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Characterization of the human intestinal CD98 promoter and its regulation by interferon- γ

Yutao Yan, Guillaume Dalmasso, Shanthi Sitaraman, and Didier Merlin

Division of Digestive Diseases, Department of Medicine, Emory University School of Medicine, Atlanta, Georgia

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Yan Y, Dalmasso G, Sitaraman S, Merlin D. Characterization of the human intestinal CD98 promoter and its regulation by interferon- γ . *Am J Physiol Gastrointest Liver Physiol* 292: G535–G545, 2007. First published October 5, 2006; doi:10.1152/ajpgi.00385.2006.—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* experiments 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 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 stimulating protein (Sp)1 binding domains, one NF- κ B binding domain, and no TATA box. Binding of Sp1 [Sp1(–874), SP1(–386), Sp1(–187), and Sp1(–177)] and NF- κ B [NF- κ B(–213)] to the promoter was confirmed by EMSA and supershift assays. Furthermore, chromatin immunoprecipitation experiments showed the *in vivo* DNA-Sp1 and DNA-NF- κ B interactions under basal and IFN- γ -stimulated conditions. Reporter genes driven by serially truncated and site-mutated CD98 promoters were used to examine basal and IFN- γ -responsive transcription in transiently transfected Caco2-BBE cells. Our results revealed that Sp1(–187), Sp1(–177), and the NF- κ B binding site were essential for basal and IFN- γ -stimulated CD98 promoter activities, whereas Sp1(–874) and Sp1(–386) were not. The results from additional site-mutated CD98 promoters suggested that Sp1(–187), Sp1(–177), and the NF- κ B site may cooperate in mediating basal and IFN- γ -stimulated CD98 promoter activities. Finally, we demonstrated that a reduction of Sp1 or NF- κ B expression reduced CD98 protein expression in unstimulated and IFN- γ -stimulated Caco2-BBE cells. Collectively, these findings indicate that the Sp1 and NF- κ B 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.

Caco2-BBE; mice; promoter; stimulating protein 1; nuclear factor- κ B

THE CD98 cell surface molecule is a 125-kDa type II membrane glycoprotein heterodimer composed of a 40-kDa nonglycosylated 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), and testis (37), and in intestinal epithelial cells (30, 31) and a wide variety of tumor cells, including hepatomas, breast cancer, and colon cancer (35, 41, 45).

In the context of intestinal inflammation, CD98 protein expression in the 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 inflammatory bowel disease (IBD) (16). In addition, 5-aminosalicylic acid (which is used for the 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 (25) have reported that epithelial CD98 also plays an important role in intestinal inflammation.

Dextran sodium sulfate (DSS) is often used to induce experimental colitis in animal models (11). We (25) 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 . Consistent with this, we (25) also found that epithelial protein expression of CD98 was enhanced by IFN- γ treatment of Caco2-BBE cells *in vitro*. DSS is believed to cause colitis by interfering with intestinal barrier function and/or stimulating local inflammation within the colon, via upregulation of both T helper type 1 (Th1) and type 2 (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 have strongly suggested that IFN- γ is capable of regulating CD98 expression, the mechanism(s) governing this activation remains unknown. Here, we characterized the 5'-flanking region of the colonic human CD98 gene promoter and investigated its mechanism of transcriptional activation by IFN- γ .

MATERIALS AND METHODS

Cell culture. Caco2-BBE (5–7) cells (passages 30–50) were grown in high-glucose DMEM (Invitrogen, Carlsbad, CA) supplemented with 14 mmol/l NaHCO₃ and 10% newborn calf serum. Cells were kept at 37°C in a humidified atmosphere containing 95% air–5% CO₂, and the medium was changed daily. Monolayers were subcultured every 7 days with 0.1% trypsin and 0.9 mmol/l EDTA in Ca²⁺/Mg²⁺-free PBS.

Address for reprint requests and other correspondence: D. Merlin, Div. of Digestive Diseases, Dept. of Medicine, Emory Univ., 615 Michael St., Atlanta, GA 30322 (e-mail: dmerlin@emory.edu).

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Animals. All experiments were carried out in female C57BL/6 mice (8 wk, 18–22 g) obtained from Jackson Laboratories (Bar Harbor, ME). Mice were group housed under a controlled temperature (25°C) and photoperiod (12:12-h 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 the experiments.

Induction of colitis and study design. Colitis was induced in all groups by the addition of DSS [40,000 Da, 3% (wt/vol), ICN Biochemicals, Aurora, OH] to the drinking water. The mean DSS-water consumption was recorded for each group. Two groups of mice ($n = 6$ mice/group) were treated for 7 days with DSS or regular water. Body weights were assessed every day during the treatment period.

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 previously

described (25). Total RNA was extracted from untreated or DSS-treated mice colonic cells and from untreated or IFN- γ -treated Caco2-BBE cells using TRIzol reagent (Invitrogen) and reverse transcribed using the ThermoScript RT-PCR System (Invitrogen). The real-time iCycler sequence detection system (Bio-Rad, Hercules, CA) was used to quantify first-strand CD98 cDNA in the presence or absence of DSS/IFN- γ in mouse colonic or Caco2-BBE cells. Briefly, 50 ng reverse-transcribed cDNA, 10 μ M gene-specific primers (CD98_{HQF}, CD98_{HQR}, CD98_{MQF}, or CD98_{MQR}; see Table 1), and iQ SYBR Green Supermix (Bio-Rad) 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. β -Actin expression levels were used as a reference, and fold inductions were calculated by the relative standard curve method. For graphical representation of quantitative PCR data, raw threshold cycle (C_t) values obtained for DSS/IFN- γ -treated cells were deducted from the C_t values obtained for internal β -actin transcript levels using the

