cell lines. Primer extension identified a single transcriptional initiation site that was identical in both colon cancer cell lines and normal colon. Although hepatic nuclear factor HNF-4 is involved in the basal activity of DRA promoter, sodium butyrate induces its activity in LS174T cells via the binding of Yin Yang 1 (YY1) and GATA transcription factors to their respective cis-elements in promoter region. We also demonstrated a reduction in DRA promoter activity in Caco-2 cells by IFN-γ, suggesting that regulation of DRA promoter by IFN-γ may contribute to the pathophysiology of intestinal inflammation. Furthermore, we showed that the DRA promoter fragment is sufficient to drive human growth hormone transgene expression specifically in villus epithelial cells of the small intestine and in differentiated upper crypt and surface epithelial cells of the colon. Our studies provide evidence for the involvement of HNF-4, YY1, and GATA transcription factors in DRA expression in intestinal differentiated epithelial cells.

SLC26A3; congenital chloride diarrhea; intestinal gene expression

THE COUPLED OPERATION of dual-ion Na+/H+ (NHE) and Cl−/HCO3− exchangers has been one of the major pathways for electroneutral sodium chloride absorption in the human intestine (1, 37, 38, 40, 42, 47, 52, 63). Genetic disruption of the genes encoding for intestinal Na+/H+ and Cl−/HCO3− exchangers has been associated with congenital sodium diarrhea and congenital chloride diarrhea (CLD), respectively (5, 31, 32, 35, 47). Also, disturbances in the function and/or dysregulation of intestinal Na+/H+ and Cl−/HCO3− exchange processes have been implicated in diarrhea associated with enteric infections and inflammatory bowel diseases (18, 26, 51, 59). In this regard, the molecular identity of the luminal intestinal Na+/H+ exchangers (NHE2 and NHE3) has been fully established and their regulation extensively investigated (9, 17, 20, 21, 43, 55, 62, 69). However, the identity of luminal intestinal Cl−/HCO3− exchange processes has slowly started to emerge (13, 17, 34, 47, 49).

Recent studies suggest that members of SLC26 family of anion exchangers might be involved in mediating intestinal luminal Cl−/HCO3− exchange process (13, 49). Of particular interest, SLC26A3 or DRA (Downregulated in adenoma) has been genetically linked to CLD, an autosomal recessive disorder, characterized by a watery stool with high chloride concentration and metabolic alkalosis (6, 28). Since DRA was found to be mutated in patients with CLD disease (30, 47) and the phenotype of DRA knockout was similar to CLD in humans (54), it was suggested that DRA anion transporter plays an essential role in intestinal luminal Cl−/HCO3− exchange activity and chloride absorption (46, 48). Interestingly, DRA polypeptide has been shown to be part of a protein complex in which DRA is linked to NHE3 via E3KARP PDZ binding protein in the apical compartment of colonocytes of the human proximal colon (39). Such an association further indicates a functional link between DRA and NHE3, suggesting their involvement in coupled Na+/H+ and Cl−/HCO3− exchange processes of the colon (39). Given an important role of DRA in intestinal luminal Cl−/HCO3− exchange process, it is critical to understand its regulation at the molecular and cellular level.

Previous studies indicated that DRA function is acutely regulated at the protein level (8, 13, 18). Interestingly, DRA expression is found to be highly abundant in the colon compared with the small intestine, indicating that it might also be subject to transcriptional regulation (29, 46). Indeed, DRA mRNA levels in the colon were shown to be reduced in animal models of inflammatory bowel disease. With respect to patients with ulcerative colitis, although Lohi et al. (41) found no changes in DRA expression, other reports, however, showed a decreased DRA expression in actively inflamed colonic epithelia (25, 68). Furthermore, previous studies demonstrated that human DRA expression is downregulated by the cytokine IL-1β at the transcriptional level (68). To date, however, nothing is known about molecular mechanisms involved in the regulation of DRA promoter activity. Therefore, the present studies were undertaken to clone human DRA pro-
moter region and to identify cis-elements and transcription factors involved in its regulation.

Our studies identified a DRA promoter region that is sufficient to drive its intestinal-specific expression both in vitro cellular models and in vivo transgenic mice. Within this core promoter, hepatic nuclear factor-4 is involved in driving basal promoter activity in LS174T cells whereas Yin Yang (YY1) and GATA zinc finger transcription factors are involved in butyrate-induced DRA promoter activity. Also, DRA promoter activity is reduced by IFN-γ, indicating its involvement in the regulation of its expression by cytokines.

