Characterization of the Human Intestinal CD98 Promoter and its Regulation by Interferon Gamma

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Abstract

Growing evidence that epithelial CD98 plays an important role in intestinal inflammation focused our interest to investigate the transcriptional regulation of CD98. Our mouse-based in vivo and in vitro studies revealed that epithelial colonic CD98 mRNA expression was transcriptionally increased in intestinal inflammation. We then isolated and characterized a 5'-flanking fragment containing the promoter region required for CD98 gene transcription. Primer extension and rapid amplification of 5'-cDNA ends (5'-RACE) were used to map a transcriptional initiation site 129 bp upstream from the translational start codon (ATG). Direct sequencing of the 5'-flanking region revealed the presence of four GC-rich Sp1 binding domains, one NF-kB binding domain and no TATA-box. Binding of Sp1 [Sp1(-874), SP1(-386), Sp1(-187) and Sp1(-177)] and NF-κB (-213)) to the promoter was confirmed by electrophoretic mobility shift (EMSA) and super shift assays. Furthermore, chromatin immunoprecipitation studies show the in vivo DNA-Sp1 and DNA-NF-kB interactions in the basal and interferon γ stimulated conditions. A reporter gene driven by serially truncated and site-mutated CD98 promoters were used to examine basal and IFN-gamma-responsive transcription in transiently transfected Caco2-BBE cells. Our results revealed that Sp1(-187), Sp1(-177) and the NF-kB binding site were essential for basal and IFN γ-stimulated CD98 promoter activities, while Sp1(-874) and Sp1(-386) were not. The results from additional site-mutated CD98 promoters suggested that Sp1(-187), Sp1(-177) and the NF-kB site may cooperate in mediating basal and IFN γ-stimulated CD98 promoter activities. Finally, we demonstrated that reduction of Sp1 or NF-kb expression reduced CD98 protein expression in un-stimulated and in interferon γ stimulated Caco2-BBE. Collectively, these findings indicate that the Sp1 and NF-kB transcription factors are likely to play a significant role in IFN-γ-mediated transcriptional regulation of CD98 in the intestinal epithelium, providing new insights into the regulation of CD98 expression in intestinal inflammation.
Introduction

The CD98 cell surface molecule is a 125 kDa type II membrane glycoprotein heterodimer composed of a 40 kDa non-glycosylated light chain and an 85 kDa glycosylated heavy chain (47). CD98 was first identified as a lymphocyte activation-related antigen that was expressed at low levels on resting peripheral T cells and showed rapid enhancement following lectin activation (9,17,19,27). The CD98 gene is a member of the family of growth-related genes and displays sequence homologies with several other inducible T-cell genes (17,24). CD98 expression was subsequently reported in activated B lymphocytes (44) and some proliferating normal tissues, including the blood brain barrier (22), basal skin layer (15), kidney proximal tubes (42,43), placenta (34,36), testis (37) intestinal epithelial cells (30,31) and also in a wide variety of tumor cells, including hepatomas, breast and colon cancers (35,41,45).

In the context of intestinal inflammation, CD98 protein expression in human colonic epithelium was shown to be upregulated by proinflammatory cytokines such as IFN-γ, and increased expression of lymphocyte activation antigens for CD98 was found on the cell surface of intestinal B cells, T cells, CD4+ T cells, and CD98+ T cells isolated from patients with Crohn’s disease and ulcerative colitis (12,13). These and other studies have revealed that markedly increased intestinal lymphocyte activation is a major immunological alteration in IBD (16). In addition, 5-aminosalicyclic acid (which is used for treatment of intestinal inflammation in IBD) inhibited the expression of the CD98 cell surface activation antigen on mitogen-activated peripheral blood lymphocytes in a dose-dependent manner, further suggesting that CD98 plays an important inflammatory role (13). Recently, we have reported that epithelial CD98 plays also an important role in intestinal inflammation (25).

DSS (Dextran Sodium Sulfate) is often used to induce experimental colitis in animal models (11). We recently demonstrated that epithelial CD98 protein expression was increased in the colonic epithelium of DSS-treated mice, and that this effect was at least partially mediated by IFN-γ (25). Consistent with this,
we also found that epithelial protein expression of CD98 was enhanced by IFN-γ treatment of Caco2-BBE cells \textit{in vitro} (25). DSS is believed to cause colitis by interfering with intestinal barrier function and/or stimulating local inflammation within the colon, via up-regulation of both Th1 and Th2 cytokines and inflammatory mediators (11). Thus, the increased expression of CD98 in inflamed intestinal epithelial cells may be due to the effect of IFN-γ. However, although the previous studies strongly suggest IFN-γ is capable of regulating CD98 expression, the mechanism(s) governing this activation remained unknown. Here, we characterize the 5'-flanking region of the colonic human CD98 gene promoter and investigate its mechanism of transcriptional activation by IFN-γ.
Materials and Methods

Cell culture
Caco2-BBE (5,6,7) cells (passages 30-50) were grown in high glucose Dulbecco’s Vogt Modified Eagle’s Media (DMEM; Invitrogen, Carlsbad, CA) supplemented with 14 mmol/L NaHCO₃ and 10% newborn calf serum. Cells were kept at 37ºC in humidified atmosphere containing 95% air and 5% CO₂ and the medium was changed daily. Monolayers were subcultured every 7 days with 0.1% trypsin and 0.9 mmol/L ethylene diamine tetraacetic acid (EDTA) in Ca²⁺/Mg²⁺-free phosphate-buffered saline (PBS).

Animals
All studies were carried out in female C57BL/6 mice (8 weeks, 18-22 g) obtained from the Jackson Laboratories (Bar Harbor, ME). Mice were group-housed under a controlled temperature (25°C) and photoperiod (12:12-hour light-dark cycle), and allowed unrestricted access to standard mouse chow and tap water. They were allowed to acclimate to these conditions for at least 7 days before inclusion in experiments.

