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Characterization of the 5′-Flanking Region and Regulation of Expression of Human Anion Exchanger SLC26A6

Seema Saksena,[1*] Alka Dwivedi,[1] Amika Singla,[2] Ravinder K. Gill,[1] Sangeeta Tyagi,[1] Alip Borthakur,[1] Waddah A. Alrefai,[1] Krishnamurthy Ramaswamy,[1] and Pradeep K. Dudeja[1]

[1] *Section of Digestive Diseases and Nutrition, Department of Medicine, University of Illinois at Chicago and Jesse Brown VA Medical Center, Chicago, Illinois 60612*

[2] *Department of Physiology and Biophysics, University of Illinois at Chicago and Jesse Brown VA Medical Center, Chicago, Illinois 60612*

ABSTRACT

SLC26A6 (putative anion transporter 1, PAT1) has been shown to play an important role in mediating the luminal $Cl^-/OH^-(HCO_3^-)$ exchange process in the intestine. Very little is known about the molecular mechanisms involved in the transcriptional regulation of intestinal SLC26A6 gene expression in the intestine. Current studies were, therefore, designed to clone and characterize the 5′-regulatory region of the human SLC26A6 gene and determine the mechanisms involved in its regulation. A 1,120 bp (p−964/+156) SLC26A6 promoter fragment cloned upstream to the luciferase reporter gene in pGL2-basic exhibited high promoter activity when transfected in Caco2 cells. Progressive deletions of the 5′-flanking region demonstrated that −214/−44 region of the promoter harbors *cis*-acting elements important for maximal SLC26A6 promoter activity. Since, diarrhea associated with inflammatory bowel diseases is attributed to increased secretion of pro-inflammatory cytokines, we examined the effects of IFNγ (30 ng/ml, 24 h) on SLC26A6 function, expression and promoter activity. IFNγ decreased both SLC26A6 mRNA and function and repressed SLC26A6 promoter activity. Deletion analysis indicated that IFNγ response element is located between −414/−214 region and sequence analysis of this region revealed the presence of potential Interferon Stimulated Responsive Element (ISRE), a binding site (−318/−300 bp) for interferon regulatory factor-1 transcription factor (IRF-1). Mutations in the potential ISRE site abrogated the inhibitory effects of IFNγ. These studies provided novel evidence for the involvement of IRF-1 in the regulation of SLC26A6 gene expression by IFNγ in the human intestine. J. Cell. Biochem. 105: 454–466, 2008.

KEY WORDS: PUTATIVE ANION TRANSPORTER 1 (PAT1) PROMOTER; IRF-1; IFNγ; HUMAN INTESTINE AND CHLORIDE ABSORPTION

Electroneutral NaCl absorption across the apical membrane of epithelial cells in the human small intestine and colon occurs via operation of dual ion antiporters: Na^+/H^+ and Cl^-/HCO_3^- antiporters [Dudeja et al., 2003; Gill et al., 2003]. The existence of two independent transporters responsible for Na^+ and Cl^- absorption in the human intestine and colon is further supported by the presence of two different genetic disorders that selectively abolish Na^+ or Cl^- absorption: Congenital sodium diarrhea with impaired Na^+/H^+ exchange (NHE) activity and congenital chloride diarrhea that affects the Cl^-/HCO_3^- exchange process [Booth et al., 1985; Holmberg, 1985, 1986]. In this regard, extensive studies have recently elucidated the molecular mechanisms of NHE isoform expression and regulation in the human intestine [Gill et al., 2002, 2005; Malakooti et al., 2002, 2006; Hecht et al., 2004; Hodges et al., 2006], however, the molecular identity of the Cl^-/HCO_3^- exchangers and their regulation in the human intestine is slowly beginning to emerge. Given that Cl^-/HCO_3^- exchangers play an important role in transepithelial Cl^- absorption, HCO_3^- secretion and maintenance of intracellular pH and Cl^- concentrations, it is critical to understand their regulation at the molecular level in the intestine.

Recent studies have shown that the two members of the SLC26 gene family: SLC26A3 or down regulated in adenoma (DRA) and SLC26A6 or putative anion transporter 1 (PAT1) are involved in mediating luminal Cl^-/HCO_3^- exchange in the intestine [Mount and

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*Correspondence to: Dr. Seema Saksena, PhD, Research Assistant Professor, University of Illinois at Chicago, Jesse Brown VA Medical Center, 820 South Damen Avenue, Chicago, IL 60612. E-mail: saksena@uic.edu

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Romero, 2004]. The expression of DRA mRNA and protein is highly abundant in the human colon compared to the ileum [Hoglund et al., 1996; Gill et al., 2003]. PAT1 functions as Cl^-/HCO_3^- or Cl^-/OH^- exchanger and is expressed in the intestine [Mount and Romero, 2004]. Studies in the mouse intestine showed the expression of PAT1 in a pattern opposite to the DRA, that is, higher expression in the small intestine compared to colon [Wang et al., 2002]. Therefore, DRA was proposed as the apical Cl^-/HCO_3^- exchanger of the colon [Hoglund et al., 1996; Melvin et al., 1999; Moseley et al., 1999; Jacob et al., 2002; Chernova et al., 2003; Lamprecht et al., 2004], whereas PAT1 was considered as the major mediator of Cl^- absorption in the small intestine [Wang et al., 2002]. Hence this region specific expression indicates that the expression of DRA and PAT1 in the intestine must be under transcriptional regulation.

In this regard, previous studies on the transcriptional regulation of DRA have shown that the pro-inflammatory cytokine, IL1β decreased DRA mRNA expression in Caco2 cells [Yang et al., 1998]. Moreover, mRNA expression of DRA was found to be significantly reduced in patients with ulcerative colitis [Yang et al., 1998] and in two animal models of colitis, the IL-10 knock-out mouse [Kuhn et al., 1993] and the HLA-B27/β2m transgenic rat [Hammer et al., 1990]. Reduced DRA expression in these intestinal inflammation models were found to be consistent with the studies of Sundaram and West [1997] showing reduced chloride absorption in chronic inflamed ileum of rabbit. However, nothing is known about the regulation of PAT1 gene expression either under basal or inflammatory states. Diarrhea associated with inflammatory bowel diseases (IBD) has been attributed to enhanced secretion of high levels of the pro-inflammatory cytokines including interferon-γ (IFN-γ) [Indaram et al., 2000; Bouma and Strober, 2003]. IFN-γ has been shown to alter intestinal ion transport and barrier properties [Hawker et al., 1980; Madara and Stafford, 1989; Sandle et al., 1990; Bell et al., 1995; Youakim and Ahdieh, 1999]. It has been shown to decrease both NHE2 and NHE3 isoform expression and function in human intestinal C2/bbe cells and rat intestine [Rocha et al., 2001]. Previous studies have shown that IFN-γ was also involved in the down-regulation of apical CFTR Cl^- channel [Besancon et al., 1994], NKCC co-transporter [Fish et al., 1999] and Na-K-ATPase [Sugi et al., 2001]. However, the effect of IFNγ on PAT1 expression and the molecular mechanisms underlying the potential modulation of PAT1 by IFNγ have not been investigated.

Therefore, the present study was undertaken to clone and characterize the 5′-flanking region of the PAT1 gene and to investigate the effect of IFN-γ on the expression and promoter activity of intestinal PAT1. Our results demonstrated that the cloned 5′-flanking region of PAT1 gene is highly active in Caco2 cells and the region between −214 and −44 harbors *cis*-acting elements that are important for maximal basal promoter activity. Furthermore, we showed that IFN-γ resulted in a decrease in the expression of PAT1 mRNA and its promoter activity. Also, our data showed the involvement of interferon regulatory factor-1 (IRF-1) in the observed regulation of intestinal PAT1 gene expression by IFN-γ. These findings provided novel evidence for the involvement of IRF-1 in the regulation of intestinal chloride absorption in diarrheal disorders associated with inflammation.