Table 1. Primers and oligonucleotides

Primer	Oligonucleotides
CD98 _{HQF}	5'-TCTTGATTGCGGGGACTAAC-3'
CD98 _{HQR}	5'-GCCTTGCCCTGAGACAACTC-3'
CD98 _{MQF}	5'-CGACCTCTCAGGATCTGCTGAAGGT-3'
CD98 _{MQR}	5'-TTCGGGACATAGAGAATCTGAAGGA-3'
CD98 _{promoter} forward primer	5'-GCAAGGTGGGTAAAGAGTCTGGCCATG-3'
CD98 _{promoter} reverse primer	5'-CATGGTGCCTGCAGAACCGGGACAC-3'
Primer 1	5'-CTCACTACAATACTTTGCTTT-3'
Primer 2	5'-AAAGCAAAGTATTGTAGTGAG-3'
Primer 3	5'-ACCTGATCTTGGGAAGGCCTG-3'
Primer 4	5'-CAGGCCCTCCCAGAATCAGGT-3'
CD98 _{R1}	5'-TTGATCTTCAACAGACCATTCTTCT-3'
CD98 _{R2}	5'-CATCCTTCTGGTTCTTGTGAATT-3'
GC-rich domain I For	5'-AGGTGCTAGCAGCTGGGGGTAATGTAGGATAAGAAG-3'
GC-rich domain I Rev	5'-CTTCTTATCCTACATTAACCCAGCTGCTAGCACCT-3'
GC-rich domain II For	5'-AGGGCCCTCGGATTTGGGTAAGGATCACAGGCCTAGT-3'
GC-rich domain II Rev	5'-ACTAGGCCTGTGATCCTTAACCCAAATCCCAGGCCCT-3'
GC-rich domain III For	5'-TTCCACCTTAAGGGGCTAACCGGGGGGGGCTCCGCTG-3'
GC-rich domain III Rev	5'-CAGCGGAGCCCCCGCCGGTTAGCCCCCTAAGGTGGAA-3'
GC-rich domain IV For	5'-TTAAGGGGCGGGCCGGGCTAAGCTCCGCTGCCCTTC-3'
GC-rich domain IV Rev	5'-GAAGGGGACGGGAGCTTAGCCCCGGCCGCCCTTAA-3'
NF- κ B _{binding} domain For	5'-CGCCACGGCTGGGGCAGTTACCGAGGTTCCACCTTAA-3'
NF- κ B _{binding} domain Rev	5'-TTAAGGTGGAACCTCGGTAAGTCCCGCCAGCCGTGGGCG-3'
GC1 _{w1}	5'-TAGCAGCTGGGGTGGGGTAGGA-3'
GC1 _{w2}	5'-TCCTACCCACCCAGCTGCTA-3'
GC1 _{M1}	5'-TAGCAGCTGGGGTAAATGTAGGA-3'
GC1 _{M2}	5'-TCCTACATTACCCAGCTGCTA-3'
GC2 _{w1}	5'-TGGGATTTGGGGCAGGATCACA-3'
GC2 _{w2}	5'-TGTGATCCTGCCCAAATCCCGA-3'
GC2 _{M1}	5'-TGGGATTTGGGTAAGGATCACA-3'
GC2 _{M2}	5'-TGTGATCCTTACCCAAATCCCGA-3'
GC3 _{w1}	5'-CCACCTAAGGGGCGGGCCGGG-3'
GC3 _{w2}	5'-CCCCGGCCGCCCTTAAGGTGG-3'
GC3 _{M1}	5'-CCACCTAAGGGGCTAACCGGGG-3'
GC3 _{M2}	5'-CCCCGGTAGCCCTTAAGGTGG-3'
GC4 _{w1}	5'-GGGGCGGGCTCCGCTGCCCTT-3'
GC4 _{w2}	5'-AAGGGGACGGGAGCCCCGCCCC-3'
GC4 _{M1}	5'-GGGGCTAAGCTCCGCTGCCCTT-3'
GC4 _{M2}	5'-AAGGGGACGGGAGCTTAGCCCC-3'
NF- κ B _{w1}	5'-ACGGCTGGGGCAGTCCCGAGGTT-3'
NF- κ B _{w2}	5'-AACCTCGGGACTGCCCGAGCCGT-3'
NF- κ B _{M1}	5'-ACGGCTGGGGCAGTTACCGAGGTT-3'
NF- κ B _{M2}	5'-AACCTCGGTAAGTCCCGAGCCGT-3'
I Sp1 For	5'-GCCCTATCAGTCTCTTCTGGATGTC-3'
I Sp1 Rev	5'-GTTAACTTTATTGAATTTAACAC-3'
II Sp1 For	5'-GGAAGAAGAGGGCGTTTGGGGG-3'
II Sp1 Rev	5'-CCTGTCTAACCTTAAAGTTCTTT-3'
III/IV Sp1 For	5'-GGGTTAGACAGGTTGGAGATGAG-3'
NF- κ B For	5'-TTGGACCATGCAGGAAGTACTGT-3'
NF- κ B Rev	5'-GCATCTGCTCAGCAGCAGGCGCG-3'

See text for details. Underlined sequences indicate mutations.

$\Delta\Delta C_t$ method as follows: $\Delta\Delta C_t = (C_{t,target} - C_{t,\beta-actin})_{treatment} - (C_{t,target} - C_{t,\beta-actin})_{nontreatment}$, and final data were derived from $2^{-\Delta\Delta C_t}$ (Livak). Quantification of CD98 transcripts was performed, as described above, in Caco2-BBE cells that were untreated or pretreated with 2 $\mu\text{g/ml}$ actinomycin D for 4 h and then stimulated with 100 U/ml IFN- γ or vehicle (-) for 24 h.