Induction of colitis and study design
Colitis was induced in all groups by the addition of dextran sodium sulfate (DSS; molecular weight 40,000 Da; 3% (w/v); ICN Biochemicals, Aurora, OH) to the drinking water. The mean DSS/water consumption was recorded for each group. Two groups of mice (n=6 per group) were treated for 7 days with DSS or regular water. Body weight was assessed every day during the treatment period.
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**Quantitative RT-PCR analysis**

Mice suffering from DSS-induced colitis or normal controls were killed, colon tissues were dissected and colonic epithelial cells were subsequently isolated as described previously (25). Total RNA was extracted from untreated or DSS treated mice colonic cells and from untreated or interferon γ treated Caco2-BBE cells using the TRIzol reagent (Invitrogen, Carlsbad, CA) and reverse transcribed using the Thermoscript™ RT-PCR System (Invitrogen, Carlsbad, CA). The real-time iCycler sequence detection system (Bio-Rad, Hercules, CA) was used to quantify the first-strand CD98 cDNA in the presence or absence of DSS/interferon γ in mouse colonic cells or in Caco2-BBE cells. Briefly, 50 ng of reverse-transcribed cDNA, 10 μM of gene-specific primers CD98HQF (see table1 #1), CD98HQR (see table1 #2), CD98MQF (see table 1 #3), CD98MQR (see table 1 #4) and the iQ SYBR Green Suppermix (Biorad, Hercules, CA) were amplified at 50 °C for 2 min and 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The β-actin expression levels were used as reference, and fold-induction was calculated by the relative standard curve method. For graphical representation of quantitative PCR data, raw cycle threshold values (Ct values) obtained for DSS/interferon γ-treated cells were deducted from the Ct value obtained for internal β-actin transcript levels, using the ΔΔCt method as follows: ΔΔCt=(Ct,Target - Ct,Actin)treatment - (Ct,Target - Ct,Actin)non-treatment, and the final data were derived from 2^-ΔΔCT (Livak). Quantification of CD98 transcripts were performed, as described above, in Caco2-BBE cells that were untreated or pretreated with 2µg/ml actinomycin D for 4 hours and then stimulated with 100U/ml interferon γ or vehicle (-) for 24hours.

**Plasmid construction**

Genomic DNA was extracted from Caco2-BBE cells using the REDExtract-N-Amp Tissue PCR Kit (Sigma, St. Louis, MI). The 5’-flanking region of the CD98 gene was PCR amplified (ThermalACE DNA polymerase; Invitrogen) in the presence of 1 M betaine (Sigma, St Louis MI) using the CD98 promoter forward
primer and CD98 promoter reverse primer listed in Table 1 (#5 and #6) based on the genomic sequence of CD98 gene (accession number NT_033903). The PCR conditions consisted of 95°C for 5 min, followed by 34 cycles of 95°C for 10 seconds, 55°C for 1 min and 72°C for 3 min, followed by a final extension at 72°C for 10 min. The expected 1034 bp-length fragment was obtained. Gel-purified fragments (Qiaquick gel extraction kit; Qiagen, Valencia, CA) were cloned into the PCR2.1/TOPO vector (Invitrogen, Carlsbad, CA) and confirmed by sequencing. The plasmid was then digested with Kpn1 and Xhol (New England Biolabs, Ipswich, MA), subcloned into the promoter-less pGL3 firefly luciferase basic vector (Promega, San Luis, CA), and confirmed by sequencing. The truncated constructs were generated by PCR using the full-length sequence as a template, along with the following primers (see Table 1 for primer sequences): CD98 promoter I _-750+4_: Primer 2 (table 1 #8) and CD98 promoter reverse primer; CD98 promoter II _-330+4_: Primer 4 (table 1 #10) and CD98 promoter reverse primer; CD98 promoter III _-1031-750_: Primer 1 (table 1 #7) and CD98 promoter forward primer (table 1 #5); CD98 promoter IV _-1030-330_: Primer 3 (table 1 #9) and CD98 promoter forward primer (table 1 #5); CD98 promoter V _-750-330_: Primers 2 and 3 (table 1 #8 and 9). Mutations in the CD98 promoter GC boxes and NF-κB site were introduced by overlapping PCR using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). The mutagenic oligonucleotide sequences are shown in Table 1, with the substitutions underlined. In detail, the first GC-rich domain mutant was generated with primer GC-rich domain I For (table 1 #13) and primer GC-rich domain I Rev (table 1 #14); the second GC-rich domain mutant with primers GC-rich domain II For (table 1 #15) and GC-rich domain II Rev (table 1 #16); the third GC-rich domain mutant with primers GC-rich domain III For (table 1 #17) and GC-rich domain III Rev (table 1 #18); the fourth GC-rich domain mutant with primers GC-rich domain IV For (table 1 #19) and GC-rich domain IV Rev (table 1 #20); the NF-κB binding domain mutant was generated with primer NF-κB binding domain For (table 1 #21) and primer NF-κB binding domain Rev (table 1 #22). Double mutants and triple mutants were generated by
using the appropriate single mutant or double mutant respectively as templates with the relevant primers described above. All mutants were confirmed by DNA sequencing.

**Transient transfection and luciferase reporter gene assay**

Caco2-BBE cells (50,000 cells/well) were cultured in 12-well plates for 1 day. At 50-70% confluence, the cells were rinsed and then incubated with 1000 µl of Opti-MEM (Gibco, Invitrogen, Carlsbad, CA). 5 ng Renilla (phRL-CMV), 2 µg of the relevant CD98 promoter construct, and 10 µg/ml Lipofectin (Invitrogen, Carlsbad, CA). In each experiment, cells were incubated with the same total amount of plasmid DNA. After 24 h, the cells were washed twice with phosphate-buffered saline and lysed with the provided buffer (Luciferase Assay Kit; Promega, San Luis Obispo, CA). Subsequently, the Caco2-BBE cells were stimulated with 1000U/ml interferon γ or vehicle for 24 hours. For the luciferase activity assay, 20 µl of cell extract was mixed with 100 µl luciferase assay substrate (Promega, San Luis Obispo, CA), and the resulting luminescence was measured for 10 s in a luminometer (Luminoskan, Thermo Labsystems, Needham Heights, MA). For normalization of luciferase activity, we used the phRL-CMV control vector containing the CMV immediate-early enhancer/promoter region, which provides strong constitutive expression of Renilla luciferase in a variety of cell types. Each extract was analyzed in duplicate, and at least two independent experiments were performed for each experiment. The observed luciferase activity reported in each figure is expressed as the percentage of the activity obtained with regards to the most active construct used in each figure. The DNAs and transfected cell lines are as indicated in the individual experiments.
Primer extension