MATERIALS AND METHODS

MATERIALS

Human recombinant IFNγ was obtained from Sigma (St. Louis, MO). JAK inhibitor I was obtained from Calbiochem (San Diego, CA). All restriction endonucleases and other modifying enzymes were obtained from New England Biolabs (Beverly, MA), Invitrogen (Carlsbad, CA) or Promega (Madison, WI). Polyclonal anti-human IRF-1 or normal rabbit IgG antibody, consensus and mutant oligonucleotides for IRF-1 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Luciferase assay system was procured from Promega. β-Galactosidase assay kit was obtained from BD Biosciences Clontech (Palo Alto, CA). γ^{32}P-ATP (3,000 ci/mmol) was from Amersham (Arlington Heights, IL).

CELL CULTURE

Caco-2 and human embryonic kidney (HEK)-293 cells were obtained from the American Type Culture Collection (ATCC) and were grown routinely in T-75-cm^2 plastic flasks in a 5% CO_2 atmosphere at 37°C. Cells were cultured in minimum essential medium (Eagle) with 2 mM L-glutamine and Earle's balanced salt solution adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM nonessential amino acids and 1.0 mM sodium pyruvate and supplemented with FBS (20% for Caco-2 cells and 10% for HEK-293 cells). Non-transformed human colonic epithelial cells, NCM460 were grown in M3:10^{TM} media (INCELL, San Antonio, TX) and were maintained in a 5% CO_2 at 37°C. Caco-2 cells were plated at a density of 1×10^5 cells/cm^2 on 12-well transwell collagen-coated inserts (permeable supports) and either transfected while still in suspension or left untransfected for RNA extraction. For promoter studies, 24 h post-transfection, cells were incubated with different doses of IFNγ (10, 30, and 50 ng/ml) at varying time points (6, 8, 12, and 24 h) in serum-reduced medium (1% FBS). For inhibitor studies, transfected cells were pre-treated with JAK inhibitor I (30 nM) for 1 h and then co-incubated with IFNγ (30 ng/ml) for another 24 h. For the uptake experiments, cells from passages between 20 and 25 were plated in 12-well transwell collagen-coated inserts at a density of 1×10^4 cells/ml. Confluent monolayers were then used for transport experiments at day 10th–12th post-plating. To ascertain the integrity of the cell monolayers grown on transwell collagen-coated inserts for transport studies, we measured transepithelial resistance (TER) in cell monolayers using an epithelial voltohmmeter (World Precision Instruments Inc., Sarasota, FL). Our data showed that TER did not change as the approximate value of TER in Caco2 cells is 300–350 $\Omega\,cm^2$ [DeMarco et al., 2003; Lynch et al., 2005]. Also in the presence of IFNγ, TER remained unchanged compared to control (control, $335 \pm 14\ \Omega\,cm^2$ vs. IFNγ $294 \pm 18\ \Omega\,cm^2$). To study the effect of IFNγ on Cl^-/OH^- exchange activity, cells were exposed to IFNγ (30 ng/ml) in serum-reduced media (1% FBS) for 24 h.

CLONING OF THE 5′-FLANKING REGION OF HUMAN PAT1 PROMOTER

The 5′-flanking region of PAT1 gene was cloned by PCR utilizing human genomic DNA, gene specific primers and the proof reading Elongase enzyme mix (Invitrogen) according to the manufacturer's instructions. The primer sequences (designed based on gene bank accession number AF279265) are:

5′ primer: GGGGTACCTGCCTTTTCCTGTTGGGGGCTGGGGA (Forward primer contains an internal site for *Kpn*I restriction enzyme which is underlined);
3′ primer: AAGAGCTCGCGCGGGGCCACCGGGAATGTGCGCT (Reverse primer contains an internal site for *Sst*I restriction enzyme which is underlined).

PCR amplified product was excised from 1% agarose gel and purified utilizing Sephaglas™ BandPrep Kit (Amersham Pharmacia Biotech, Piscataway, NJ). The amplified 5′-flanking region was then digested with *Kpn*I and *Sst*I enzymes and cloned into luciferase reporter gene vector, pGL-2 basic (Promega). The orientation and the sequence of the insert were determined by sequencing. This confirmed that the 1.12 kb fragment (p−964/+156) represented the 5′-flanking region of the PAT1 gene.

REPORTER PLASMID CONSTRUCTION

Plasmids used for functional analysis of the PAT1 promoter activity were generated using pGL2 basic vector (Promega, Madison, WI) that contains a promoter-less luciferase reporter gene. Four 5′-deletion constructs of p−714/+156, p−414/+156, p−214/+156, and p−44/+156 were generated by PCR amplification method. Four different forward primers contained an internal site for *Kpn*I restriction enzyme and their sequences are:

primer-1: 5′-GGGGTACCTTCCTAATTCTTCAGGGTCTGCAGCC-3′;
primer-2: 5′-GGGGTACCAGTCGCAGAAAGCCTGCGGGTGGCG-CT-3′;
primer-3: 5′-GGGGTACCCGCGGGTGCTTAGTTCCAGTTCTGCAG-3′; and
primer-4: 5′-GGGGTACCTTGGCCCAAGCCTCCCCGTCCTGCCC-3′.
The sequence of the reverse primer contained site for *Sst*I enzyme: 5′-AAGAGCTCGCGCGGGGCCACCGGGAATGTGCGCT -3′.

The amplifications were performed using proof reading Elongase enzyme mix (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. PCR products were then digested with *Kpn*I and *Sst*I enzymes and cloned into luciferase reporter gene vector, pGL-2 basic (Promega). The fidelity of the constructs were then confirmed by sequencing, and plasmids were prepared for transfection using a kit from Qiagen (Valencia, CA).

RNA EXTRACTION AND REAL-TIME PCR ANALYSIS

Total RNA was prepared from Caco-2 cells treated with 30 ng/ml of IFNγ for 24 h using Absolutely RNA RT-PCR Miniprep Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Equal amounts of RNA from both treated and control samples were reverse transcribed and amplified in a one-step reaction using Brilliant SYBR Green QRT-PCR Master Mix kit (Stratagene). Real-time PCR was performed using Mxp3000 (Stratagene). PAT1 was amplified with gene-specific primers (sense primer: 5′-AGATGCCCCACTACTCTGTCCT-3′; antisense primer: 5′-ATCCACACCACACCTCTGCTT-3′). Histone was amplified as an internal control using gene-specific primers (sense primer: 5′-ACCGACCTTCGTTTCCAGAG-3′; antisense primer: 5′-CTTGGCG-TGAATAGCACAGA-3′). Because the amplification efficiencies for both PAT1 and histone were shown to be approximately equal, the quantitation was expressed as a ratio of $2^{\Delta C_T} - \text{PAT1}/2^{\Delta C_T} -$ histone, where $\Delta C_T - \text{PAT1}$ and $\Delta C_T -$ histone represent the differences between the threshold cycles (C_T) of amplification of treated and control RNA for PAT1 and histone, respectively.

CL^-/OH^- EXCHANGE ACTIVITY

Cl^-/OH^- exchange activity was determined by measuring DIDS-sensitive $^{36}Cl^-$ uptake in base loaded cells as previously described by Olsnes et al. [1987] and further established by us with some modifications [Alrefai et al., 2001; Saksena et al., 2005]. Cl^-/OH^- exchange activity (DIDS-sensitive $^{36}Cl^-$ uptake) was expressed as nmol/mg protein/5 min.pt?>

SITE-DIRECTED MUTAGENESIS

Site-directed mutations corresponding to the critical potential binding site (ISRE) for transcription factor IRF-1 (−324 to −293 bp) was made using the QuickChange Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions and confirmed by sequencing. The Bold letters indicate the mutations. The mutant oligonucleotide used is:

ISRE mutant: 5′-CGGCCAGTGGAGGGTCCTGGTCCCACTCCG-TG-3′!