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) using CD98_{promoter forward primer} and CD98_{promoter reverse primer} shown in Table 1 based on the genomic sequence of the CD98 gene (Accession No. NT_033903). PCR conditions consisted of 95°C for 5 min, followed by 34 cycles of 95°C for 10 s, 55°C for 1 min, and 72°C for 3 min, with a final extension at 72°C for 10 min. The expected 1,034-bp-long fragment was obtained. Gel-purified fragments (Qiaquick Gel Extraction Kit, Qiagen, Valencia, CA) were cloned into the PCR2.1/TOPO vector (Invitrogen) and confirmed by sequencing. The plasmid was then digested with *KpnI* and *XhoI* (New England Biolabs, Ipswich, MA), subcloned into the promoterless pGL3 firefly luciferase basic vector (Promega, San Luis, CA), and confirmed by sequencing. Truncated constructs were generated by PCR using the full-length sequence as a template along with the following primers (see Table 1): CD98 promoter I_(-750,+4), *primer 2* and CD98_{promoter reverse primer}; CD98 promoter II_(-330,+4), *primer 4* and CD98_{promoter reverse primer}; CD98 promoter III_(-1031,-750), *primer 1* and CD98_{promoter forward primer}; CD98 promoter IV_(-1030,-330), *primer 3* and CD98_{promoter forward primer}; and CD98 promoter V_(-750,-330), *primer 2* and *primer 3*. Mutations in CD98 promoter GC boxes and the NF- κ B 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 primers GC^{-rich domain I} For and GC^{-rich domain I} Rev; the second GC-rich domain mutant with primers GC^{-rich domain II} For and GC^{-rich domain II} Rev; the third GC-rich domain mutant with primers GC^{-rich domain III} For and GC^{-rich domain III} Rev; and the fourth GC-rich domain mutant with primers GC^{-rich domain IV} For and GC^{-rich domain IV} Rev (see Table 1). The NF- κ B binding domain mutant was generated with primers NF- κ B_{binding domain} For and NF- κ B_{binding domain} Rev (see Table 1). 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, cells were rinsed and then incubated with 1,000 μl Opti-MEM (GIBCO, Invitrogen), 5 ng *Renilla* (pRL-CMV), 2 μg of the relevant CD98 promoter constructs, and 10 $\mu\text{g/ml}$ Lipofectin (Invitrogen). In each experiment, cells were incubated with the same total amount of plasmid DNA. After 24 h, cells were washed twice with PBS and lysed with the provided buffer (Luciferase Assay Kit, Promega). Subsequently, Caco2-BBE cells were stimulated with 1,000 U/ml IFN- γ or vehicle for 24 h. For the luciferase activity assay, 20 μl cell extract was mixed with 100 μl luciferase assay substrate (Promega), 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 pRL-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 the figures is expressed as the percentage of the activity obtained

with regard to the most active construct used in the figures. 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 the 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 was extracted from Caco2-BBE cells and normal human colonic mucosal biopsies using TRIzol reagent (Invitrogen) as recommended by the manufacturer. The integrity of the purified total RNA was determined by formaldehyde agarose gel electrophoresis. CD98_{promoter reverse primer} (see Table 1), located upstream of the translation start site (TSS), was labeled with 6,000 $\mu\text{Ci/mmol}$ [γ -³²P]dATP (Perkin-Elmer, Boston, MA) using the KinaseMarx kit (Ambion, Austin, TX) and purified using NucAway Spin Columns (Ambion). The 100-bp marker (New England Biolabs) was radiolabeled with the same isotope just before use. For primer extension, reactions consisting of 10 or 20 μg total RNA, 1 pmol radiolabeled primer, 10 μl of 2 \times AMV primer extension buffer, 2 mM sodium pyrophosphate, and 1 μl (15 units) AMV reverse transcriptase (Invitrogen) were heated to 60°C for 20 min for annealing and then cooled to room temperature for 10 min. The mixture was then incubated at 42°C for 45 min, and the reaction was stopped by the addition of 20 μl loading dye. Samples were then heated at 90°C for 10 min and loaded directly onto 5% Tris-borate-EDTA (TBE) gels (Bio-Rad). Gels were run at 200 V for 90 min, vacuum dried, and autoradiographed overnight at -80°C.

Rapid amplification of 5'-cDNA ends. Initial rapid amplification of 5'-cDNA ends (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) as recommended by the manufacturer. Briefly, gene-specific reverse primers were designed to be complementary to nt 113–137 and 486–508 of the CD98 gene (CD98_{R1} and CD98_{R2}, respectively; Table 1). Primary and nested PCRs were performed using Platinum *Taq* DNA polymerase (Invitrogen). The products of the nested PCR were cloned into the PCR 2.1 TOPO vector (Invitrogen) and then sequenced.

Nuclear protein extraction. Caco2-BBE cells were washed once in ice-cold 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 MgCl₂, and 0.1% Nonidet P-40; pH 7.9 at 4°C) for 10 min on ice. For the isolation of nuclei, the lysate was vigorously mixed and microcentrifuged for 5 min at 12,000 g and 4°C, and the nuclear pellet was washed once with 1 ml of Nonidet P-40-free lysis buffer. For the extraction of nuclear proteins, the nuclear pellet was resuspended in 1 ml of protein extraction buffer (420 mM NaCl, 20 mM HEPES, 1.5 MgCl₂, 0.2 mM EDTA, and 25% glycerol; pH 7.9) for 10 min at 4°C. After being vigorously mixed, the nuclear suspension was microcentrifuged for 5 min at 4°C, the pellet was discarded, and the supernatant was microcentrifuged (12,000 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). DTT (0.5 mM), PMSF (0.5 mM), and leupeptin (10 $\mu\text{g/ml}$) 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 PMSF. Samples were stored at -70°C until use.

EMSA. Probes for EMSAs were prepared using a standard protocol. The primers are shown in Table 1. In detail, GC1_{W1} and GC1_{W2} are complementary oligonucleotides for the first Sp1 binding site, GC1_{M1} and GC1_{M2} are complementary oligonucleotides for the first Sp1 binding site mutant, GC2_{W1} and GC2_{W2} are complementary oligonucleotides for the second Sp1 binding site, GC2_{M1} and GC2_{M2} are complementary oligonucleotides for the second Sp1 binding site mutant, GC3_{W1} and GC3_{W2} as well as GC3_{M1} and GC3_{M2} correspond to the third Sp1 binding site and its mutant; GC4_{W1} and GC4_{W2} as