MATERIALS AND METHODS

Cell Culture

All animal studies were performed in accordance with an animal protocol approved by IACUC at the University of Pennsylvania. LS174T and Caco-2 cells (ATCC) were plated in 10-cm dishes containing DMEM supplemented with 10 or 20% fetal bovine serum for LS174T and Caco-2 cells, respectively, as recommended by the supplier.

Northern Blots and Primer Extension Analysis

Total RNA for Northern blots were isolated by the guanidinium thiocyanate-CsCl gradient method. Ten micrograms of total RNA for each sample were electrophoretically separated, transferred to a nylon membrane, and hybridized to probes for either DRA, human growth hormone (hGH), or 7S as previously described (45).

Primer extension analysis was performed essentially as previously described (45). The oligonucleotide primer named DRA +77 (5'-GACACATACTCTGTGAGG-3') was end labeled with [γ-32P]ATP and incubated with total RNA isolated from normal human colon or postconfluent Caco-2 cells at 50°C for 3 h in a solution containing 1.5 M NaCl, 50 mM Tris-HCl (pH 7.5), and 10 mM EDTA. After reverse transcription, the RNA was hydrolyzed with 0.1 M NaOH, the DNA precipitated with ethanol, and the product separated in an 8% polyacrylamide urea gel. Controls included labeled oligonucleotide primer alone and Escherichia coli transfer RNA.

Isolation of DRA Promoter

A 290-bp fragment of the human cDNA for DRA beginning at the ATG translational initiation codon was isolated by Not I/Sal I restriction digest of the previously described cDNA clone. This cDNA was then sent to Research Genetics (Huntsville, AL) for hybridization screening of a human bacterial artificial chromosome (BAC) clones that contained each containing a 100- to 150-kb DNA insert.

5'-RACE “anchored” PCR was performed as previously described (67) to isolate the 5’ transcribed/untranslated region of the DRA cDNA. Total RNA isolated from postconfluent Caco-2 cells was reverse transcribed by random hexamer oligonucleotides. After poly G-tailing and purification, the DRA product was amplified by PCR using a poly C-tailed primer named SI-CCC previously described (67) and a primer specific for the human DRA cDNA called DRA+191 (5'-TACTGATTCCAAAGGGTTCC-3'). A single 250-bp product was amplified, isolated, and cloned into a TA cloning vector (Invitrogen). After verification of the identity of the PCR product by dideoxy sequencing, the anchored PCR product was used as a Southern probe to identify a restriction fragment of the human BAC clone that contained the promoter region of the DRA gene. In this fashion, a single 4-kb Hind III restriction fragment was isolated and cloned into pKS (vector) to give plasmid pBAC-4kb. Comparison of the anchored PCR product sequence to that of the pBAC-4kb tentatively identified the start of transcription for the DRA gene 400 bp from the 3’ end of the genomic clone, which demonstrated that pBAC-4kb contained 3765 bp of the promoter region for the human DRA gene.

RT-PCR and Real-Time PCR Analysis

RT-PCR was performed essentially as described (3). Briefly, 2–4 μg of total RNA was used for reverse transcription with sequence-specific primers and SuperScript II reverse transcriptase ( Gibco-BRL, Gaithersburg, MD). The reaction was carried out in a 20 μl reaction containing 100 mM Tris-HCl pH 8.3, 2.5 mM MgCl2, 10 mM DTT, 50 mM KCl, 1 μM dNTPs, 10 μM of antisense primer, and 1 μl of SuperScript II reverse transcriptase and incubated at 42°C for 1 h. The reaction was terminated by heating at 70°C for 15 min, and then 1 μl of RNAse H was added to the reaction and incubated for 20 min at 37°C.

Two microliters of the RT reaction were diluted into a final volume of 50 μl of a PCR mix containing 100 mM Tris-HCl (pH 8.3), 2.5 mM MgCl2, 50 mM KCl, 200 μM dNTPs, 1 μM each of antisense and sense primers, 0.5 μl of a mix of Taq DNA polymerase (Gibco-BRL) and Taq Pfu (Stratagene, La Jolla, CA) with the ratio of 16:1 (unit/unit). PCR reaction was carried out using a Microcycler programmable heating/cooling dry block (Perkin-Elmer, Norfolk, CT) for 30–40 cycles of amplification (94°C, 30 s; 58°C, 1 min; 72°C, 30 s) followed by 10 min at 72°C. PCR products were separated by electrophoresis on a 1% agarose gel containing ethidium bromide (0.5 μg/ml). Bands of expected sizes were visualized under UV light utilizing Eagle eye II Still Video System (Stratagene). The sequence of DRA primers used for RT-PCR reactions are forward, 5'-TCTGGAATCATCAGTGTGGC-3'; reverse, 5'-ATACACCTGTGAATCACGACG-3'.