TRANSIENT TRANSFECTION AND LUCIFERASE ASSAYS

For transfection studies, Caco-2 cells (1.0×10^5) were seeded on 12 well transwell collagen-coated inserts (permeable supports) and transfected while still in suspension with one of the PAT1 promoter-luciferase constructs and pCMVβ, (β-galactosidase mammalian expression vector, BD Biosciences Clontech, Palo Alto, CA) using Lipofectamine 2000 reagent (Invitrogen, CA). The latter plasmid served as an internal control for transfection efficiency. A total of 3 μg DNA/well at a ratio of 5:1 for experimental versus pCMVβ was used for each transfection. After 48 h, cells were washed with 1× PBS and cell lysates prepared in Reporter Lysis Buffer (Promega). The activities of both firefly luciferase and β-galactosidase were measured by luminometer according to the manufacturer's instruction using kits from Promega and Clontech, respectively. The promoter activity was expressed as a ratio of luciferase to β-galactosidase activity (Relative Luciferase Activity) in each sample.

NUCLEAR EXTRACTS AND ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

Nuclear extracts from control or IFNγ (30 ng/ml) treated Caco2 cells were prepared as previously described [Hadjiagapiou et al., 2005]. The sequence of the top strand of the probe containing ISRE motif (shown in bold) 5′-**AGTGGAGTTTCCTTTTCCCACT**-3′ spanned from nucleotides −318 to −298. ISRE double-stranded oligonucleotide was end-labeled with T4-polynucleotide kinase and γ^{32}P-ATP (Amersham, IL). DNA/protein binding reactions were performed as previously described [Malakooti et al., 2002, 2006].

CHROMATIN IMMUNOPRECIPITATION (CHIP)

ChIP assays were performed using the ChIP-IT Express kit from Active Motif (Carlsbad, CA) according to the manufacturer's instructions. Briefly, untreated or IFNγ treated confluent Caco2 cells (grown in 6-well transwell collagen-coated inserts) were cross-linked with 1% formaldehyde for 10 min at room temperature (21–23°C). Chromatin fragments were isolated from nuclei by enzymatic shearing (200–800 bp). Shearing efficiency was confirmed by gel electrophoresis and protein concentration of the sheared chromatin lysate was determined by the method of Bradford [Bradford, 1976]. Five hundred micrograms each of the sheared chromatin protein (untreated and IFNγ-treated) was used for immunoprecipitation with 3 μg of IRF-1 or normal rabbit IgG antibody and 25 μl of Protein G magnetic beads. A no antibody control was also used. Immunoprecipitation was carried out for 4 h on a rotator at 4°C. Magnetic beads along with the co-precipitated immuno-complexes were collected, washed and resuspended in 100 μl of elution buffer. Cross-linking of DNA proteins was reversed by incubation at 65°C in the presence of NaCl and proteins were removed by proteinase K treatment at 42°C. Ten microliters aliquot of the sheared chromatin was used for input DNA preparation. Immunoprecipitated DNA samples were purified (Qiagen) and used for real time PCR amplification.

The resulting purified DNA fragments (5 μl) were subjected to real time PCR for the quantification of association of IRF-1 with the PAT1 promoter in untreated and IFNγ treated samples using primers (ISRE) flanking the potential ISRE binding sequence (forward: 5′-GAGATGCGTAGTCGCAGAAA-3′ and reverse: 5′-CCCTGAG-TAGGTTGGAGCTG-3′) and primers (non-ISRE) that do not contain the ISRE site and are 0.4 kb away from this site (forward: 5′-GGAGGTAGGATGGAAGCTGT-3′ and reverse: 5′-ATCAGGCCC-CAATTACAAAG-3′). The quantification of IRF-1 protein binding to the promoter region was expressed as ratio of $2^{\Delta C_T}_{(\text{untreated})}/2^{\Delta C_T}_{(\text{IFN}\gamma)}$ as previously described [Kotlo et al., 2007; Li and Chiang, 2007]. ΔC_T was obtained by subtracting the threshold cycles (C_T) of IRF-1 DNA immunoprecipitates amplified from untreated and IFNγ-treated samples using non-ISRE primers (away from ISRE site) from the same corresponding samples using ISRE primers flanking the ISRE site (−318/−300 bp). The relative binding association was expressed as Fold Increase in arbitrary unit with control set as "1."

STATISTICAL ANALYSIS

Results are expressed as means ± SE. Student's *t*-test was used in statistical analysis. $P < 0.05$ or less was considered statistically significant.

RESULTS

PAT1 PROMOTER IS HIGHLY ACTIVE IN CACO2 CELLS

The 1,120 bp promoter fragment (−964/+156, where +1 is the transcription initiation site) was cloned in pGL2 basic, upstream from the luciferase reporter gene. Nucleotide sequence of the promoter fragment shown in Figure 1A was screened for potentially important *cis* elements. A number of putative binding sites for various transcription factors are present immediately upstream of the transcription initiation site. Functional analysis of PAT1 promoter was performed in Caco-2 cells. PAT1 promoter construct was transiently co-transfected into intestinal Caco2 cells along with the β-galactosidase mammalian expression vector as an internal control to measure transfection efficiency. As shown in Figure 1B, PAT1 promoter was highly active (>400-fold) in Caco2 cells when compared with cells transfected with the pGL2 basic empty vector alone. When the promoter activity was assessed in different human cell lines, such as kidney HEK293 and colonic NCM460 cells, all the cell lines showed increased activity compared with the empty vector, however highest activity was observed in the intestinal Caco2 cells followed by NCM460 and HEK293 cells.

PAT1 PROMOTER ANALYSIS IN CACO2 CELLS

In an effort to determine the regions that play an important role in the promoter activity, a number of consecutive deletions were generated at the 5′-end of the PAT1 promoter. The newly generated fragments were placed upstream from the luciferase reporter gene in pGL2-basic and transiently transfected into Caco-2 cells. As shown in Figure 2, the deletion constructs, p−714/+156, p−414/+156, and p−214/+156 showed promoter activity similar to the full-length PAT1 promoter construct, p−964/+156. However, deletion of ∼150 bases (p−44/+156) drastically decreased PAT1 promoter activity compared to p−964/+156. These results suggested that the basal regulatory elements important for maximal promoter activity are present between −214 and −44 bp region.

IFNγ INHIBITS PAT1 EXPRESSION AND PROMOTER ACTIVITY

Previous studies have shown that IFNγ down-regulated the expression of both NHE2 and NHE3 in C2BBe1 cells [Rocha et al., 2001]. Also recently, we have shown that IFNγ repressed NHE3 promoter activity in C2BBe1 cells [Amin et al., 2006]. Since the apical Cl^-/HCO_3^- exchanger, PAT1 is predominantly expressed in the small intestine [Wang et al., 2002] and its coupling with NHE3 maybe involved in ileal NaCl absorption, we hypothesized that similar to NHE3, IFNγ may also attenuate PAT1 expression and promoter activity. We first examined the effect of various doses of IFNγ on PAT1 promoter activity in Caco2 cells. As shown in Figure 3A, IFNγ significantly decreased PAT1 promoter activity at all doses, but the inhibition was more pronounced at 30 and 50 ng/ml (∼50%). Hence, 30 ng/ml was used in all subsequent experiments. The time course showed that IFNγ was not effective until 8 h, but a significant decrease was observed after 12 and 24 h (Fig. 3B). These results provided further evidence that IFNγ regulates PAT1 at the transcriptional level. Subsequently the effect of IFNγ was also examined on PAT1 mRNA level utilizing real-time PCR. Caco2 cells were treated with 30 ng/ml of IFNγ for 24 h in serum-reduced medium. Real time PCR was carried out using total RNA extracted from control and IFNγ-treated Caco2 cells and PAT1 specific primers. As shown in Figure 4, IFNγ (30 ng/ml, 24 h) significantly decreased the relative abundance of PAT1 mRNA by ∼50% as compared to control.