well as GC_{4M1} and GC_{4M2} correspond to the fourth Sp1 binding site and its mutant, and primers NF- κ B_{W1} and NF- κ B_{W2} as well as NF- κ B_{M1} and NF- κ B_{M2} correspond to the NF- κ B binding site and its mutant (Table 1). Probes were synthesized with HPLC purification and represent the transcription factor binding sites, and they were first end labeled with a Biotin 3' End DNA Labeling Kit (Pierce). Briefly, 0.2 U/ μ l of terminal deoxynucleotidyl transferase (TdT), 0.5 μ M biotin-11-UTP, and 100 nM of each single-stranded oligonucleotide were incubated in 1 \times TdT reaction buffer for 30 min at 37°C. EDTA (2.5 μ l of 0.2 M) was added to stop the reaction, and 50 μ l 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 min. 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). Briefly, 20 fmol of each biotin end-labeled target oligonucleotide pair were incubated in EMSA binding buffer (100 mM Tris, 500 mM KCl, and 10 mM DTT; pH 7.5) containing 2.5% glycerol, 5 mM MgCl₂, 50 ng/ μ l poly(dI-dC), 0.05% Nonidet P-40, and 5 μ g 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 μ g related Sp1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and anti-NF- κ B antibody (Abcam, Cambridge, MA). Complexes were resolved by electrophoresis on native 5% TBE Criterion Precast Gels (Bio-Rad) in 0.5 \times TBE buffer at 110 V/gel. Gels were semidry transferred to Biotinylated Pre-Cut Modified Nylon Membranes (0.45 μ M, Pierce) using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad). Membranes were then cross-linked (UVC-508 Ultraviolet Cross-linker, Ultra LUM, Claremont, CA) and visualized using the Chemiluminescent Nucleic Acid Detection System (Pierce).

Chromatin immunoprecipitation assay. Sp1 and NF- κ B 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 IFN- γ for 24 h. Cells were then fixed with 1% formaldehyde for 10 min at 37°C (to initiate cross-linking), scraped off the plate, washed with ice-cold PBS, and resuspended in 200 μ l of SDS lysis buffer for 10 min on ice. Cells were then sonicated with three sets of 12-s pulses at 35% power to shear the DNA into 200- to 1,000-bp fragments. Samples were centrifuged, and the supernatant (a sample of this was used as total DNA input) was diluted in ChIP dilution buffer and precleared with a protein A agarose-salmon sperm DNA slurry to reduce the nonspecific background. Samples were then immunoprecipitated with rabbit anti-Sp1 (Santa Cruz Biotechnology) or rabbit anti-NF- κ B antibody (Santa Cruz Biotechnology) overnight at 4°C. Complexes were collected in a protein A agarose-salmon sperm DNA slurry for 1 h at 4°C, washed once each with the provided low-salt, high-salt, and LiCl wash buffers, and then washed twice in Tris-EDTA buffer [10 mM Tris·HCl (pH 8.0) and 1 mM EDTA]. The immunoprecipitated chromatin was eluted from protein A using freshly prepared elution buffer (100 mM NaHCO₃ and 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. Sp1 (I, II, and III/IV) and NF- κ B promoter elements in immunoprecipitates were detected by PCR using the following specific primers (see Table 1): I Sp1 For and I Sp1 Rev, II Sp1 For and II Sp1 Rev, III/IV Sp1 For and CD98_{promoter} reverse primers, and NF- κ B For and NF- κ B Rev. The products were resolved on a 1% agarose gel and visualized with ethidium bromide.

Transfection of small inhibitory RNA. Subconfluent (80%) Caco2-BBE cells plated on six-well plates (Costar, Corning, NY) were transfected with small inhibitory (si)RNA duplexes directed against

Sp1 and NF- κ B, and a nontargeting siRNA was used as the control for nonsequence-specific effects of siRNAs (Ambion) using Lipofectin (Invitrogen) in serum-free medium. Serum was added after 20 h, and, 24 h later, Caco2-BBE cells underwent IFN- γ treatment for 24 h; cells were then used for Western blot analysis.

Western blot analysis. Cells were lysed in 1% (wt/vol) Triton X-100, 20 mM Tris (pH 8.0), 50 mM NaCl, 5 mM EDTA, 0.2% (wt/vol) BSA, and protease inhibitors (One Tablet Complete, Mini/10 ml lysate buffer, Roche Diagnostic, Penzberg, Germany). Cell lysates (50 μ g total protein) were then boiled in sample buffers containing 2% SDS and 20% glycerol without β -mercaptoethanol (for nonreducing conditions) or with 10 mM β -mercaptoethanol (reducing conditions to analyze CD98 expression as a monomer) at 100°C for 5 min. Samples were resolved by 7.5% SDS-PAGE and transferred overnight at 4°C to 0.2- μ m nitrocellulose membranes (Bio-Rad). Blots were blocked for 1 h in blocking buffer (5% nonfat dry milk, Tris-buffered saline, and 0.1% Tween 20), washed with blocking buffer, and then incubated for 1 h at room temperature with 1:1,000 dilutions of rabbit anti-Sp1 antibody (Santa Cruz Biotechnology) or rabbit anti-NF- κ B (p65) antibody (Santa Cruz Biotechnology). Blots were then washed twice for 30 min in blocking buffer and further incubated for 1 h at 37°C with the appropriated peroxidase-conjugated antibody diluted 1:1,000. Finally, blots were washed twice for 30 min in blocking buffer and then probed using an ECL system (Amersham).

Statistical analysis. Results are expressed as means \pm SE. Data were evaluated by ANOVA when multiple groups were compared, using Tukey's test as a post hoc test.

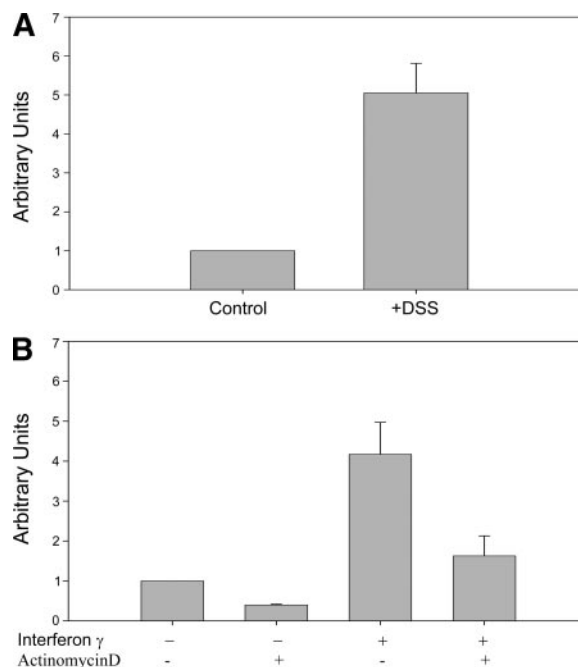


Fig. 1. *A*: CD98 mRNA expression was increased in colonic tissues from dextran sodium sulfate (DSS)-treated mice. Quantification of CD98 transcripts in intestinal tissues from mice treated with or without DSS is shown. For graphical representation of quantitative PCR data, raw threshold cycle (C_t) values obtained from the experimental groups were deducted from C_t values obtained from the control group using the $\Delta\Delta C_t$ method, and values were normalized using $2^{-\Delta\Delta C_t}$ with β -actin gene levels serving as the internal standard. Values were normalized as fold differences relative to transcript levels in untreated controls. Presented results are representative of 3 different experiments. *B*: IFN- γ transcriptionally enhanced CD98 expression in Caco2-BBE cells. Quantification of CD98 transcripts was performed, as described above, in Caco2-BBE cells that were untreated (–) or pretreated (+) with 2 μ g/ml actinomycin D for 4 h and then treated with 100 U/ml IFN- γ (+) or vehicle (–) for 24 h.