For real-time quantitative RT-PCR, total RNA was prepared from Caco-2 cells treated with IFN-γ as indicated above utilizing Absolutely RNA RT-PCR Miniprep Kit (Stratagene) according to the manufacturer’s instructions. Equal amounts of RNA from both treated and control samples were reverse transcribed and amplified in one-step reaction utilizing Brilliant SYBR Green QRT-PCR Master Mix Kit (Stratagene). Real-time PCR was performed utilizing Mx3000 (Stratagene). DRA was amplified with gene-specific primers (sense primer, 5'-TTCAATGGTACGCTGCTAC-3'; antisense primer, 5'-ATGGTGGTGTGCTGCTGCTG-3'). β-Actin was amplified as an internal control utilizing gene-specific primers (sense primer, 5'-CATGTTTGAGAATCCTGAAAC-3'; antisense primer, 5'-CCAGGAAGCTCAGCAAA-3'). Since the amplification efficiencies for both DRA and β-actin were approximately equal, the quantitation was expressed as a ratio 2ΔCt-DRA/2ΔCt-β-actin, where ΔCt-NPC1L1 and ΔCt-β-actin represent the difference between the threshold cycle of amplification of treated and control RNA for NPC1L1 and β-actin, respectively.

DNase I Footprinting

−688(DRA)LUC was digested with Hind III and end labeled with 33P by using Klenow. After releasing the probe by digestion with Smal I, the probe was gel purified, mixed with various concentrations of bovine serum albumin (BSA) or nuclear proteins isolated from LS174T cells (Control) or from cells treated with butyrate (2.5 mM for 24 h) and digested with RNase-free DNase I (BMB) for 90 s at room temperature. After termination of the reaction, the products were precipitated and electrophoresed on a 6% acrylamide gel along with a sequencing ladder (not shown). The details of the methods used for this assay have been described previously (61).

Electrophoretic Mobility Shift Assays

Nuclear proteins were isolated from LS174T of the method of Dignam et al. (11). The protein concentrations of the extracts were determined by the methods of Bradford and aliquots were stored at −80°C. Complementary oligonucleotides with overlapping ends spanning DNA regions identified by DNase I footprinting (FP1, FP2) were synthesized, annealed, and labeled with 33P by using Klenow

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enzyme. Binding reactions, each containing 10 µg of nuclear proteins, were performed as previously described (66) and subsequently separated on a 4% polyacrylamide gel. The sequences of the oligonucleotides used for EMSA are shown below (sense strand only); lower case “m” denotes an oligonucleotide with a site-directed mutation (underlined): FP-1, 5′-CCTCAAGTCTCAAGTGACACCCAACTA- TGAGCT-3′; mFP-1, 5′-TCAAGTCTCAAGTGACACCCAACTA- TGAGCT-3′; FP-2, 5′-CTAGGCAGGTTATGTTGCTTCTTAGAGCT-3′; mFP-2, 5′-TAGGCAGGTTATGTTGCTTCTTAGAGCT-3′. The morulae were transferred to the uteri of pseudopregnant female mice. Tail DNA was fractionated by formaldehyde-agarose gel electrophoresis and fractionated by formaldehyde-agarose gel electrophoresis and blotting onto Magnacharge nylon membrane by standard capillary transfer methods. Ugn cDNA or transgene-specific SV40 poly(A) sequence was radioabeled with [α-32P]-dATP by use of the Random Primer Labeling system. Blots were visualized and quantitated with a Molecular Dynamics PhosphorIager system.

Statistical Analysis

Results are expressed as means ± SE. Student’s t-test was utilized for statistical analysis. A P value of 0.05 or less was considered statistically significant.

RESULTS

Cloning of DRA Promoter and Identification of Transcription Initiation Site of DRA Gene

To delineate the molecular mechanisms involved in the regulation of DRA promoter activity, we cloned the promoter by isolating a 4-kb fragment of DRA gene through hybridization screening of human BAC genomic library as described in MATERIALS AND METHODS. Sequence analysis confirmed that the isolated clone contained 3765 bp of the 5′ regulatory region of DRA gene identical to a previously published sequence (24).
Although genomic structure of DRA has been previously established, the identity of its transcription initiation site remains uncertain (24). We therefore identified the DRA transcription initiation site by primer extension analysis utilizing total RNA isolated from normal human colon and from human intestinal Caco-2 cells. As shown in Fig. 1, a single 111-bp primer extension product was detected, indicating a similar site for the initiation of DRA transcription in normal human colon and Caco-2 cells. The transcription initiation site was further confirmed by 5′-RACE PCR utilizing RNA extracted from human intestinal Caco-2 cells and a reverse primer complementary to an area in the coding sequence of DRA cDNA. A restriction map of the 4 kb of DRA genomic clone is depicted and the transcription initiation site (denoted as +1) is also shown in Fig. 1.