IDENTIFICATION OF THE IFNγ-RESPONSIVE REGION IN PAT1 PROMOTER

To determine which region of PAT1 promoter is responsible for IFNγ-mediated inhibition of PAT1 promoter activity, a series of

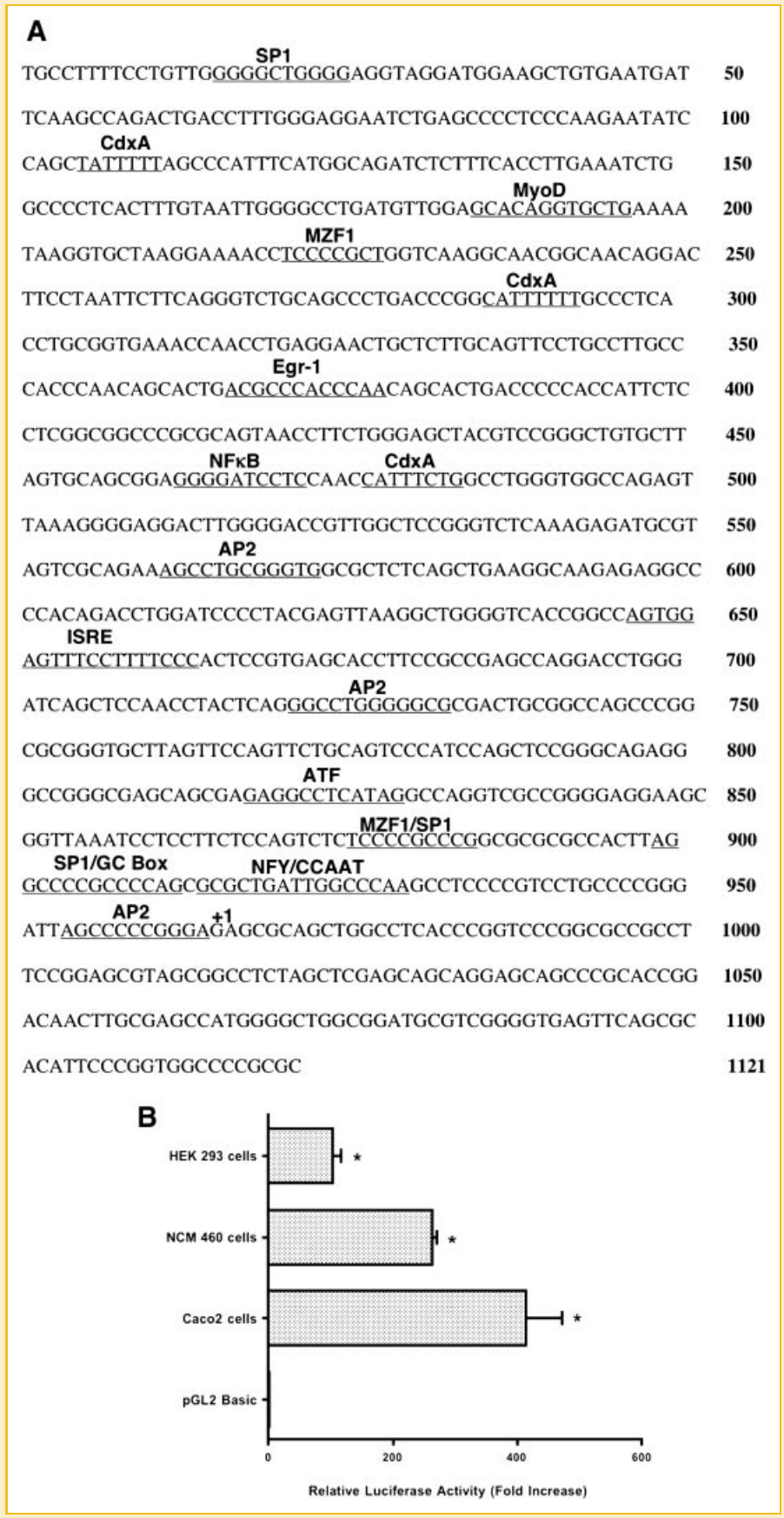

Fig. 1. PAT1 promoter is highly active in Caco2 cells: A: Nucleotide sequence of the 1.12 kb PAT1 promoter region. The transcription initiation site is marked as +1 and location of the putative binding sites for various transcription factors are underlined. B: The PAT1 promoter (−964/+156) was inserted upstream from the luciferase gene in pGL2 vector, and its activity was determined by transient transfection. The different human cell lines, intestinal Caco2 cells, colonic NCM460 cells and kidney HEK293 cells were transiently cotransfected with the PAT1 promoter construct along with pCMVβ vector. Relative promoter activity was measured by the firefly luciferase assay and normalized to β-galactosidase activity to correct for transfection efficiency. The activity of the promoter is expressed as fold increase compared with the pGL2 basic vector alone. Results were obtained from six separate experiments performed in triplicate and are expressed as mean ± SEM. $^{*}P < 0.05$ compared with empty vector.

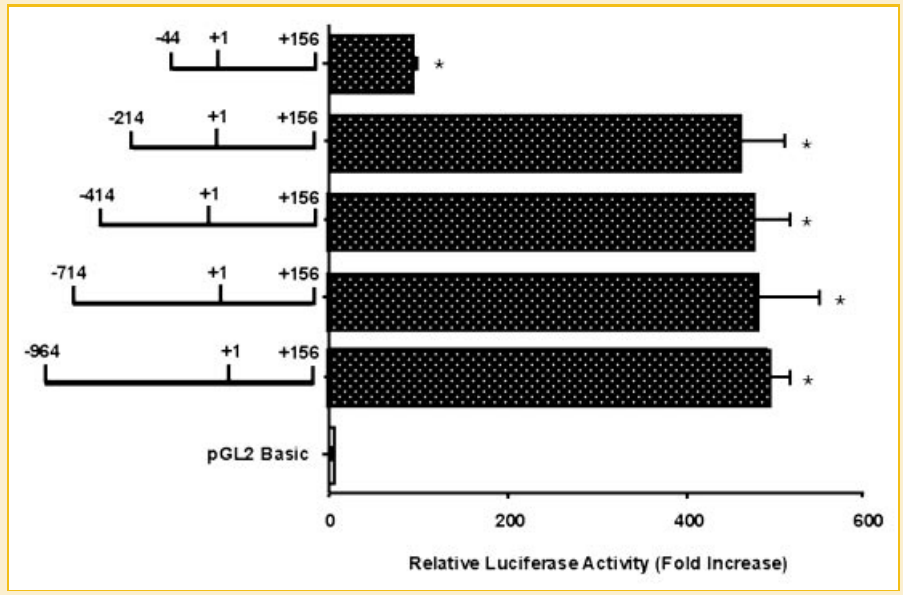

Fig. 2. Deletion analysis of PAT1 promoter. Deletions of the 5′-end of PAT1 promoter were inserted upstream from the luciferase gene in pGL2 vector and transient transfection of Caco-2 cells was performed. Relative promoter activity was measured by the firefly luciferase assay and normalized to β-galactosidase activity to correct for transfection efficiency. The activity of the promoter is expressed as fold increase compared with the pGL2 basic vector alone. Results were obtained from four separate experiments performed in triplicate and are expressed as mean ± SEM. $^{*}P < 0.05$ compared with empty vector.

5′-truncated PAT1-reporter constructs were generated in pGL2 basic vector containing progressive deletions from the 5′-end of the full length PAT1 promoter construct, p−964/+156. Figure 5 depicts the promoter activity of various 5′-deletion constructs in response to IFNγ (30 ng/ml, 24 h). The full-length promoter construct, p−964/+156 exhibited 46% inhibition in promoter activity in response to IFNγ treatment compared to untreated control. Deletion constructs, p−714/+156 and p−414/+156 showed similar inhibition (54%) indicating that deletion from −964 to −414 did not alter the inhibitory effects of IFNγ on PAT1 promoter activity. However further deletion to −214 (p−214/+156) abolished the inhibitory effects of IFNγ. These results suggested that IFNγ-responsive element (s) is located between −414 and −214 region. Further sequence analysis of this promoter region (p−414/−214) indicated potential binding sites for transcription factors, AP2 and interferon regulatory factor 1 (IRF-1).