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 the relative CD98 mRNA expression in DSS-treated and -untreated mice. As shown in Fig. 1A, colonic tissues from DSS-treated mice showed approximately fivefold 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.

IFN- γ transcriptionally enhances CD98 expression in Caco2-BBE cells. To determine whether or not IFN- γ directly increased CD98 mRNA at the transcriptional 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 (Fig. 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 that spanned positions -1030 to +4 bp relative to the A of the CD98 gene start codon ATG (Fig. 2). This fragment (Accession No. DQ125482) was identical to the previously reported sequence from human HPB-MLT T cell tumor cells (17). We then used 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 Sp1, alcohol dehydrogenase gene regulator 1 member D1, homeo domain factor Nkx-2, stress response element, and NF- κ B transcription factors as well as common heat shock factor transcription factors. As previously reported (17), we identified four potential Sp1 binding sites but no potential TATA or CAAT boxes.

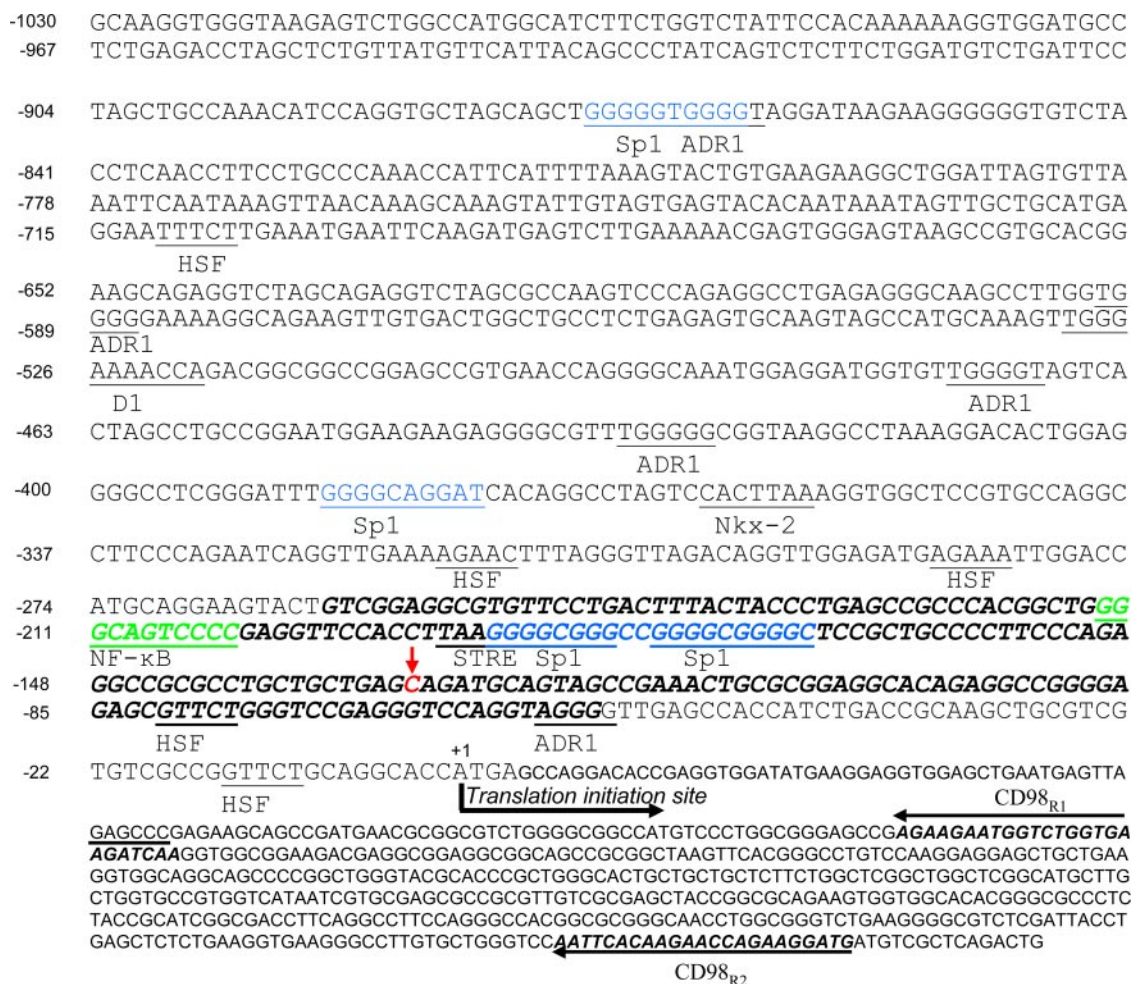


Fig. 2. Sequence of the cloned 5' flanking region of the human CD98 gene. Putative transcription factor binding sites were identified by the TFsearch program. Our analysis indicated putative binding sites for stimulating protein 1 (Sp1), alcohol dehydrogenase gene regulator 1 member D1 (ADR1), homeo domain factor Nkx-2, stress response element (STRE), and NF- κ B transcription factors as well as common heat shock factor (HSF) 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 (5'-GGGGTGGG-3') at positions -187 and -177, one consensus sequence (5'-GGGGTGGG-3') at position -874, and one consensus sequence (5'-GGGGCAGG-3') at position -386 (blue). The consensus sequence (5'-GGGCAGTCCC-3') of the NF- κ B transcription factor binding site was located at position -213 (green). Two gene-specific primers (CD98_{R1} and CD98_{R2}) for rapid amplification of 5'-cDNA ends (5'-RACE) are shown. The source of the additional nucleotide sequence downstream of position +4 was provided by the cloning of the CD98 coding sequence from Caco2-BBE cells that was identical to the one reported under Accession No. J02939.