Colonic mucosal biopsies were collected from patients who underwent colonoscopy for polyp or cancer surveillance. All subjects were given informed consent, as approved by Human Investigations Committee of Emory University Atlanta, GA. Human colonic mucosa biopsies and mouse colonic mucosa (the muscle layer was carefully removed using pointed forceps). Primer extension experiments were performed. Briefly, total RNA were extracted from Caco2-BBE cells and normal human colonic mucosal biopsies using the TRIzol reagent (Invitrogen, Carlsbad, CA) as recommended by the manufacturer. The integrity of the purified total RNA was determined by formaldehyde agarose gel electrophoresis. The CD98 promoter reverse primer (see table 1 #6), located upstream of the translation start site, was labeled with 6000 µCi/mmol \( [\gamma-^{32}p] \) dATP (Perkin Elmer, Boston, MA) using the KinaseMarx kit (Ambion, Austin, TX) and purified using NucAway Spin Columns (Ambion, Austin, TX). The 100-bp marker (New England Biolabs, Ipswich, MA) was radio-labeled with the same isotope just before use. For primer extension, reactions consisting of 10 or 20 µg total RNA, 1pmol radio-labeled primer, 10µl of 2X AMV primer extension buffer, 2mM sodium pyrophosphate and 1 µl (15u) AMV RT (Invitrogen, Carlsbad, CA) were heated 60°C for 20 min for annealing, and then cooled at room temperature for 10 min. The mixture was then incubated at 42°C for 45 minutes and the reaction was stopped by addition 20 µl loading dye. The samples were then heated at 90°C for 10 min and loaded directly onto 5% TBE gels (Bio-Rad, Hercules, CA). The gels were run at 200 V for 90 min, vacuum-dried and autoradiographed overnight at -80°C.

5’-Rapid Amplification of cDNA Ends

Initial 5’-RACE experiments were performed on 5 µg of total RNA from Caco2-BBE cells, using the 5’ RACE System for Rapid Amplification of cDNA Ends (Invitrogen, Carlsbad, CA), as recommended by the manufacturer. Briefly gene-specific reverse primers were designed to be complementary to nucleotides 113-
137 and 486-508 of the CD98 gene (CD98\textsubscript{R1} and CD98\textsubscript{R2}, respectively; Table 1 #11 and 12). The primary and nested PCR reactions were performed using Platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA). The products of the nested PCR reaction were cloned into the PCR 2.1 TOPO vector (Invitrogen, Carlsbad, CA), and then sequenced.

**Nuclear protein extraction**

Caco2-BBE cells were washed once in ice-cold phosphate-buffered saline (PBS), dislodged from the plates by scratching, transferred in PBS to 50 ml Falcon tubes and centrifuged at 800 rpm for 5 min. The resulting pellets were resuspended in 5 ml of cold lysis buffer (10 mM HEPES, 10 mM KCl, 1.5 mM MgCl\textsubscript{2} and 0.1% NP-40, pH 7.9, at 4°C) for 10 min on ice. For isolation of nuclei, the lysate was vigorously mixed and microcentrifuged for 5 min at 12,000 X g and 4°C, and the nuclear pellet was washed once with 1 ml of Nonidet P-40-free lysis buffer. For extraction of nuclear proteins, the nuclear pellet was resuspended in 1 ml of protein extraction buffer (420 mM NaCl, 20 mM HEPES, 1.5 MgCl\textsubscript{2}, 0.2 mM EDTA and 25% glycerol, pH 7.9) for 10 min at 4°C. After vigorous mixing, the nuclear suspension was microcentrifuged for 5 min at 4°C, the pellet was discarded, and the supernatant was microcentrifuged (12000 rpm) again for 5 min at 4°C.

The protein content in the final supernatant (nuclear protein extract) was measured using the Bradford method (Bio-Rad, Hercules, CA). Dithiothreitol (0.5 mM), phenylmethylsulfonyl fluoride (PMSF; 0.5 mM), and leupeptin (10 pg/ml) were added to the lysis and extraction buffers just before use. The diluting buffer contained the same amounts of DTT and leupeptin, but only 0.2 mM phenylmethylsulfonyl fluoride. Samples were stored at -70°C until use.
Electrophoretic Mobility Shift Assays (EMSA)

Probes for the electrophoretic mobility shift assays (EMSAs) were prepared using a standard protocol. Primers are listed in the table, in detail, GC1W\textsubscript{1} and GC1W\textsubscript{2} (table 1 #23 and 24) are complementary oligonucleotides for the first Sp1 binding site, GC1M\textsubscript{1} and GC1M\textsubscript{2} (table 1 #25 and 26) are complementary oligonucleotides for the first Sp1 binding site mutant; GC2W\textsubscript{1} and GC2W\textsubscript{2} (table 1 #27 and 28) are complementary oligonucleotides for the second Sp1 binding site, GC2M\textsubscript{1} and GC2M\textsubscript{2} (table 1 #29 and 30) are complementary oligonucleotides for the second Sp1 binding site mutant; primers GC3W\textsubscript{1} and GC3W\textsubscript{2} (table 1 #31 and 32), GC3M\textsubscript{1} and GC3M\textsubscript{2} (table 1 #33 and 34) correspond to the third Sp1 binding site and its mutant; primers GC4W\textsubscript{1} and GC4W\textsubscript{2} (table 1 #35 and 36), GC4M\textsubscript{1} and GC4M\textsubscript{2} (table 1 #37 and 38) correspond to the fourth Sp1 binding site and its mutant; primers NF-\textit{kB}W\textsubscript{1} and NF-\textit{kB}W\textsubscript{2} (table 1 #39 and 40), NF-\textit{kB}M\textsubscript{1} and NF-\textit{kB}M\textsubscript{2} (table 1 #41 and 42) correspond to the NF-\textit{kB} binding site and its mutant. The probes were synthesized with HPLC purification and represent the transcription factor binding sites and were first end-labeled with a Biotin 3′ End DNA Labeling Kit (Pierce, Rockford, IL). Briefly, 0.2 U/µl of TdT, 0.5 µM biotin-11-UTP and 100 nM of each single-stranded oligonucleotide were incubated in 1x TdT reaction buffer for 30 min at 37°C. EDTA (2.5 µl of 0.2 M) was added to stop the reaction, and 50 µl of chloroform:isoamyl alcohol was added to extract the labeled oligonucleotides. For annealing, concentrated complementary oligonucleotides were mixed together at a 1:1 molar ratio and incubated at 95°C for 5 minutes. The heat was then gradually reduced over hours until the oligonucleotides reached room temperature. Standard EMSAs were performed using the LightShift Chemiluminescent EMSA Kit (Pierce, Rockford, IL). Briefly, 20 fmol of each biotin end-labeled target oligonucleotide pair was incubated in EMSA binding buffer (100 mM Tris, 500 mM KCl, 10 mM DTT, pH 7.5) containing 2.5% glycerol, 5 mM MgCl\textsubscript{2}, 50 ng/µl poly(dI-dC), 0.05% NP-40 and 5 µg Caco2-BBE nuclear proteins for 15 min at room temperature. For the competition EMSA, we added 400-fold (8 pmol) of unlabeled paired oligonucleotides.
For the supershift EMSA, we added 2 µg related Sp1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-NF-κB antibody (Abcam, Cambridge, MA). Complexes were resolved by electrophoresis on native 5% TBE Criterion Precast Gels (Bio-Rad, Hercules, CA) in 0.5 x TBE buffer at 110 V/gel. Gels were semi-dry transferred to Biodyne B Pre-cut Modified Nylon Membranes (0.45 µM; Pierce, Rockford, IL), using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad, Hercules, CA). The membranes were then cross-linked (UVC-508 Ultraviolet Cross-linker; Ultra LUM, Claremont, CA), and visualized using the Chemiluminescent Nucleic Acid Detection System (Pierce, Rockford, IL).