**DRA mRNA Expression in Human Intestinal Cell Lines**

Promoter analysis depends on transient transfection of promoter constructs into preconfluent cells. To establish a suitable in vitro cellular model for DRA promoter analysis, we first examined DRA expression in preconfluent LS174T and Caco-2 human colonic cell lines. As shown in Fig. 2A, DRA is expressed in preconfluent LS174T and Caco-2 cells as determined by RT-PCR. However, these preconfluent colonic cell lines demonstrated low level of DRA expression since its mRNA was undetectable by Northern blotting (Fig. 2B). In this regard, sodium butyrate has been shown to induce differentiation and gene expression in colonic epithelial cells (10, 53). Therefore, we examined whether sodium butyrate also induced DRA expression in preconfluent LS174T and Caco-2 cells. As depicted in Fig. 2B, 24 h incubation with sodium butyrate in preconfluent LS174T but not in preconfluent Caco-2 cells induced DRA expression in a dose-dependent manner with a maximum effect occurring at 2.5 mM concentration. Butyrate-treated LS174T have been previously shown to exhibit biochemical characteristics and phenotype similar to those of differentiated intestinal epithelial cells (23, 27, 53). Therefore, preconfluent butyrate-treated LS174T cells with high levels of DRA expression appear to represent an excellent model for DRA promoter analysis.

**DRA Promoter Analysis in LS174T Cells**

Several promoter constructs of DRA gene representing progressive 5′ deletions cloned in pGL2 luciferase reporter vector were transiently transfected into LS174T cells. Twenty-four hours later, cells were treated with 2.5 mM sodium butyrate for an additional 24 h, or with vehicle, and DRA promoter activity was assessed by luciferase assay. As shown in Fig. 3, the −3765(DRA)LUC promoter construct was significantly active with about sixfold more luciferase activity over the empty vector. Progressive 5′ deletions to −398 bp did not alter DRA promoter activity. However, basal activity of the promoter was significantly reduced by −50% in the −102(DRA)LUC construct, indicating that the region between −398 and −102 harbors cis-elements responsible for maximal basal activity of DRA promoter. Also, −3765(DRA)LUC exhibited no significant promoter activity in nonintestinal cell lines HepG2 and NIH 3T3 (data not shown). These findings demonstrate that 3765 bp of the DRA promoter can direct intestinal cell line-specific transcription. Data depicted in Fig. 3 further show that sodium butyrate stimulated the activity of −3765(DRA)LUC and −688(DRA)LUC but not −398(DRA)LUC and −102(DRA)LUC DRA promoter constructs, indicating that butyrate response elements are located in the region between −688 and −398 bp of DRA promoter. Incubation with sodium butyrate had no effect on the activity of CMV-β galactosidase expression vector used in these experiments as a control to normalize for transfection efficiency (data not shown).

**Analysis of DNA-Protein Interactions in the DRA Promoter**

**Mapping of DNA-protein interactions.** To further identify putative cis-elements and transcription factors involved in the basal activity as well as the stimulation of DRA promoter by butyrate, we utilized DNase I footprint analysis with nuclear extracts from wild-type LS174T cells or cells treated with sodium butyrate. As shown in Fig. 4A, a DNase I-protected region (referred to as FP1) was identified when a 32P-radioactive-labeled probe, representing a fragment of DRA promoter flanking between base pairs −118 to −114, was incubated with increasing amounts of LS174T nuclear extracts from both control or butyrate-treated cells. The same region was not identified when the probe was incubated with same amount of BSA as a negative control, indicating the specificity of the results. Sequence analysis of this region indicated the presence of a potential binding site for the hepatic nuclear factor-4 (HNF-4) transcription factor. Since the protected fragment is located in the region responsible for the maximal activity of DRA promoter, these observations implicated the involvement
of HNF-4 in driving the basal activity of DRA promoter in LS174T human colonic cells.

To determine DNA-protein interactions in the DRA promoter region that elicit the effect of butyrate, we performed DNase I footprint analysis with a probe flanking the area between $-688$ and $-398$ bp of DRA promoter. As depicted in Fig. 4B, a protected fragment was identified (designated as FP2) when the probe was incubated with nuclear extracts from LS174T cells but not BSA. Sequence analysis of the FP2 fragment shows that it encompasses the region $-468$ to $-487$ bp where two overlapping potential binding sites for binding to YY1 and GATA zinc finger transcription factors were identified. These findings suggest the possible involvement of YY1 and GATA zinc finger transcription factors in butyrate-induced stimulation of DRA promoter activity.