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 (5'-GGGGCGGG-3') at positions -187 and -177, one consensus sequence (5'-GGGGTGGG-3') at position -874, and one consensus sequence (5'-GGGGCAGG-3') at position -386 (Fig. 2, blue). The consensus sequence (5'-GGGCAGTCCC-3') of the NF- κ B transcription factor binding site was located at position -213 (Fig. 2, green).

Mapping the 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 CD98_{promoter reverse primer} (Table 1) located downstream of the start codon ATG (Fig. 3A, arrow), indicating the existence of a single TSS near nt 150. To determine the exact location of the TSS of the human CD98 gene, we used 5'-RACE followed by PCR mapping of the human genomic DNA region surrounding the putative TSS. 5'-RACE was performed on total RNA from Caco2-BBE cells with a primer corresponding to CD98_{R2} (Table 1), and subsequent PCR was performed using a primer corresponding to CD98_{R1} (Table 1). A single DNA fragment of 300 bp was produced (Fig. 3B). Sequencing revealed that this fragment

mapped to the National Center for Biotechnology Information reference sequence 129 bp upstream of 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 TSS of the CD98 gene, and its first base is referred to as nt -128 hereafter in this work.

Functional mapping of 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), *construct I* (nt -750 to +4), *construct II* (nt -350 to +4), *construct III* (nt -1030 to -750), *construct IV* (nt -1030 to -330), and *construct V* (nt -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). Transfected Caco2-BBE cells were left unstimulated or were stimulated with 100 U/ml IFN- γ for 24 h. 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 with the control empty pGL3 basic vector (Fig. 4, A and B). Transfection of the

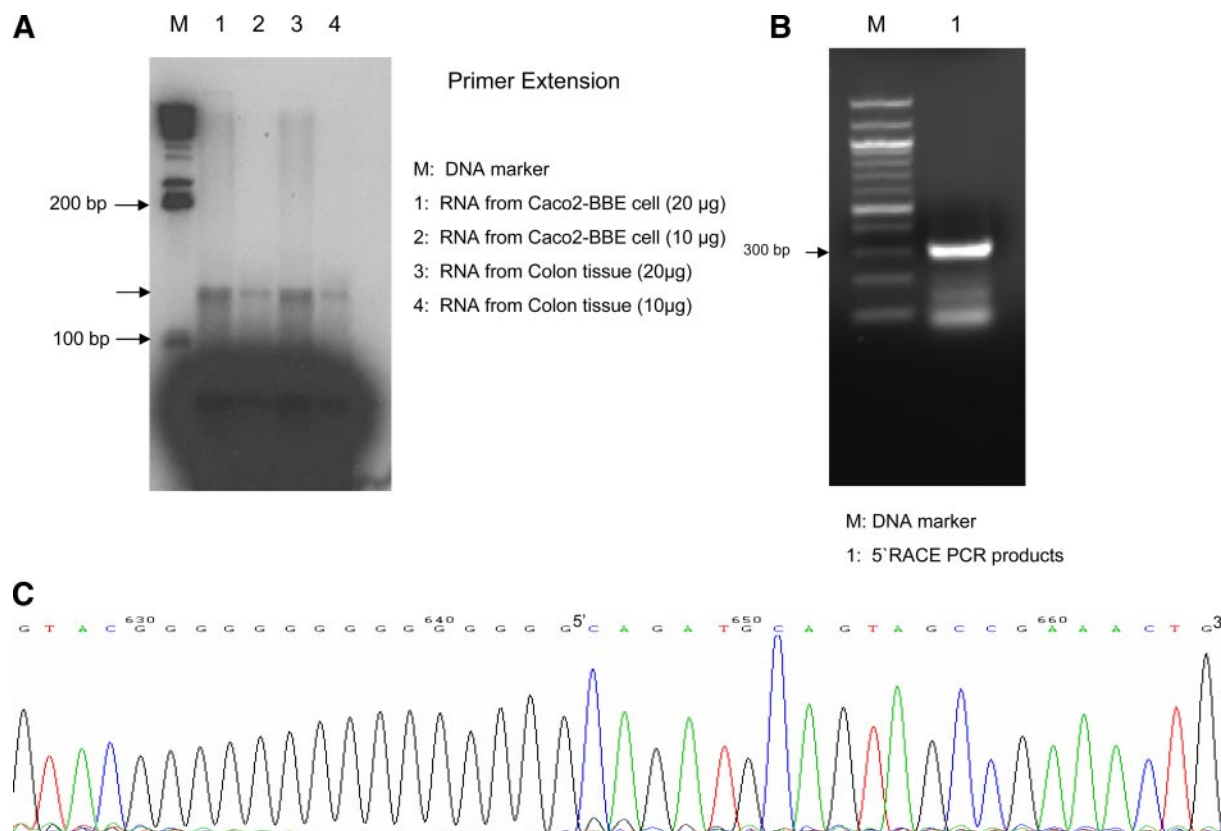


Fig. 3. Localization of the transcription initiation start site (TSS). **A:** mapping of the TSS by primer extension analysis. An oligonucleotide complementary to the TSS of the CD98 mRNA was 5'-end labeled with 32 P and hybridized to 20 and 10 μ g of total RNA extracted from Caco2-BBE cells (lanes 1 and 2, respectively) and human colon tissue (lanes 3 and 4, respectively). cDNA was extended as described in MATERIALS AND METHODS and then analyzed on a 5% Tris-borate-EDTA gel. M, molecular weight marker. **B:** gel electrophoresis of PCR products. Human Caco2-BBE cell cDNA was retranscribed by the gene-specific primer CD98 RACE 2, and another PCR step was applied to identify the TSS of the human CD98 gene using the abridged universal amplification primer as the sense DNA primer complementary to the 5'-RACE abridged anchor primer, which is a poly G primer (g-oligo), and CD98 RACE 1 as the gene-specific antisense primer. Nested PCR products were electrophoresed on 1% agarose gels and stained with ethidium bromide. M, 100-bp DNA ladder (molecular size marker); lane 1, 5'-RACE PCR product. **C:** mapping of the TSS of CD98. Gel-purified 5'-RACE PCR products were cloned into PCR2.1 and sequenced. The Chromas software package was used for sequence analysis.