**Chromatin immunoprecipitation assay**

Sp1 and NF-κB chromatin immunoprecipitation (ChIP) assays were performed using the ChIP Assay Kit (Upstate Cell Signaling Solutions, Lake Placid, NY), according to the manufacturer's instructions, with some modifications. Briefly, Caco2-BBE cells were grown to confluence, and then treated with or without interferon γ for 24 hours. The cells were then fixed with 1% formaldehyde for 10 minutes at 37° (to initiate cross-linking), scraped off the plate, washed with ice-cold PBS and resuspended in 200 µl of SDS lysis buffer for 10 minutes on ice. The cells were then sonicated with 3 sets of 12 s pulses at 35% power, to shear the DNA into 200-1000 bp fragments. The samples were centrifuged, and the supernatant (a sample of this was used as total DNA input) was diluted in ChIP dilution buffer and pre-cleared with a protein A Agarose/Salmon Sperm DNA slurry to reduce the non-specific background. The samples were then immunoprecipitated with rabbit anti-Sp1 (Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit anti-NF-κB antibody (Santa Cruz Biotechnology, Santa Cruz, CA) overnight at 4°C. The complexes were collected in a protein A Agarose/Salmon Sperm DNA slurry for 1 h at 4°C, and then washed once each with the provided low salt, high salt and LiCl wash buffers, and twice in TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). The immunoprecipitated chromatin was eluted from the protein A using freshly prepared elution buffer (100
mM NaHCO₃, 1% SDS), and the protein-DNA cross-links were reversed by treatment with NaCl (200 mM) at 65°C for 4 h. The DNA was purified by incubation with proteinase K at 45°C for 1 h, followed by phenol/chloroform extraction and ethanol precipitation with glycogen. The Sp1 (I, II, III/IV) and NF-kB promoter elements in the immunoprecipitates were detected by PCR using specific primers: see table 1: ISp1For, ISp1Rev (table 1 #43 and 44); IIISp1For, IIISp1Rev (table 1 #45 and 46); III/IV Sp1For, CD98 promoter reverse primer (table 1 #47 and 6); NF-kBFor (table 1 #48), NF-kBRev (table 1 #49). The products were resolved in a 1% agarose gel and visualized with ethidium bromide.

Transfection of small inhibitory RNA

Subconfluent (80%) Caco2-BBE cells plated on 6 well plates (Costar, Corning, NY) were transfected with siRNA duplexes directed against Sp1, NF-kB and a non-targeting siRNA was used as a control for non-sequence-specific effects of siRNAs (Ambion, Austin, TX) using Lipofectin (Invitrogen, Carlsbad, CA) in serum-free medium. Serum was added after 20 h, and 24 hours later Caco2-BBE underwent interferon γ for 24, then cells were used for Western blotting.

Western blot analysis

Cells were lysed in 1% (w/v) Triton X-100, 20 mM Tris, pH 8.0, 50 mM NaCl, 5 mM EDTA, 0.2% (w/v) bovine serum albumin and protease inhibitors (One tablet Complete, Mini /10 ml lysate buffer; Roche Diagnostic, Penzberg, Germany). The cell lysates (50 µg of total protein) were then boiled in sample buffers containing 2% SDS and 20% glycerol without β-mercaptoethanol (for non-reducing conditions) or with 10 mM β-mercaptoethanol (Reducing conditions to analyze CD98 expression as monomer) at 100°C for 5 min. The samples were resolved by 7.5% SDS-PAGE and transferred overnight at 4°C to 0.2 µm nitrocellulose membranes (Bio-Rad, Hercules, CA). The blots were blocked for 1 hour in blocking buffer (5% non-fat dry
milk, TBS and 0.1% tween-20), washed with blocking buffer, and then incubated for 1 hour at room temperature with 1:1000 dilutions of rabbit anti Sp1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or rabbit anti- NF-kB (p65) (Cruz Biotechnology, Santa Cruz, CA). The blots were then washed twice for 30 min in blocking buffer, and further incubated for 1 h at 37°C with the appropriate peroxidase-conjugated antibody diluted 1:1000. Finally, the blots were washed twice for 30 min in blocking buffer and then probed using an enhanced chemiluminescence system (ECL, Amersham, Piscataway, NJ).

**Statistical analysis**

Results are expressed as mean ± S.E. Data were evaluated by ANOVA when comparing multiple groups, using Tukey as a post-hoc test.
Results

DSS enhances CD98 mRNA expression in mice

As a preliminary step toward a functional study of CD98, we used real-time RT-PCR to examine relative CD98 mRNA expression in DSS-treated and –untreated mice. As shown in Figure 1A, colonic tissues from DSS-treated mice showed approximately 5-fold greater levels of CD98 transcript versus those from untreated mice. This result suggests that epithelial CD98 mRNA expression is enhanced by experimentally induced colitis in mice.