POTENTIAL ISRE SITE IS ESSENTIAL FOR IFNγ-MEDIATED INHIBITION OF PAT1 PROMOTER ACTIVITY

Previous studies have shown that most of the effects of IFNγ on gene expression occur via the activation of transcription factor IRF-1 [Kumatori et al., 2002; Hisamatsu et al., 2003; Oshima et al., 2004; Vila-del Sol and Fresno, 2005]. To further investigate the role of IRF-1 in the inhibition of PAT1 promoter activity by IFNγ, we generated mutations in the potential ISRE binding site. As shown in Figure 6, mutations in the ISRE site (shown in grey box) significantly abrogated the inhibitory effects of IFNγ on PAT1 promoter activity. These results clearly demonstrated that the ISRE *cis* element may be involved in the modulation of PAT1 promoter in response to IFNγ.

IFNγ INDUCES IRF-1 TRANSCRIPTION FACTOR THAT BINDS TO POTENTIAL ISRE SITE IN THE PAT1 PROMOTER

To further confirm the ability of the potential ISRE *cis* element to bind IRF-1, we performed electrophoretic mobility shift analysis (EMSA) using the ISRE *cis* element as an end-labeled probe. As shown in Figure 7, the incubation of the ^{32}P-labeled ISRE *cis* element with the nuclear extracts from untreated (control) or IFNγ-treated cells resulted in a band representing binding of the probe to IRF-1. The binding of labeled potential ISRE site to Caco2 proteins (DNA–protein complex) was significantly increased in the presence of IFNγ (lanes 3 and 9) compared to control (lanes 2 and 8). The binding specificity of this complex was examined by competition experiments where excess of unlabeled specific probe (cold ISRE oligonucleotide) or consensus or mutant IRF-1 oligo was used. The DNA–protein complex was eliminated in the presence of excess of cold probe (lanes 4 and 5), or consensus IRF-1 oligonucleotide (lanes 6 and 7) but not in the presence of mutant IRF-1 oligo (lanes 12 and 13). To confirm the identity of the protein in this complex, we added the specific IRF-1 antibody. Although no supershift band was observed, addition of the anti-IRF-1 antibody in high concentration blocked the formation of the DNA–protein complex (lanes 10 and 11). Addition of an antibody in EMSAs may result in blocking the formation of the DNA–protein complex if the antibody binds to a site on the transcription factor that is essential for DNA binding [Latchman, 1999]. Thus, our results (lanes 10 and 11) suggested a blocking of the complex by the antibody, indicating that IRF-1 binds to the potential ISRE site of PAT1 promoter. These results further indicate the role of IRF-1 in IFNγ-mediated inhibition of PAT1 promoter activity.

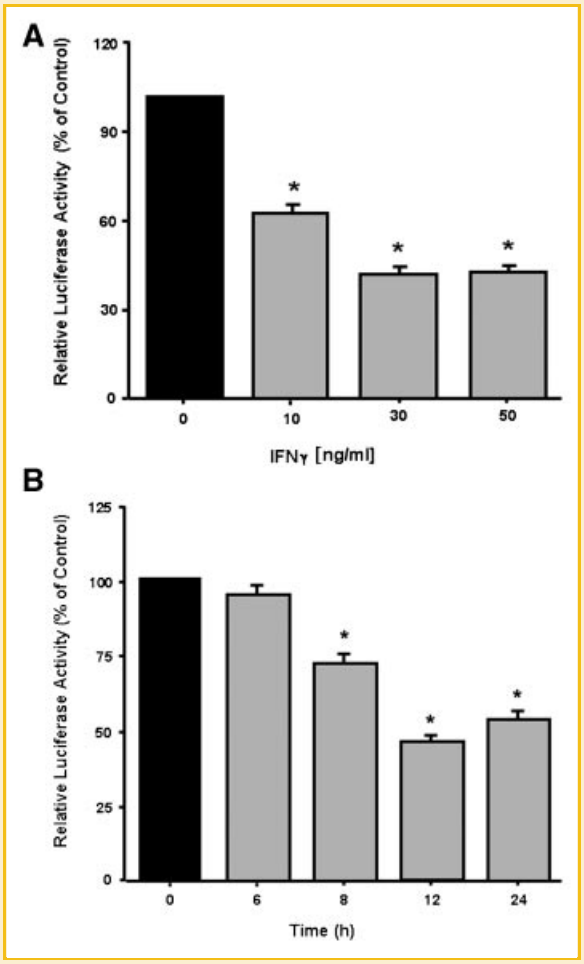

Fig. 3. Effect of IFNγ on PAT1 promoter activity: Caco-2 cells were transiently transfected with the PAT1 luciferase promoter construct (p−964/+156) along with pCMVβ vector. Twenty-four hours post-transfection, cells were then treated with varying doses of IFNγ for 24 h in media containing 1% FBS (A). Transfected cells were treated with IFNγ (30 ng/ml) at different time points (B). Cells were then harvested 48 h post-transfection and the promoter activity was measured by luciferase assay. Values were normalized to β-galactosidase activity to correct for transfection efficiency. Results represent mean ± SEM of six separate experiments performed in triplicate and are expressed as % of control comparing transfected cells treated with IFNγ with untreated cells (control). $^{*}P < 0.05$ compared with control.

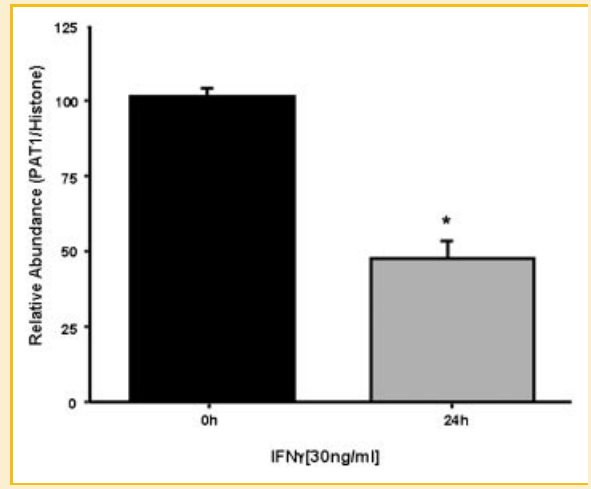

Fig. 4. Effect of IFNγ on human putative anion transporter 1 (PAT1) expression in Caco-2 cells: Post-confluent Caco-2 cells were treated with 30 ng/ml of IFNγ for 24 h in media containing 1% FBS. Total RNA was then extracted from the cells, and 100 ng were amplified with PAT1 or histone gene-specific primers using 1-step RT-PCR mix containing SYBR Green fluorescence dye for real-time PCR quantitation. The relative abundance of PAT1 mRNA normalized to the level of histone mRNA (internal control) was calculated as described in Materials and Methods Section. $^{*}P < 0.05$ compared with control.

IFNγ INDUCES ASSOCIATION OF IRF-1 WITH THE SLC26A6 PROMOTER IN VIVO

We have demonstrated that IRF-1 binds to the potential ISRE site of PAT1 promoter region. To examine the interactions of IRF-1 transcription factor with the PAT1 promoter in vivo, ChIP assays were performed. Untreated and IFNγ-treated Caco2 cells were cross-linked using formaldehyde and sheared chromatin was isolated and subjected to immunoprecipitation using anti-IRF-1 or normal rabbit IgG antibody. Immunoprecipitated DNA was purified and subjected to real time PCR using ISRE primers flanking the ISRE site and non-ISRE primers (0.4 kb away from the ISRE site). Our results showed that the value of $\Delta C_{T(IgG\text{-}IRF\text{-}1)}$, that is, differences in the threshold cycles (C_T) of amplification between IgG and IRF-1 DNA-immunoprecipitates with the non-ISRE primers in both untreated and IFNγ-treated samples was "0," indicating that there is no enrichment of the DNA fragment away from the IRF-1 binding region. However, with the ISRE primers, $\Delta C_{T(IgG\text{-}IRF\text{-}1)}$ value was "1" in untreated and "2" in IFNγ-treated samples, indicating a two- and four-fold enrichment of the DNA fragment flanking the IRF-1 binding region in untreated and IFNγ-treated samples, respectively. Therefore, our results showed that IFNγ increased the association of IRF-1 with PAT1 promoter region (contain ISRE site) by ~2-fold compared to untreated (Fig. 8). These findings further confirm the role of IRF-1 in the inhibitory effects of IFNγ effects on PAT1 promoter activity.