cells with *construct I*_(-750,+4) and *construct 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, *construct III*_(-1030,-750), *construct IV*_(-1030,-330), and *construct V*_(-750,-330) had significantly lower promoter activities (Fig. 4, A and B). Following IFN- γ stimulation, the promoter activities of *construct I*_(-750,+4), *construct II*_(-330,+4), and full-length CD98 were similar (~250-fold increase; Fig. 4, A and B), whereas *construct III*_(-1030,-750), *construct IV*_(-1030,-330), and *construct V*_(-750,-330) did not show significant increases in promoter activity compared with controls (Fig. 4, A and B). 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 *construct I*_(-750,+4) and *construct II*_(-330,+4) (Fig. 4, A and B). As the minimal effective region contains two Sp1 binding sites (at the -187 and -177 positions) and the NF- κ B binding site (-213), these results suggest that the Sp1 and NF- κ B transcription factors are essential for basal and IFN- γ -stimulated CD98 promoter activities.

EMSA analysis of putative Sp1 and NF- κ B binding sites. To further confirm the importance of Sp1 and NF- κ B 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- κ B 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- κ B antibodies. 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 wild-type or mutant boxes or to a random sequence. 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- κ B antibodies shifted the migrating bands in an upward direction, indicating the specificity to Sp1 and NF- κ B proteins. Collectively, these results indicate the importance of Sp1 and NF- κ B for activation of the CD98 gene.

In vivo analysis of putative Sp1 and NF- κ B binding sites in untreated and IFN- γ -treated Caco2-BBE cells. To examine whether or not Sp1 and NF- κ B binding sites are important in the response to IFN- γ in Caco2-BBE cells, we performed ChIP analysis. As shown in Fig. 6, in Caco2-BBE cells at the resting level, transcription factor Sp1 binds to the I Sp1 (lane 1), II Sp1 (lane 3), and III/IV Sp1 (lane 5) binding sites and transcription factor NF- κ B binds to the NF- κ B binding site (lane 7). After IFN- γ treatment, the DNA binding activities of I Sp1 (lane 2), II Sp1 (lane 4), III/IV Sp1 (lane 6), and NF- κ B (lane 8) were increased. As a control, no band was detected in the absence of the DNA template. Together, these results suggest that Sp1 and NF- κ B may play a role in the IFN- γ induction of CD98 promoter activity.

Role of Sp1 and NF- κ B 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- κ B binding mutants (see Table 1). We then used a transient

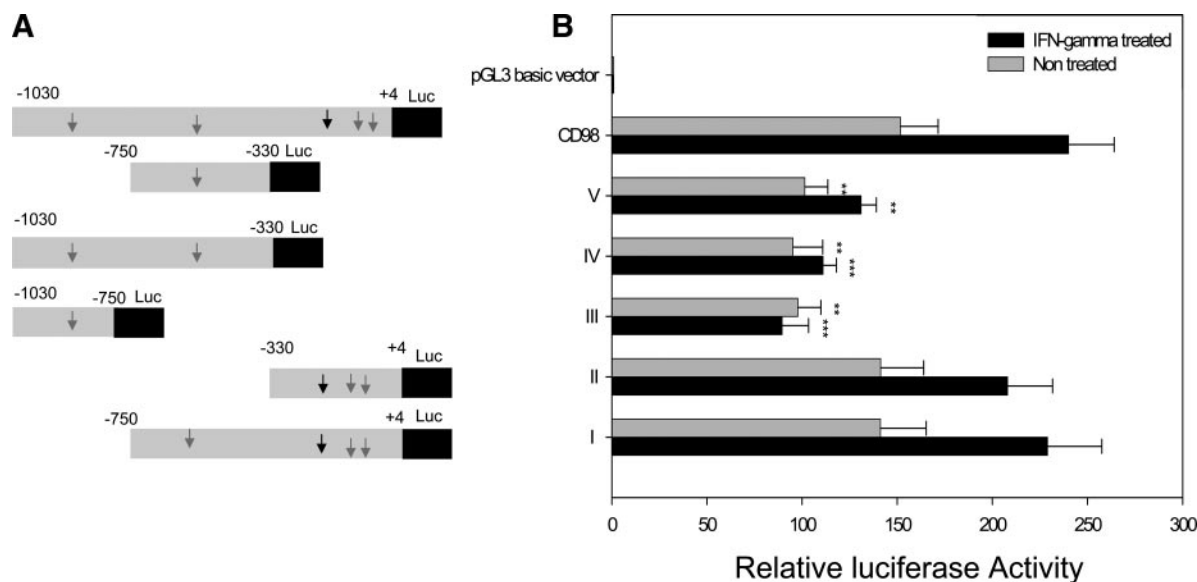


Fig. 4. A: schematic representation of human CD98 gene promoter constructs. Utilized CD98 gene promoter constructs contained sequences corresponding to the full-length CD98 promoter (nt -1030 to +4) as well as various deleted fragments (*construct I*, nt -750 to +4; *construct II*, nt -330 to +4; *construct III*, nt -1030 to -750; *construct IV*, nt -1030 to -330; and *construct V*, nt -750 to -330) inserted upstream of the luciferase (Luc) coding region (represented as solid bars) in the pGL3-basic plasmid. Numbers are given in relation to the translational start codon (+1) and indicate 5'-ends of the deletion constructs. The location of the identified positive regulatory region is indicated by a green box. Positions of the putative Sp1 and NF- κ B sites are indicated by arrows. B: functional activities of the 5' deleted promoter constructs in unstimulated (nontreated) or IFN- γ -stimulated Caco2-BBE cells. Luc activities for Caco2-BBE cells transfected with each promoter construct were normalized to *Renilla* Luc activities driven by the phRL-CMV control vector. Activities are expressed as fold inductions over those seen in cells transfected with the empty pGL3-basic vector. Each value represents the mean \pm SD of at least 3 independent sets of transfection experiments performed in triplicate. * P < 0.05, ** P < 0.01, and *** P < 0.001 vs. full-length promoter with or without IFN- γ treatment.