Interferon-γ transcriptionally enhances CD98 expression in Caco2-BBE cells

To determine whether or not interferon γ directly increased CD98 mRNA at the transcription level, we examined the effects of an RNA synthesis inhibitor, actinomycin D, on CD98 mRNA expression. Pretreatment of Caco2-BBE cells with actinomycin D significantly reduced CD98 mRNA expression because actinomycin D inhibited new production of mRNA (Figure 1B).

Cloning and characterization of the human CD98 promoter

To confirm the previously reported sequence and identify conserved elements within the promoters and 5` flanking regions of the human CD98 gene, we PCR amplified and sequenced a fragment of genomic DNA from Caco2-BBE and normal human colonic cells, spanning positions -1030 to +4 bp relative to the A of the CD98 gene start ATG (Figure 2). This fragment (Accession number DQ125482) was identical to the previously reported sequence from human HPB-MLT T-cell tumor cells (17). We then used the TFSEARCH software to identify putative transcription factor binding sites; those scoring > 95 were considered positive (data not shown). Our analysis indicated putative binding sites for the Sp1 (Stimulating
Protein 1), ADR1 (Alcohol Dehydrogenase gene Regulator 1), D1, Nkx-2 (Homeo domain factor Nkx-2), STRE (Stress-Response Element) and NF-κB (Nuclear Factor-kappaB) transcription factors, as well as common HSF (Heat Shock Factor) transcription factors. As previously reported (17), we identified four potential Sp1 binding sites, but no potential TATA or CAAT boxes. Thus, the CD98 promoter can be considered a TATA-less promoter with four GC-rich domains. The four potential GC-box/Sp1 transcription factor-binding elements included two consensus sequences (GGGGCGGG) at positions -187 and -177, one consensus sequence (GGGGTGGG) at position -874 and one consensus sequence (GGGGCAGG) at position -386 (Figure 2; Blue color). The consensus sequence (GGGCAGTCCCC) of the NF-κB transcription factor-binding site was located at position -213 (Figure 2; Green color).

Mapping the transcription start site (TSS) of the human CD98 gene

Primer extension experiments were carried out with total RNA from Caco2-BBE cells and colonic tissue (Fig. 3A). A single ~150 bp extension product was produced from the CD98 promoter reverse primer (table 1 #6) located downstream of the start ATG (Fig. 3A, see arrow), indicating the existence of a single transcription initiation site near nt 150. To determine the exact location of the transcription site of the human CD98 gene, we used 5'-RACE followed by PCR mapping of the human genomic DNA region surrounding the putative TSS. The 5'-RACE was performed on total RNA from Caco2-BBE cells with a primer corresponding to Primer CD98R2 (Table 1 #12), and subsequent PCR was performed using a primer corresponding to CD98R1 (see Table 1 #11). A single DNA fragment of 300 bp was produced (Fig. 3B). Sequencing revealed that this fragment mapped to the NCBI reference sequence 129 bp upstream of the translation initiation codon (ATG) (Fig. 3C), even though this ATG is not embedded in the optimal Kozak sequence. Our results indicated that this should be considered the transcription start site (TSS) of the CD98 gene, and its first base is referred to as nt - 128 hereafter in this work.
Functional mapping of the CD98 promoter basal and IFN-γ-stimulated activities

To localize the core promoter region for CD98 gene expression, we generated six partial deletion constructs of the ~1 kb region upstream of the start codon, fused with the luciferase reporter gene. These included full-length CD98 (nt -1030 to +4); I, -750 to +4; II, -350 to +4; III, -1030 to -750; IV, -1030 to -330; and V, -750 to -330. The transcriptional activities of these promoter-luciferase constructs were studied by transient transfection of human Caco2-BBE cells with the Dual-Luciferase Reporter Assay System (Promega, San Luis, CA). Transfected Caco2-BBE cells were left un-stimulated or were stimulated with 100 U/ml IFN-γ for 24 hours. The 1 kb wild-type CD98 promoter construct displayed both basal and IFN-γ-stimulated promoter activities of ~150-fold and 250-fold, respectively, compared to the control empty pGL3 basic vector (Fig. 4A and 4B). Transfection of cells with constructs I (-750, +4) and II (-330, +4) elicited 140-fold higher promoter activity than the pGL3 vector, a response that was very similar (93%) to that of the full-length CD98 promoter. In contrast, constructs III (-1030, -750), IV (-1030, -330) and V (-750, -330) had significantly lower promoter activities (Fig. 4A and 4B). Following IFN-γ stimulation, the promoter activities of constructs I (-750, +4), II (-330, +4) and full-length CD98 were similar (~250-fold increase) (Fig. 4A and 4B), while constructs III (-1030, -750), IV (-1030, -330) and V (-750, -330) did not show significant increases in promoter activity when compared to controls (Fig. 4A and 4B). Together, these results suggest that elements responsible for the basal and IFN-γ-stimulated promoter activities of the CD98 gene are contained in the full-length CD98 promoter, as well as deletion constructs I (-750, +4) and II (-330, +4) (Fig. 4A and 4B). As the minimal effective region contains two Sp1 binding sites (at the -187 and -177 positions) and the NF-κB binding site (-213), these results suggest that the Sp1 and NF-κB transcription factors are essential for basal and IFN-γ-stimulated CD98 promoter activities.
EMSA analysis of the putative Sp1 and NF-kB binding sites

To further confirm the importance of Sp1 and NF-kB for activation of the CD98 gene we used EMSA to characterize their binding to Sp1(-874) (Fig. 5A), Sp1(-386) (Fig. 5B), Sp1(-187) (Fig. 5C), Sp1(-177) (Fig. 5D) and the NF-kB binding site (Fig. 5E). Gel shifts were carried out with Caco2-BBE nuclear extracts as a source of protein, along with biotin-labeled double-stranded oligonucleotides containing the respective consensus-binding sites, mixed with a series of unlabeled double-stranded oligonucleotide competitors or relevant Sp1 and NF-kB antibodies supershift analysis. To ensure that the low mobility complexes we observed were sequence-specific, we also carried out EMSA experiments in the presence of a 400-fold molar excess of unlabeled competitor oligonucleotides corresponding to the wild-type or mutant boxes, or a random sequence. The unlabeled wild-type oligonucleotides markedly diminished formation of all three retardation complexes, whereas the random and mutant oligonucleotides did not, indicating that this effect was sequence-specific. Finally, gel shift analysis revealed that incubation of the DNA–protein complexes with anti-Sp1 or anti-NF-kB antibodies shifted the migrating bands in an upward direction, indicating the specificity to Sp1 and NF-kB proteins. Collectively, these results indicate the importance of Sp1 and NF-kB for activation of the CD98 gene.