Identification of transcription factors that bind DRA promoter. Binding of nuclear proteins to the footprinted fragments of DRA promoter were further confirmed by the EMSA utilizing FP1 and FP2 regions as probes. Shown in Fig. 5A, the incubation of 32P-labeled FP1 with nuclear extracts of LS174T cells resulted in a band indicating the binding of nuclear proteins to FP1 (lane 2). The presence of 100-fold excess of unlabeled probe abolished the binding (lane 3) indicating its specificity. Figure 5A also shows that 100-fold excess of...

Fig. 2. DRA mRNA expression in LS174T and Caco-2 cells. Total RNA was extracted from human colonic LS174T and Caco-2 cells. A: 2 μg of RNA were utilized for RT-PCR reaction using DRA gene-specific primers. PCR products were separated by electrophoresis on 1% agarose gel. The predicted size band of 355 bp is shown in the figure. B: Northern blot analysis for DRA using RNA isolated from cells treated with sodium butyrate for 24 h at the concentrations indicated. Stripping and reprobing these blots for 7S RNA was performed as a loading control.
mutated FP1 (lane 4) failed to effectively compete the shifted bands of FP1, demonstrating that such mutation is sufficient to block the binding of nuclear proteins. Additionally, supershift analysis with antibodies against HNF-4α transcription factor showed that binding to FP1 is effectively disrupted, indicating that HNF-4α binds to FP1 region of DRA promoter, as depicted in Fig. 5B.

The EMSA analysis utilizing FP2 fragment, as depicted in Fig. 5C, revealed two separate bands indicating the presence of two distinct DNA–protein complexes. Furthermore, competition with a YY1 consensus oligonucleotide (lane 5) led to a loss of the lower band, and incubation with two different antibodies specific for YY1 (lanes 8 and 9) resulted in a disruption of the binding. These findings demonstrate the binding of YY1 transcription factor with FP2 region of DRA promoter. Intriguingly, a 20-bp oligonucleotide sequence that silences expression of the Fabpl gene in crypt epithelial cells (57) also effectively competed the YY1 band (lane 6). The other DNA–protein complex appears to involve a binding of GATA zinc-finger transcription factor binding since the presence of GATA consensus sequence (lane 7 in Fig. 5C) completely abolished the upper band of the EMSA gel. Additionally, 100-fold competition with an oligonucleotide containing a 2-bp mutation in the YY1 and GATA site in FP2 (lane 4) failed to eliminate the binding of both GATA and YY1. It is interesting that YY1 binding increased when GATA binding was reduced by competition (lane 7) and that decreased GATA binding was observed when a YY1 consensus oligonucleotide was used as a competitor. These results suggest that YY1 and GATA may influence the binding of each other.

**Activation of the DRA Promoter by Sodium Butyrate is Dependent on GATA and YY1 Binding**

Both YY1 and GATA transcription factors demonstrate specific binding to partially overlapping sequences within a footprinted region in the butyrate response region of DRA promoter. To determine the functional significance of such binding on the stimulation of DRA promoter by butyrate, we generated a mutated −688(DRA-mYY1/mGATA)LUC construct of DRA promoter harboring the same 2-bp mutation that was sufficient to disrupt the binding of both YY1 and GATA transcription factors (Fig. 5C, lane 4). As shown in Fig. 6, basal activity of mutated −688(DRA-mYY1/mGATA)LUC is similar to that of wild-type −688(DRA)LUC DRA promoter construct. However, treatment with sodium butyrate failed to stimulate the activity of −688(DRA-mYY1/mGATA)LUC, clearly indicating that the binding of YY1 and GATA transcription factors is essential for butyrate-induced activation of DRA promoter.

**Regulation of DRA Expression and Promoter Activity by IFN-γ**

Since DRA expression was shown to be altered in intestinal inflammation, we examined whether DRA promoter activity is also regulated by inflammatory mediators such as IFN-γ. For these studies, we utilized Caco-2 cells because they have been shown to be a suitable model to study the effect of cytokines on intestinal epithelial cells (16, 65). Although DRA promoter was modestly active in transiently transfected subconfluent Caco-2 cells, however, its activity, as depicted in Fig. 7A, was significantly higher than empty vector. Similar to the findings in LS174T cells, deleting the fragment between −398 and −102 bp caused a significant reduction in DRA promoter activity in Caco-2 cells. These results further support the observation that the area between −398 and −102 harbors cis-elements essential for maximal basal activity of the DRA promoter. To assess the effects of IFN-γ, Caco-2 cells plated on permeable supports were treated from the basolateral side with IFN-γ (30 ng/ml) for 24 h and the expression of DRA was evaluated. As shown in Fig. 7B, the relative abundance of DRA mRNA was significantly reduced by IFN-γ as assessed by real-time RT-PCR using β-actin as an internal control. Figure 7C shows that IFN-γ treatment results in parallel decrease in the promoter activity of DRA gene. Collectively, these findings indicate that the decrease in DRA expression by IFN-γ cytokine occurs via a reduction in its promoter activity.
DRA Promoter-Driven Expression of hGH and Ugn in Transgenic Mice is Restricted to the Differentiated Epithelium of Both Small Intestine and Colon