IFNγ EFFECTS ARE JAK DEPENDENT

Since IFNγ mediates its effects through the Janus Kinase (JAK)/Signal Transducer and Activator of Transcription 1 (STAT1) [Levy and Darnell, 2002], we investigated the role of JAK in mediating the effects of IFNγ on PAT1 promoter activity. Transiently-transfected Caco2 cells were treated with the specific JAK 1 and 2 inhibitor, JAK inhibitor I (30 nM) for 1 h prior to the addition of IFNγ (30 ng/ml), followed by co-incubation at 24 h. JAK inhibitor I significantly blocked the IFNγ-mediated effects (Fig. 9) indicating that JAK pathway is involved in IFNγ effects.

IFNγ DECREASES CL^-/OH^- EXCHANGE ACTIVITY

To examine the effects of IFNγ at the functional level (Cl^-/OH^- exchange activity), Caco-2 cells were incubated with IFNγ in serum-reduced medium at a concentration of 30 ng/ml for 24 h and

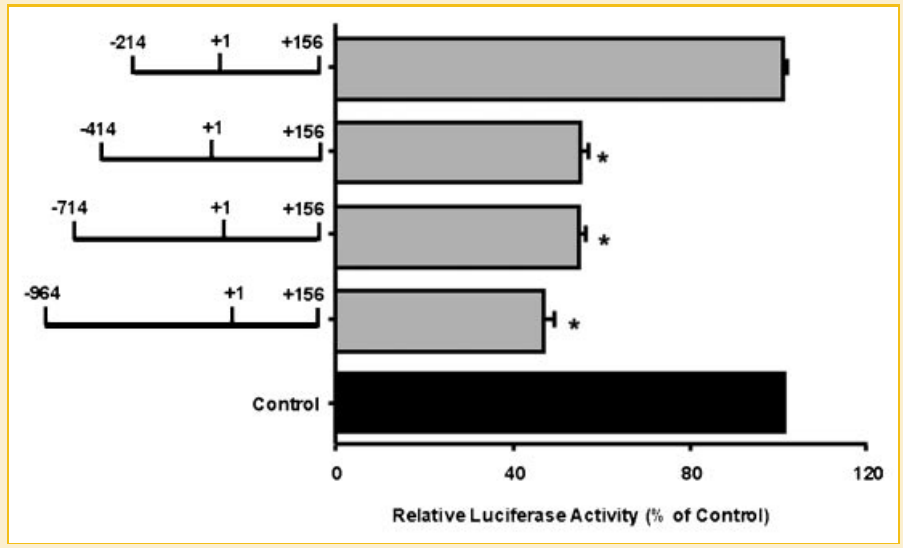

Fig. 5. Functional analysis of various deletion constructs in response to IFNγ: Caco-2 cells were transiently transfected with different 5′-deletion constructs of PAT1 promoter along with pCMVβ vector. Twenty-four hours post-transfection, cells were then treated with 30 ng/ml of IFNγ for 24 h in media containing 1% FBS. Cells were then harvested 48 h post-transfection and the promoter activity was measured by luciferase assay. Values were normalized to β-galactosidase activity to correct for transfection efficiency. Results represent mean ± SEM of five separate experiments performed in triplicate and are expressed as % of control comparing transfected cells treated with IFNγ with untreated cells (control). $^{*}P < 0.05$ compared with respective control.

DIDS-sensitive Cl^-/OH^- exchange activity was assessed as described in Methods. As shown in Figure 10, consistent with mRNA levels and promoter activity, a significant decrease in Cl^-/OH^- exchange activity (~50%) was also observed at 24 h. However, JAK1 inhibitor significantly abolished the inhibitory effects of IFNγ on Cl^-/OH^- exchange activity (Fig. 11). These findings suggest that IFNγ-mediated inhibition of SLC26A6 expression and function is dependent on JAK mediated pathway.

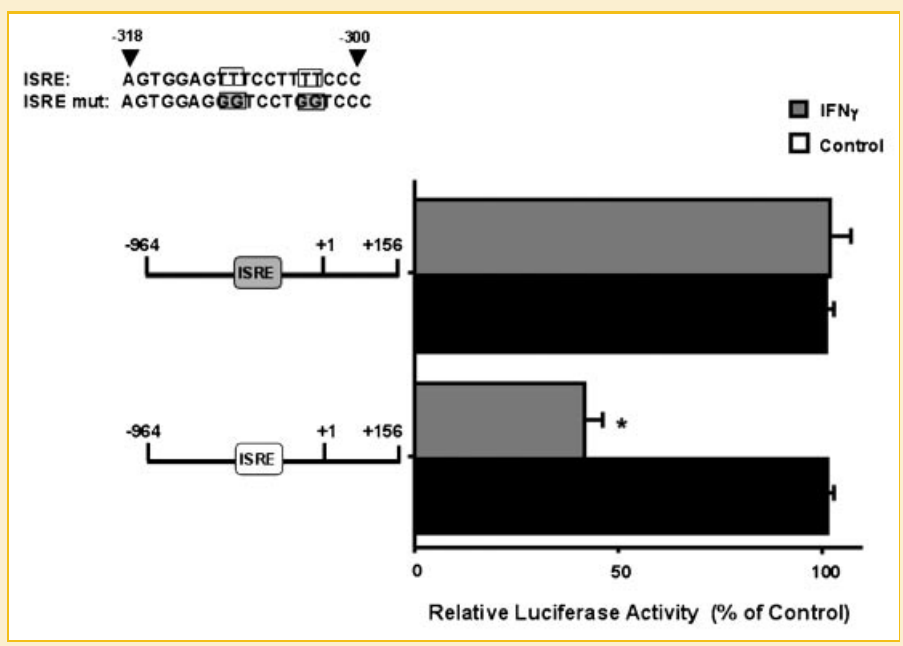

Fig. 6. Potential ISRE site is the IFNγ-response motif: Caco-2 cells were transiently transfected with PAT1 promoter construct (−964/+156) and a construct with a mutated ISRE site (ISRE mutant, mutated sites are shown as grey box) along with pCMVβ vector. Twenty-four hours post-transfection, cells were then treated with 30 ng/ml of IFNγ for 24 h in media containing 1% FBS. Cells were then harvested 48 h post-transfection and the promoter activity was measured by luciferase assay. Values were normalized to β-galactosidase activity to correct for transfection efficiency. Results represent mean ± SEM of four separate experiments performed in triplicate and are expressed as % of control comparing transfected cells treated with IFNγ with untreated cells (control). $^{*}P < 0.05$ compared with control.