In vivo analysis of the putative Sp1 and NF-kB binding sites in untreated and interferon-γ treated Caco2-BBE cells

To examine whether or not Sp1 and NF-kB binding sites are important in response to interferon γ in Caco2-BBE cells, we performed Chromatin immunoprecipitation analysis. As shown in Figure 6, Caco2-BBE at the resting level, transcription factor Sp1 binds to the ISp1 (lane 1), IISp1 (lane 3) and III/IVSp1 (lane 5) binding sites and NF-kB transcription factor bind to NF-kB binding site (lane 7). After interferon γ
treatment, the DNA binding activity of IISp1 (lane 2), III/IVSp1 (lane 6), NF-κB (lane 8) were increased. As controls, no band was detected in absence of DNA template. Together these results suggest that Sp1 and NF-κB may play a role in interferon γ induction of CD98 promoter activity.

The role of Sp1 and NF-κB- binding sites in basal and IFN-γ-stimulated CD98 promoter activities

To investigate the functional role of the three relevant binding motifs in regulating CD98 promoter activity, we generated various Sp1 and NF-κB binding mutants (see Table 1). We then used a transient reporter assay to compare the activities of the mutant and wild-type CD98 promoter constructs in the presence or absence of IFN-γ stimulation. Our results revealed that Sp1(-874)Mut and Sp1(-386)Mut had near wild-type basal and IFN-γ-stimulated promoter activities (Fig. 7), whereas Sp1(-187)Mut, Sp1(-177)Mut and NF-κB Mut showed significantly reduced basal and IFN-γ-stimulated promoter activities (by ~25%) (Fig. 7). Interestingly, when we examined double mutant constructs, NF-κB Mut/Sp1(-187)Mut, NF-κB Mut/Sp1(-177)Mut, Sp1(-187)Mut/Sp1(-177)Mut, and the triple mutant, NF-κB Mut/Sp1(-187)Mut/Sp1(-177)Mut, the basal and IFN-γ-stimulated promoter activities were further reduced by ~ 40% (Fig. 7). Together, these results demonstrate that the Sp1(-187), Sp1(-177) and NF-κB sites, but not the Sp1(-874) and Sp1(-386) sites play roles in basal and IFN-γ-stimulated CD98 promoter activities. Furthermore, the increased reduction of promoter activities in the double and triple mutants suggest that there may be additive effects among the Sp1(-187), Sp1(-177) and NF-κB sites or their binding partners in determining basal and IFN-γ-stimulated CD98 promoter activities.
Reduction of Sp1 or NF-kb expression reduced CD98 protein expression in un-stimulated and in stimulated Caco2-BBE cells by interferon γ.

One approach to study the functional role of a specific protein is to knock down its expression. As shown in Figure 8, we found that Caco2-BBE cells transfected with siRNA against Sp1 and NF-kB show lower expression of targeted proteins Sp1 (Sp1 lane 1) and NF-kB (NF-kB lane 5) when compared to Caco2-BBE transfected with the scrambled control Sp1 siRNA (Sp1 lane 4) and NF-kB (NF-kB lane 8). Interestingly, the basal CD98 protein expression was reduced in Caco2-BBE cells transfected with siRNA Sp1 (CD98 lane 1 vs CD98 lane 4) or NF-kB (CD98 lane 5 vs CD98 lane 8). Thus, siRNA-Sp1 and siRNA-NF-kB could effectively reduce the basal expression of CD98 protein expression in Caco2-BBE cells via the decrease of expression of their respective target proteins SP1 and NF-kB. It was of interest to investigate whether siRNA against Sp1 and/or against NF-kB reduced interferon γ induction of CD98 protein expression in Caco2-BBE. As shown in Figure 8, interferon γ stimulated Caco2-BBE cells transfected with Sp1 siRNA or NF-kB siRNA display lower CD98 protein expression (CD98 lane 2 and CD98 lane 6 respectively) when compared to interferon γ stimulated Caco2-BBE cells transfected with the scrambled control Sp1 siRNA (CD98 lane 3) and NF-kB (CD98 lane 7).
Discussion

CD98 is constitutively expressed by a variety of tissues, including intestinal epithelial cells (25,30,31). We previously demonstrated that DSS-induced colitis is a useful model for examining the role of CD98 in the colonic mucosa (25), and found that the effect of DSS on epithelial CD98 expression is mediated via IFN-\(\gamma\) (25). IFN-\(\gamma\) is present at high levels in irritable bowel disease (IBD) tissues, where it prepares enterocytes to function in host defense (16). Antibody-based inhibition of endogenous IFN-\(\gamma\) has been shown to ameliorate the chronic stage of colitis, indicating that IFN-\(\gamma\) is likely to act as a key mediator for intestinal inflammation (16). Here, we showed that CD98 expression is activated at the transcriptional level in interferon \(\gamma\)-treated intestinal epithelial cells, and examined the mechanisms responsible for IFN-\(\gamma\)-mediated activation of CD98 gene transcription in the colonic epithelium.

We first isolated and functionally characterized the 5’ flanking region of the CD98 gene from the Caco2-BBE epithelial cell line. Computer analysis indicated the presence of four GC/GT boxes potentially capable of binding Sp1 transcription factors, along with an NF-\(\kappa\)B transcription factor binding site. The latter result is in agreement with the cloned CD98DNA sequence analysis from HPB-MLT human T-cell tumor line that revealed that the 5’ flanking region of the gene encoding CD98 contains a hypomethylated CpG island and four potential binding sites for Sp1 transcription factors, but did not contain TATA or CCAAT boxes (24). Both competition EMSA and antibody supershift experiments revealed that Sp1 and NF-\(\kappa\)B both interacted with the promoter region. We found that different Sp1 binding sites generated different DNA-protein interaction profiles that suggest that each of these binding sites have distinct functional properties. Furthermore, chromatin immunoprecipitation studies show in vivo DNA-Sp1 and DNA-NF-kB interactions in the basal and interferon \(\gamma\) stimulated conditions. The 5’ flanking region does not contain TATA or CCAAT boxes, and primer extension and RACE assays revealed that the major transcriptional initiation site was located 129 bases upstream from the first ATG codon. This is consistent
with the previous identifications of single start sites for TATA-less promoters in other genes, including the genes encoding thymidine kinase (10), dihydrofolate reductase (1) and adenine deaminase (46).