Previous studies have shown DRA to be predominantly expressed in small intestine and colon (29, 46). To further investigate the molecular elements responsible for DRA tissue-specific expression, and to establish an in vivo model to study the regulation of DRA promoter activity, we generated transgenic mice in which the expression of hGH is driven by the DRA promoter. A hGH reporter construct was created by subcloning the fragment of DRA promoter containing the region between base pairs −3765 and −12 into the pØGH vector. The expression of hGH along the gastrointestinal tract is shown in Figure 5.

Fig. 5. Gel shift analysis of DRA promoter.

A: nuclear extracts from LS174T cells were incubated with 32P-labeled FP1 double-stranded oligonucleotide representing the DRA promoter. Protein-DNA complexes were competed with excess of unlabeled probes (lanes 3, 4). Lane 1: probe alone. Lane 2: probe with nuclear extract. Lane 3: competition with 100-fold excess of unlabeled oligonucleotide. Lane 4: competition with 100-fold excess of mutated oligonucleotide.

B: nuclear extracts were incubated with 32P-labeled FP1 in the presence (lane 4) or the absence (lane 2) of anti-HNF-4 antibodies. The complexes were competed by excess of unlabeled probes (lanes 3).

C: nuclear extracts were incubated with labeled FP2 double-stranded oligonucleotide. Lane 1: probe alone. Lane 2: probe with nuclear extract. Lane 3: competition with 100-fold excess of unlabeled oligonucleotide. Lane 4: competition with 100-fold excess of unlabeled mutated oligonucleotide. Lane 5: competition with 100-fold excess of Yin Yang 1 (YY1) consensus sequence. Lane 6: competition with 100-fold excess of GATA consensus sequence. Lane 7: extracts were incubated in the presence of an antibody against the COOH terminal of YY1. Lane 8: extracts were incubated in the presence of an antibody against the NH2 terminal of YY1.

Fig. 6. Disruption of YY1 and GATA binding blocks the activation of DRA promoter by butyrate. LS174T cells were transiently transfected with either −688 (DRA) promoter construct of DRA promoter along with pCMV β-galactosidase mammalian expression vector. After 24 h, cells were treated with 2.5 mM butyrate or left untreated (control) for additional 24 h. Promoter activity was then measured by luciferase assay and the values were normalized to β-galactosidase to adjust for the transfection efficiency. Data are shown as mean ± SE from at least 3 experiments performed at 3 separate occasions. Results are expressed as % of control compared with cells treated with vehicle alone. *P < 0.05 compared with control.

Transcriptional Regulation of Intestinal Anion Transporter

DRA Promoter-Driven Expression of hGH and Ugn in Transgenic Mice is Restricted to the Differentiated Epithelium of Both Small Intestine and Colon
was assessed by Northern blot analysis (Fig. 8) from F1 offspring of a female founder. Similar results have been observed with the analysis of a second founder line (not shown). As shown in Fig. 8A, the expression of hGH transgene mRNA was detected at significant levels only in the small and large intestine (Northern blot, 1-h exposure). hGH was undetectable in other tissues (liver, lung, heart, spleen, kidney, testis, skeletal muscle, skin, brain, fat, pancreas), except for testis, where hGH mRNA was barely detectable after prolonged exposure (data not shown). The intestinal expression of endogenous DRA mRNA is also shown in Fig. 7 (18-h exposure). As depicted in Fig. 8A, the hGH transgene shows higher levels of expression in the small intestine compared with endogenous DRA. Also, the hGH mRNA was not detected in the cecum, where the endogenous DRA mRNA is in high abundance. These findings indicate that the −3765/+12 promoter fragment lacks cis-elements responsible for activating DRA gene expression in the cecum and cis-elements involved in attenuating its expression in the small intestine. This same promoter region also directed intestine-specific transgene expression in both the small intestine and distal colon, but not the proximal colon or cecum, when the Ugn cDNA was used as the reporter in transgenic mice (Fig. 8, B and C). As was the case with the hGH transgene, transgenically expressed Ugn was not expressed in nonintestinal tissues (data not shown). Although it is possible that the variability in hGH and Ugn transgene expression in the colon may be due to copy number-dependent and/or insertion site-dependent effects, the Ugn transgenic animals confirm that the core DRA promoter is capable of directing high level intestine-specific gene expression that is reporter gene independent.