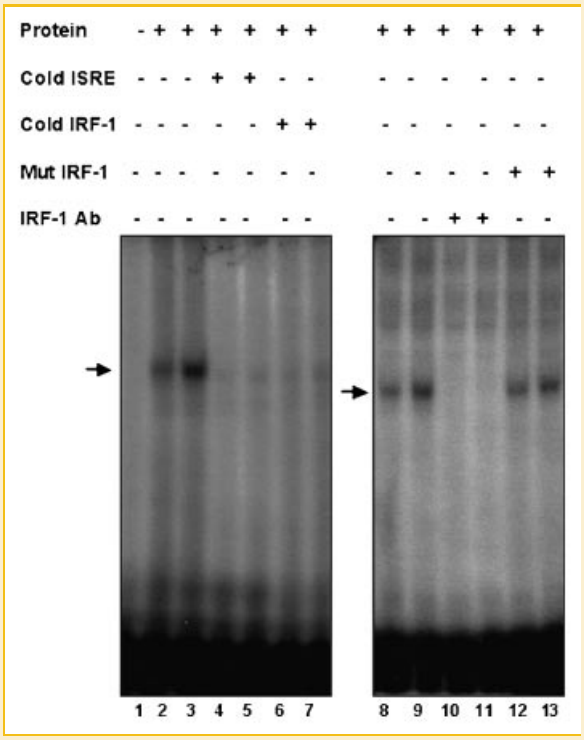

Fig. 7. IFNγ-induced IRF-1 interacts with the potential ISRE motif: EMSA was performed using a double-stranded oligonucleotide (−324 to −293 bp) as end-labeled probe and nuclear extracts from untreated (control) or IFNγ (30 ng/ml) treated Caco2 cells. A total of 10 μg of nuclear proteins were combined with 50,000 cpm probe per reaction and after 30 min incubation at room temperature resolved on a 4% non-denaturing polyacrylamide gel and visualized by autoradiography. Lane 1 depicts only probe. DNA–protein binding in control (lanes 2 and 8) was significantly increased (lanes 3 and 9) in response to IFNγ. Competition experiments were performed in the presence of unlabeled probe (lanes 4 and 5), an oligonucleotide containing IRF-1 consensus binding site (lanes 6 and 7) and IRF-1 mutant oligonucleotide (lanes 12 and 13). Anti-IRF-1 antibody (2 μg, lanes 10 and 11) blocked the formation of the DNA–protein complex. + and − signs indicate the presence or absence of reaction components in the reaction mixture (shown on top). Gels are shown as a representative of three separate experiments with similar results.

DISCUSSION

Diarrhea generally results from either increased secretion or impairment in NaCl absorption or both. Electroneutral NaCl absorption has been suggested to involve coupling of apical membrane Na^+/H^+ (NHE3/NHE2) with $Cl^-/OH^-(HCO_3^-)$ exchangers (PAT1/DRA). Recent evidence has demonstrated repression of NHE3 promoter activity in human intestinal epithelial cells by the pro-inflammatory cytokine IFNγ [Amin et al., 2006]. However, whether IFNγ directly regulates PAT1 (apical Cl^-/HCO_3^- exchanger) gene expression in the intestine has not been examined. In the current study, we have cloned and characterized the human PAT1 promoter region and have shown its regulation by IFNγ. Nucleotide sequence analysis of the 1.12 kb promoter region revealed multiple potential *cis*-acting elements for various transcription factors. These included Sp1, AP2, CdxA, Mzf1, MyoD, and Egr1. PAT1 promoter contains

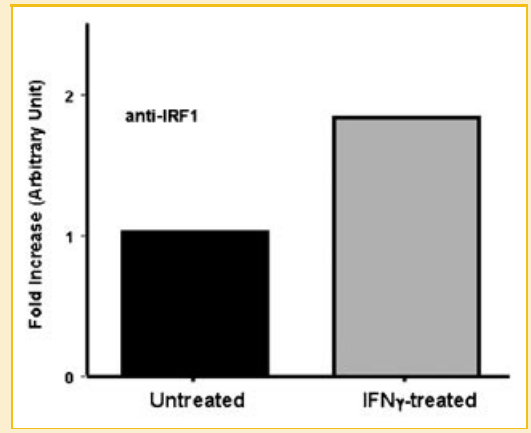

Fig. 8. IFNγ induces binding of IRF-1 to endogenous PAT1 promoter: Cross-linked chromatin was isolated from untreated and IFNγ treated Caco2 cells subsequent to formaldehyde treatment. ChIP assays were performed with anti-IRF-1 antibody. Co-immunoprecipitated DNA as template and Primers were used to amplify the PAT1 promoter region as described in Materials and Methods Section. Results represent three separate experiments and are expressed as Fold Increase.

features that are common in regulated genes. This is evident by the presence of the potential transcription factor binding sites that mediate tissue-specific and developmental regulation of the genes. For example, the presence of CdxA, which is involved in gene expression and differentiation in the intestine [Suh et al., 1994; Lambert et al., 1996; Chawengsaksophak et al., 1997; Qualtrough et al., 2002], MyoD, which is implicated in myocyte differentiation [Blackwell and Weintraub, 1990; Winter et al., 1993; Lluis et al., 2005] and Mzf1, which is involved in erythrocyte-specific gene

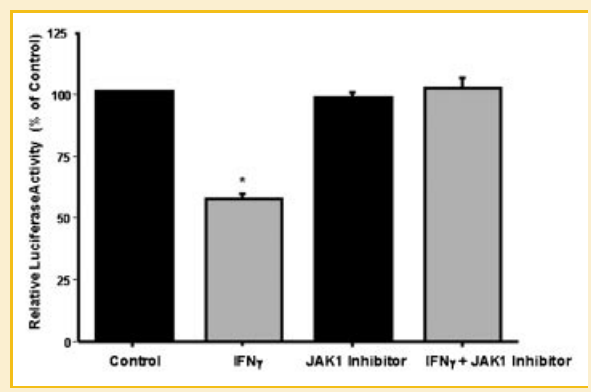

Fig. 9. IFNγ-mediated inhibition of PAT1 promoter activity is JAK-dependent: Caco-2 cells were transfected with the PAT1 luciferase promoter construct (p−964/+156) along with pCMVβ vector. Twenty-four hours post-transfection, Caco2 cells were pre-treated with the specific JAK 1 and 2 inhibitor, JAK inhibitor I (30 nM) for 60 min prior to the addition of 30 ng/ml IFNγ for another 24 h. Cells were then harvested 48 h post-transfection and the promoter activity was measured by luciferase assay. Values were normalized to β-galactosidase activity to correct for transfection efficiency. Results represent mean ± SEM of three separate experiments performed in triplicate and are expressed as % of control comparing transfected cells treated with IFNγ with untreated cells (control). $^*P < 0.05$ compared with control.

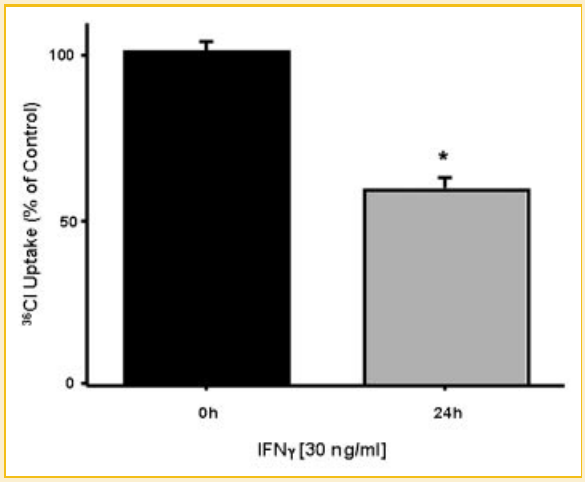

Fig. 10. Effect of IFNγ on apical Cl^-/OH^- exchange activity in Caco-2 cells: Caco2 cells were incubated with 30 ng/ml of IFNγ in media containing 1% FBS for 24 h. Cells were then washed with 1× PBS and were base loaded with Hepes/KOH medium adjusted to pH 8.5 for 30 min. Cl^-/OH^- exchange activity was measured as DIDS-sensitive (300 μM) ^{36}Cl uptake at 5 min. Results are expressed as % of control and represent mean ± SEM of three separate experiments performed in triplicate. $^*P < 0.05$ compared to control.

expression and regulation [Morris et al., 1994; Hromas et al., 1995; Hui et al., 1995] across different species including rat, mouse and human would suggest developmental expression of PAT1 and tissue-specific regulation of PAT1 promoter. Our studies showed that the PAT1 promoter activity was relatively high in the intestinal Caco2 cells compared to colonic NCM460 and kidney HEK293 cells. The results are consistent with the fact that PAT1 is predominantly expressed in the small intestine [Wang et al., 2002].