Transfection of Caco2-BBE cells with luciferase reporter constructs fused to the CD98 gene promoter region or its serially truncated derivatives revealed that specific DNA regulatory elements are located within 0.33 kb of the transcription start site. This region contains the NF-κB site at -213, along with Sp1(-187) and Sp1(-177), suggesting that these three sites are important for basal and IFN-γ-stimulated transcriptional activity of CD98 in the tested intestinal cell line. These findings are consistent with previous studies indicating that IFN-γ treatment can enhance NF-κB binding (32) and activity (4,33), and that IFN-γ treatment can induce formation of an Sp1-like complex in HT-29 cells (8). However, this is the first report showing that IFN-γ increases the colonic human CD98 promoter activity.

Sp1 was originally identified as a ubiquitous transcription factor implicated in the constitutive expression of several genes (40). More recently, the Sp1 family been shown to be involved in inducible gene transcription in response to stimulators such as glucose (3,21), epidermal growth factor (28) and the membrane glycoprotein CD14 (29). Furthermore, increasing evidence suggests that the Sp1 family plays an important regulatory role during proliferation and differentiation (23,26). NF-κB, a heterodimeric protein complex consisting of 50 kDa and 65 kDa subunits, plays crucial roles in the regulation of immunoglobulin gene expression and the response to cellular activation (2). Interestingly, the increased transcriptional repression seen in our double and triple deletion mutants suggests that the NF-κB, Sp1(-187) and Sp1(-177) sites may cooperate in mediating basal and IFN-γ-stimulated CD98 transcription. Interaction between NF-KB and Sp1 has been reported to be important and may serve as a regulatory mechanism to activate specific viral and cellular genes (18, 38,39). Accordingly, we demonstrated that reduction of Sp1 or NF-kb expression reduced CD98 protein expression in un-stimulated and in interferon γ stimulated Caco2-BBE.
It has been demonstrated that the increased epithelial expression of cell surface proteins in patients suffering IBD occurs through a number of different pathways, including the production of cytokines by activated mucosal lymphocytes. Interferon gamma is present at high levels in IBD tissues. Here, we show that interferon gamma transcriptionally enhances CD98 glycoprotein expression via the Sp1 and NF-kB transcription factors in intestinal epithelial cells. We suggest that during intestinal inflammation CD98 expression is up-regulated in immune cells but also in intestinal epithelial cells. The up-regulation of CD98 in intestinal epithelial cells could affect functions such as β1 integrin mediated events (14, 20), that have been implicated in the etiology of various pathologic conditions, including inflammatory disorders such as inflammatory bowel disease.
ACKNOWLEDGMENTS

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Reference:


Figure Legends

Figure 1. A) **CD98 mRNA expression is increased in colonic tissues from DSS-treated mice**

Quantification of CD98 transcripts in intestinal tissues from mice treated with or without DSS. For graphical representation of quantitative PCR data, the raw cycle threshold values (Ct values) obtained from the experimental groups were deducted from the Ct value obtained from the control group using the ΔΔCt method, and the methods were normalized using $2^{-\Delta \Delta Ct}$, with β-actin gene levels serving as the internal standard. Values are normalized as fold-differences relative to transcript levels in untreated controls. The presented results are representative of three different experiments. B) Interferon-γ transcriptionally enhances CD98 expression in Caco2-BBE cells. Quantification of CD98 transcripts were performed, as described above, in Caco2-BBE cells that were untreated (-) or pretreated (+) with 2µg/ml actinomycin D for 4 hours and then treated with 100U/ml interferon γ (+) or vehicle (-) for 24 hours.

Figure 2. **Sequence of the cloned 5'-flanking region of the human CD98 gene**

Putative transcription factor binding sites were identified by the TFsearch program. Our analysis indicated putative binding sites for the Sp1 (Stimulating Protein 1), ADR1 (Alcohol Dehydrogenase gene Regulator 1), D1, Nkx-2 (Homeo domain factor Nkx-2), STRE (Stress-Response Element) and NF-κB (Nuclear Factor-kappaB) transcription factors, as well as common HSF (Heat Shock Factor) transcription factors. We identified four potential Sp1 binding sites, but no potential TATA or CAAT boxes. Thus, the CD98 promoter can be considered a TATA-less promoter with four GC-rich domains. The four potential GC-box/Sp1 transcription factor-binding elements included two consensus sequences (GGGGCGGGG) at positions -187 and -177, one consensus sequence (GGGGTGCGG) at position -874 and one consensus
sequence (GGGGCAGG) at position -386 (Figure 2; Blue color). The consensus sequence (GGGCAGTCCCC) of the NF-κB transcription factor-binding site was located at position -213 (Figure 2; Green color). Two gene-specific primers (CD98_{R1} and CD98_{R2}) for 5’-Rapid Amplification of cDNA Ends. The source of additional nucleotide sequence downstream the position +4 was provided by the cloning of the CD98 coding sequence from Caco2-BBE cells that is identical to the one reported under the accession # J02939.