Immunohistochemical staining of the intestinal tissues depicted in Fig. 9 showed the hGH transgene to be strictly expressed in the differentiated villus epithelium in the small intestine (A: ×100; B: ×200) and the differentiated upper crypt and surface epithelium in the colon (C: ×100; D: ×200). Collectively, these findings show that 3765 bp of the DRA promoter region contains cis-acting elements capable of activating intestine-specific and colonic gene expression.

**DISCUSSION**

The regulation of DRA gene represents a valuable model system to examine the physiological consequences of gene expression in differentiated intestinal epithelial cells due to its abundant expression primarily in the colonic epithelium and its critical role in intestinal chloride absorption (13, 29, 46, 48). DRA expression has been shown to be minimal or absent in adenocarcinoma and several colon cancer cell lines (56, 68). In the present study, the use of butyrate to induce DRA gene expression specifically in LS174T colon cancer cell line, therefore, represents a unique in vitro cellular model to investigate transcriptional activation of the DRA gene. These studies demonstrate that DRA promoter is regulated at two levels:

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**Fig. 7.** Modulation of DRA expression and promoter activity by IFN-γ. **A:** Caco-2 cells were transiently cotransfected with the indicated DRA promoter constructs along with mammalian expression vector for β-galactosidase (pCMVβ). After 48 h, cells were harvested and the promoter activity was measured by luciferase assay. Values were normalized to β-galactosidase activity to adjust for the transfection efficiency. Data were obtained from 3 different experiments and are shown as means ± SE. Results are expressed as light units/β-galactosidase. *P < 0.05 compared with control. **B:** Caco-2 cells were plated on permeable support of Transwell inserts and incubated for 24 h with 30 ng/ml of IFN-γ that was added to the basal compartment of the insert. Total RNA was extracted and real-time RT-PCR was performed utilizing SYBR Green fluorescent dye as described in MATERIALS AND METHODS. The level of DRA mRNA was calculated based on the ΔCt method and normalized to level of human β-actin mRNA. Data are expressed as % of control and represent means ± SE of values obtained from 3 different experiments. *P < 0.05 compared with control. **C:** Caco-2 cells were plated on Transwell inserts and transiently transfected with −1300(DRA)LUC construct along with pCMVβ mammalian expression vector. The second day, IFN-γ was added to the basal compartment at final concentration of 30 ng/ml for 24 h. Promoter activity was then assessed by luciferase assay and the values were normalized to β-galactosidase to adjust for the transfection efficiency. Data are presented as means ± SE from at least 3 different experiments. Results are expressed as % of control. *P < 0.05 compared with control.
basal level of transcription as well as transcriptional regulation by butyrate and IFN-γ.

The basal activity of DRA promoter construct containing 3765 nucleotides upstream of transcription initiation site was similar to the construct containing only 398 nucleotides of the promoter region. However, a 50% decrease in the basal activity was observed in a construct harboring 102 nucleotide of DRA promoter, indicating that the basal level of DRA transcription is conferred by a fragment flanking the area between −398 bp and −102 bp of promoter region of DRA gene. In part, our findings showed that this is due to a cis-acting element capable of binding to HNF-4 transcription factor present in this region. HNF-4 is an orphan nuclear receptor that is highly expressed in liver, kidney, and intestine, where it regulates the expression of various genes such as apolipoprotein CIII and apolipoprotein AIV (4, 58, 70). The transcriptional activity of HNF-4 has been shown to be modulated by intracellular signaling molecules such as AMP-activated protein kinase (33). It is plausible, therefore, to speculate that the binding of HNF4 to DRA promoter is essential for its basal activity and may also mediate the regulation of DRA expression by the activated second messenger.