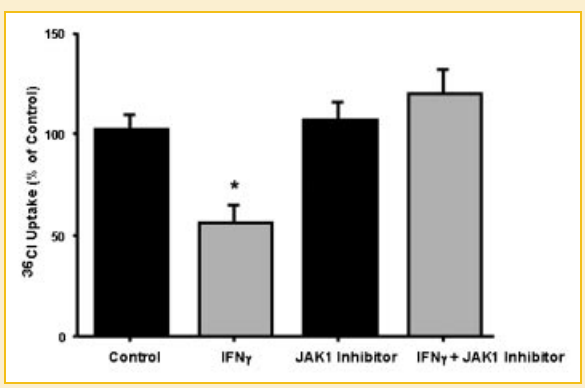

Fig. 11. Effect of JAK1 and 2 inhibitor on IFNγ induced inhibition of Cl^-/OH^- exchange activity in Caco-2 cells: Caco2 cells were pre-incubated with JAK1 inhibitor (30 nM) for 60 min in media containing 1% FBS and then co-incubated with 30 ng/ml of IFNγ for 24 h. Cells were then washed with 1× PBS and were base loaded with Hepes/KOH medium adjusted to pH 8.5 for 30 min. Cl^-/OH^- exchange activity was measured as DIDS-sensitive (300 μM) ^{36}Cl uptake at 5 min. Results are expressed as % of control and represent mean ± SEM of three separate experiments performed in triplicate. $^*P < 0.05$ compared to control.

Progressive deletions from the 5′-flanking region of PAT1 promoter showed that deletions from −714 to −214 bp exhibited similar activity compared to the full-length promoter construct (p−964/+156). However, further deletion to −44 bp significantly decreased promoter activity by ∼10 fold indicating that the region flanking the area between −214 and −44 bp harbors *cis* elements sufficient to drive the basal activity of the promoter in Caco2 cells. Interestingly, in the presence of the pro-inflammatory cytokine, IFNγ, the inhibitory effects of IFNγ on promoter activity of the deletion constructs, p−714/+156 and p−414/+156 were similar to the full-length p−964/+156. However, further deletion to −214 bp significantly abrogated the inhibitory effects of IFNγ on promoter activity. These findings indicated the presence of potential IFNγ response element(s) in the region flanking the area between −414 and −214 bp in the PAT1 promoter.

In general, IFNγ elicits its effects via the induction of signal transduction pathway involving the janus kinases (JAK 1 and 2) and signal transducer and activator transcription factor 1 (STAT1). The binding of IFNγ to its surface receptor activates the receptor-associated tyrosine kinases, JAK1 and JAK2. JAKs tyrosine phosphorylate and activate the latent cytosolic STAT1, which then dimerizes, translocates to the nucleus and activates several IFNγ response genes by binding to IFNγ-response elements within their promoters [Sims et al., 1993; Coccia et al., 1995]. Also, among the IFNγ response genes are several IFN regulatory factors (IRFs), a growing family of transcription factors (up to nine members have been characterized) that contains among others, IRF-1 and IRF-2 [Mamane et al., 1999]. They contain the characteristic helix-turn-helix motif [Taniguchi et al., 2001]. IRF-1 is strongly inducible by IFNγ and binds IFN-stimulated responsive elements (ISREs) within promoters to regulate transcription, while IRF-2 is constitutively expressed factor and competes with IRF-1 for the same *cis* element. These ISREs (IRF-E), usually defined by pallindromic TTTC sequences separated by two or three nucleotides [Levy, 1998] are located in the promoter region of many IFNγ inducible genes. IFNγ-dependent activation of IRF-1 has been previously shown in human intestinal epithelial cells [Oshima et al., 2004] further lending support to its potential role in the observed inhibition of PAT1 promoter activity by IFNγ as shown in this study.

The involvement of JAK/STAT1 pathway was confirmed using the specific JAK1 and 2 inhibitor, JAK inhibitor I. The inhibitory effects of IFNγ on PAT1 promoter activity were completely abrogated in the presence of the inhibitor. Our study also provided evidence for the role of IRF-1 in the regulation of PAT1 promoter activity. Sequence analysis of the IFNγ responsive region identified one potential ISRE *cis* element flanking the region of −318 to −300 bp. Interestingly, mutation in the potential ISRE *cis* element attenuated the inhibitory effects of IFNγ on promoter activity (Fig. 6) in parallel with blocking the IRF-1 binding to ISRE probe by the mutant IRF-1 oligo (Fig. 7, lanes 12 and 13). Additionally, EMSAs showed increased protein binding to the oligonucleotide spanning the potential ISRE site (−318 to −300 bp) under IFNγ-stimulated conditions (Fig. 7, lanes 3 and 9). The DNA–protein complex was blocked by IRF-1 antibody suggesting that the identified protein is IRF-1 (Fig. 7, lanes 10 and 11). These findings were validated in vivo by performing CHIP assays coupled with real

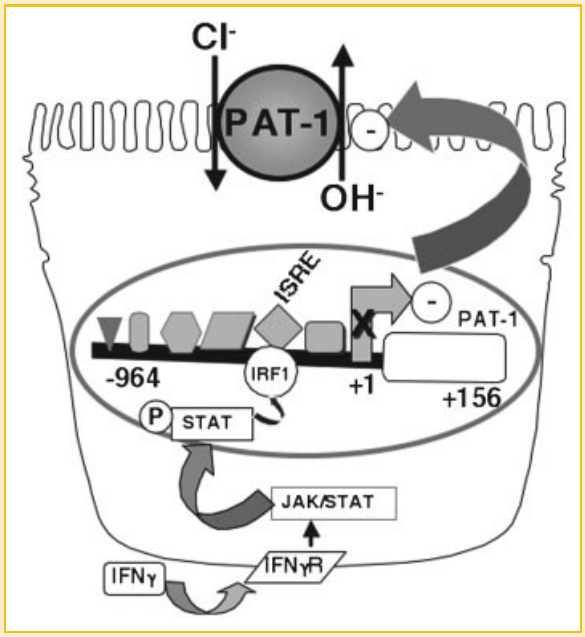

Fig. 12. Proposed model of the potential mechanisms of IFNγ-mediated inhibition of PAT1 gene expression in human intestinal epithelial cells.

time PCR analysis in untreated and IFNγ-treated Caco2 cells. The results showed that IFNγ significantly increased the association of IRF-1 with endogenous PAT1 promoter, which contain the ISRE site (Fig. 8) compared to untreated. It should be noted that the IFNγ-mediated decrease in PAT1 promoter activity was parallel to decrease in both PAT1 mRNA levels and function (Cl^-/OH^- exchange activity). However, Cl^-/OH^- exchange activity in these cells could be a function of both PAT1 and DRA expression. Previous studies have shown that DRA mRNA expression was reduced in patients with ulcerative colitis [Yang et al., 1998] and in animal model of intestinal inflammation [Kuhn et al., 1993]. Therefore, a potential decrease in DRA expression in response to IFNγ treatment in the observed decrease in Cl^-/OH^- exchange activity cannot be ruled out. Taken together, these observations indicate that the ISRE element on PAT1 promoter appears to play an important role in the regulation of PAT1 gene expression by IRF-1. In contrast to present studies, our studies [Amin et al., 2006] of regulation of NHE3 promoter activity by IFNγ showed involvement of Sp1 and Sp3 transcription factors rather than IRF-1. Although, PAT1 might be involved in coupled NaCl absorption along with NHE3 in the small intestine, it seems that their regulation by IFNγ occurs via distinct molecular mechanisms. The significance of these dispartate pathways of regulation of Na^+ and Cl^- transport in the intestine is not clear at present and will be the subject of future investigation.

A schematic representation of regulation of PAT1 expression by IFNγ is shown in Figure 12, which shows the involvement of IRF-1 in the regulation of intestinal PAT1 gene expression. This inhibition occurs via a putative ISRE *cis* element in the PAT1 promoter. We speculate that the repression of PAT1 promoter activity along with NHE3 promoter may lead to decreased NaCl absorption in the ileum and might contribute to the pathophysiology of diarrhea associated with IBD.

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