Figure 3. *Localization of the transcription initiation start site*

(A) Mapping of the transcription initiation start site by primer extension analysis. An oligonucleotide complementary to the translation initiation start site of the CD98 mRNA was 5'-end labeled with ^{32}\text{P} and hybridized to 20 μg and 10 μg of total RNA extracted from Caco2-BBE cells (lanes 1 and 2, respectively) and human colon tissue (lanes 3 and 4, respectively). The cDNA was extended as described in Materials and Methods and then analyzed on a 5% TBE gel. Lane M shows molecular weight marker. (B). Gel electrophoresis of PCR products. Human Caco2-BBE cell cDNA was re-transcribed by the gene-specific primer, CD98 RACE 2, and another PCR step was applied to identify the transcriptional start site of the human CD98 gene, using Abridged Universal Amplification Primer (AUAP) as the sense DNA primer complementary to 5' RACE abridged anchor primer (AAP), which is a poly G primer (g-oligo) and CD98 RACE 1 as the gene-specific antisense primer. The nested PCR products were electrophoresed on 1% agarose gels and stained with ethidium bromide. Lane M, 100 bp DNA ladder (molecular size marker); lane 1, 5’ RACE PCR product. (C) Mapping the transcript start site of CD98. Gel purified 5' RACE PCR products were cloned into PCR2.1 and sequenced. The Chromas software package was used for sequence analysis.
Figure 4. (A) Schematic representation of the human CD98 gene promoter constructs. The utilized CD98 gene promoter constructs contained sequences corresponding to the full-length promoter, -1030 to +4 (CD98), as well as various deleted fragments (I, -750 to +4; II, -330 to +4; III, -1030 to -750; IV, -1030 to -330; and V, -750 to -330), inserted upstream of the luciferase coding region (represented as black) in the pGL3-basic plasmid. The numbers are given in relation to the translational start codon (+1), and indicate the 5' ends of the deletion constructs. The location of the identified positive regulatory region is indicated by a green box. The positions of the putative SP1 and NF-κB sites are indicated by arrows. (B) Functional activities of the 5' deleted promoter constructs in unstimulated (Non-treated) or IFN-γ-stimulated (IFN-γ-treated) Caco2-BBE cells. The luciferase activities for Caco2-BBE cells transfected with each promoter construct were normalized to the Renilla luciferase activities driven by the phRL-CMV control vector. Activities are expressed as fold-induction over that seen in cells transfected with empty pGL3-basic vector. Each value represents the mean ±S.D. of at least three independent sets of transfection experiments performed in triplicate. The gray bar represents the mean relative value in the absence of IFN-γ treatment, while black bars show the mean relative values in the presence of IFN-γ treatment. *P<0.05, **P<0.01 and ***P<0.001 vs full-length promoter with or without IFN-γ treatment.

Figure 5. **EMSA-based characterization of the Sp1 and NF-κB binding sites within CD98 promoter**

The binding of the four Sp1 sites and NF-κB site [A, Sp1(-874), Oligonucleotides used: GC1_1 and GC1_2 (wild type), GC1_1M and GC1_2M (Mutant); B, Sp1(-386), Oligonucleotides used: GC2_1 and GC2_2 (wild type), GC2_1M and GC2_2M (Mutant); C, Sp1(-187), Oligonucleotides used: GC3_1 and GC3_2 (wild type) and GC3_1M and GC3_2M (Mutant); D, Sp1(-177), Oligonucleotides used: GC4_1 and GC4_2 (wild type), GC4_1M and GC4_2M (mutant) ; E, NF-κB, Oligonucleotides used: NF-kB_1 and NF-kB_2 (wild type), NF-kB_1M and NF-kB_2M (Mutant)] were analyzed by EMSA. Lane 1, biotin-labeled oligonucleotide alone; lane
2, biotin-labeled oligonucleotides incubated with 5 µg Caco2-BBE nuclear extracts; lane 3, biotin-labeled oligonucleotides incubated with 5 µg Caco2-BBE nuclear extracts in the presence of anti-Sp1 (A, B, C, D) or -NF-kB (E) antibodies; lane 4, biotin-labeled oligonucleotides incubated with 5 µg Caco2-BBE nuclear extracts in the presence of a 200-fold excess of cold competitor oligonucleotide; lane 5, biotin-labeled oligonucleotides incubated with Caco2-BBE 5 µg nuclear extracts in the presence of a 200-fold excess of non-specific competitor oligonucleotide; lane 6, biotin-labeled binding site-mutated oligonucleotides incubated with 5 µg Caco2-BBE nuclear extracts; lane 7, biotin-labeled binding site-mutated oligonucleotides incubated with 5 µg Caco2-BBE nuclear extracts in the presence of cold competitor oligonucleotide. In figure 5A, lanes 8 to 10 are the controls provided with the kit; lane 8, biotin-EBNA control DNA; lane 9, biotin-EBNA control DNA + EBNA extract; lane 10, biotin-EBNA control DNA + EBNA extract + unlabeled EBNA DNA.

Figure 6. **Sp1 and NF-kB may play a role in interferon γ induction of CD98 promoter activity.** ChIP assays were performed as described in the “Material and Methods”. The antibodies indicated were incubated with crosslinked DNA isolated from Caco2-BBE cells that had been treated in the absence (-) or in the presence (+) of interferon γ. The Sp1 (I, II, III/IV) and NF-kB promoter elements in the immunoprecipitates were detected by PCR using specific primers as described in the table 1.

Figure 7. **Effects of GC box mutations on CD98 promoter activity**

Arrows depict the mutations introduced into the CD98 promoter constructs. The various GC boxes were mutated singly or in combination, and the expression constructs were transiently transfected into Caco2-BBE cells. (A) Basal or IFN-γ-stimulated CD98 promoter activities of the full-length wild-type construct
were set to 100% (Control). The presented values represent the mean ±S.D. of at least three independent sets of transfection experiments performed in triplicate. *P<0.05, **P<0.01 and ***P<0.001 vs controls.

**Figure 8. Reduction of Sp1 or NF-kb expression reduced CD98 protein expression in un-stimulated and in Caco2-BBE cells stimulated by interferon γ.** Caco2-BBE cells were transfected with the indicated siRNA and then cells were un-stimulated or stimulated by interferon γ for 24 hours as described in the “Material and Methods”. Cells were harvested and subjected to Western blotting using the Sp1, NF-kB(p65) and CD98 antibodies described in the “Material and Methods”. As a loading control, β-actin was also detected in the same membrane using an anti-β-actin antibody. The siRNA-Ctrl used was an siRNA-scramble sequence from Ambion.
Table 1: Primers and oligonucleotides

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Figure 3

A

B

C

Primer Extension

M: DNA marker
1: RNA from Case 2-808 cell (20 μg)
2: RNA from Case 2-808 cell (10 μg)
3: RNA from Colon tissue (20 μg)
4: RNA from Colon tissue (10 μg)

M: DNA marker
1: SYBR-PCR products
Figure 6

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DNA input

[Image of gel electrophoresis with bands indicating binding and interference results]
Figure 8

[Diagram showing protein expression levels for Sp1, CD8, and β-actin with different conditions marked as 1, 2, 3, 4, 5, 6, 7, and 8.]

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