In contrast to activation of basal transcription, deletional analysis of the DRA promoter region also identified a 290-bp region between −398 and −688 that is necessary for high-level transcriptional activation induced by sodium butyrate. The response element for butyrate was mapped to a region on DRA promoter that contains overlapping consensus sequences for two zinc finger transcription factors, YY1 and GATA. YY1 is a widely expressed GLI-Krüppel-type zinc finger transcription factor that can act as a transcriptional repressor, an activator, or an initiator element (22). YY1 has been shown to modulate the expression of genes that are targets to cytokines or stress response and genes that are associated with differentiation (22). Interestingly, DRA expression has been shown to be modulated by both cytokines and differentiation status of intestinal epithelial cells (68). Since butyrate induces differentiation in colonic epithelial cells (27), it is not surprising, therefore, that butyrate-induced activation of DRA promoter is mediated by YY1 transcription factors. Interestingly, a 20-bp fragment from the promoter region of fatty acid binding protein fabp1 gene, which has been previously shown to be responsible for silencing its expression in the crypt epithelium of the small intestine (57), effectively competed for YY1 binding in the DRA promoter. In view of these observations, it is possible to hypothesize that YY1 may be critical for silencing the expression of intestinal genes such as DRA and fabp1 in proliferating undifferentiated intestinal epithelial cell and may play, therefore, a critical role in generating their expression pattern along the crypt-surface axis. GATA transcription factors have also been shown to be involved in determining the spatial and temporal expression pattern of several intestinal genes including liver fatty acid binding protein (L-Fabp) and lactase-phlorizin hydrolase (LPH) (12, 64). A family of GATA transcription factors have been described consisting of at least six members, of which GATA-4, -5, and -6 are expressed in the intestine (14). GATA-5 and -6 are expressed throughout the gastrointestinal tract (14). In this regard, GATA-5 transcription factor was shown to modulate the promoter activity of rat NHE3 and suggested to play a critical role in maintaining its differential expression along the crypt-villus axis (36). The fact
that GATA is involved in butyrate-induced stimulation of DRA promoter activity also suggests a role for this transcription factor in inducing DRA expression in differentiated intestinal epithelial cells. Although the upstream mechanism(s) by which butyrate induces DRA expression specifically in LS174T cells remain to be elucidated, one plausible mechanism may involve the ability of butyrate to inhibit histone deacetylases, leading to alterations in chromatin structure and subsequent activation of gene expression in a cell line-dependent fashion (50).

Along with the analysis of basal and butyrate-induced stimulation of DRA promoter activity, our data demonstrated that the inflammatory mediator IFN-γ also elicits its effect on DRA expression by modulating the promoter activity. Interestingly, IL-1β cytokine has been previously shown to modulate DRA expression at the level of gene transcription (68). Our present findings also show the modulation of DRA promoter activity by another cytokine, IFN-γ. Taken together, these observations suggest the importance of regulation or dysregulation of DRA promoter activity in the pathogenicity of intestinal inflammation underlying the development of diarrhea associated with intestinal inflammatory disorders.

To further elaborate on the findings of DRA promoter regulation in the in vitro models and further define their physiological significance, we developed transgenic mice in which the expression of hGH or Ugn transgenes were driven by human DRA promoter. Our initial studies demonstrated that a fragment containing 3765 bp of the DRA promoter recapitulated some of the features of its endogenous gene expression. These include intestine specificity, expression in the differentiated intestinal epithelium, and expression in the colonic epithelium. Surprisingly, human DRA-promoter driven transgene was expressed at the same levels in both the small intestine and the colon. Since DRA expression has been previously shown to be more abundant in human colon compared with small intestine (29, 46), it appears that elements responsible for suppressing DRA expression in the small intestine are lacking in the 3765-bp fragment of DRA promoter utilized in the transgenic mice. It should be noted that hGH transgenic mice were larger than wild-type mice, consistent with elevated circulatory levels of this hormone when it is ectopically expressed in the intestinal epithelium.

With respect to the mechanisms of intestinal gene expression, several transcription factors such as HNF-1, Cdx 1/2, and GATA have been shown to play critical roles in the expression of intestinal genes such as sucrase-isomaltase, LPH, and I-FABP (15). However, despite the fact that these transcription factors are expressed in both the small and the large intestine, the endogenously expressed sucrase-isomaltase and I-FABP genes and the expression of transgene constructs driven by the regulatory elements for these genes are not found in the epithelial cells of the colon (44, 60). In contrast to these previous transgenic models, the DRA promoter-driven transgenic mice represent a unique model in which the expression of the transgene is strictly targeted to the surface colonocytes and villus enterocytes.

In conclusion, our studies indicate that the transcriptional regulation of the DRA gene can be used as a model to characterize mechanisms that regulate colonic gene expression. Since DRA plays a critical role in the pathogenesis of a congenital diarrheal disease in humans and may also be involved in the pathogenesis of diarrhea observed in inflammatory states of the intestine, these studies may provide important information as to how DRA expression is altered in intestinal pathobiology.

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REFERENCES


29. Liedke C, Hopfer U. Mechanism of chloride translocation across small intestinal brush border membrane. II. Demonstration of Cl−/OH− ex-


43. Markowitz AJ, Wu GD, Birkenmeier EH, Traber PG. G934 TRANSCRIPTIONAL REGULATION OF INTESTINAL ANION TRANSPORTER