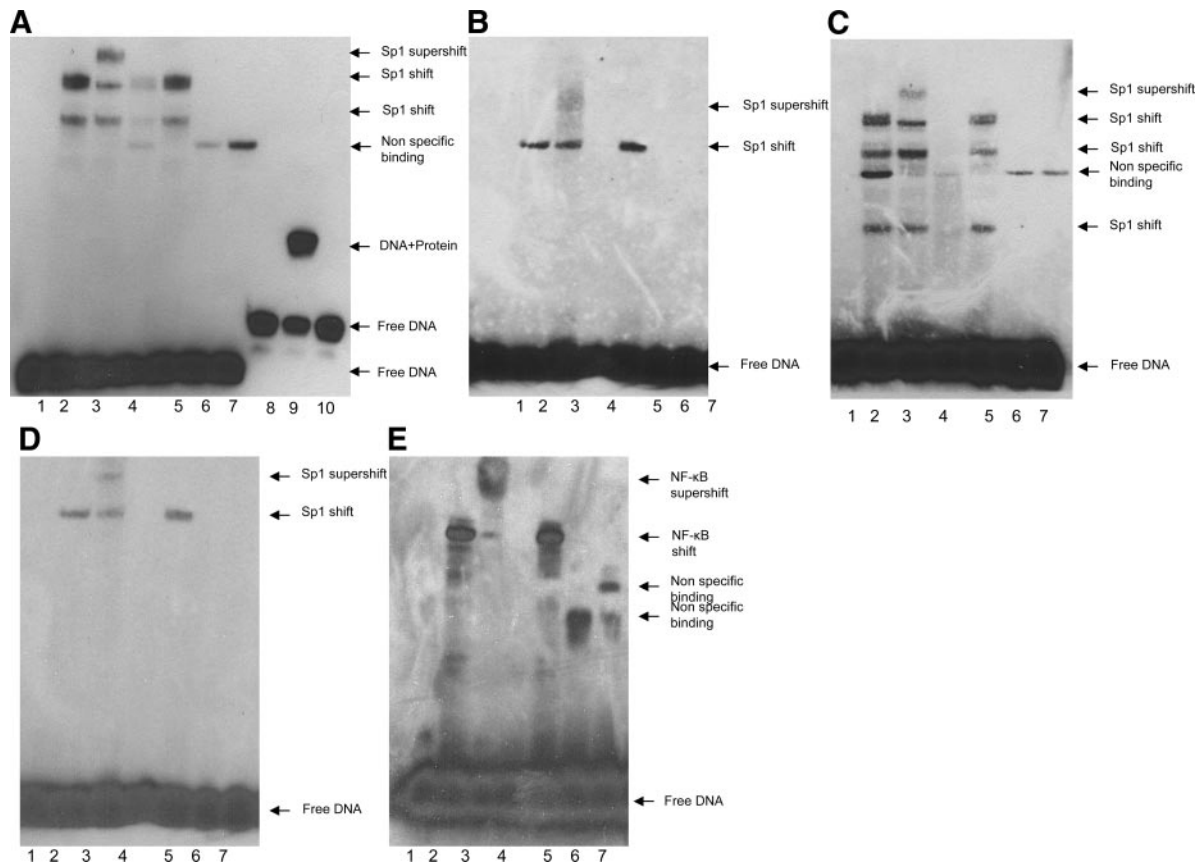
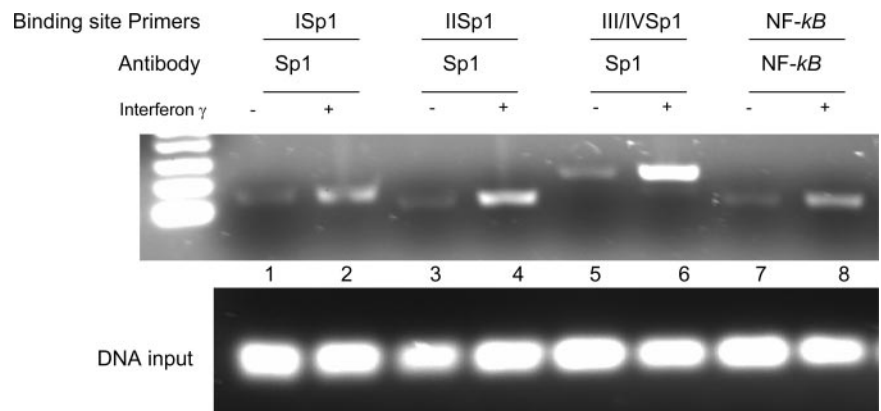


Fig. 5. EMSA-based characterization of Sp1 and NF- κ B binding sites within the CD98 promoter. The binding of the four Sp1 sites and the NF- κ B site are shown. **A:** Sp1(-874). The oligonucleotides used were GC1_{W1} and GC1_{W2} (wild type) as well as GC1_{M1} and GC1_{M2} (mutant). **B:** Sp1(-386). The oligonucleotides used GC2_{W1} and GC2_{W2} (wild type) as well as GC2_{M1} and GC2_{M2} (mutant). **C:** Sp1(-187). The oligonucleotides used were GC3_{W1} and GC3_{W2} (wild type) as well as GC3_{M1} and GC3_{M2} (mutant). **D:** Sp1(-177). The oligonucleotides used were GC4_{W1} and GC4_{W2} (wild type) as well as GC4_{M1} and GC4_{M2} (mutant). **E:** NF- κ B. The oligonucleotides used were NF- κ B_{W1} and NF- κ B_{W2} as well as NF- κ B_{M1} and NF- κ B_{M2} (mutant). Lane 1, biotin-labeled oligonucleotide alone; lane 2, biotin-labeled oligonucleotides incubated with 5 μ g Caco2-BBE nuclear extracts; lane 3, biotin-labeled oligonucleotides incubated with 5 μ g Caco2-BBE nuclear extracts in the presence of anti-Sp1 (A-D) or -NF- κ B (E) antibodies; lane 4, biotin-labeled oligonucleotides incubated with 5 μ g Caco2-BBE nuclear extracts in the presence of a 200-fold excess of cold competitor oligonucleotide; lane 5, biotin-labeled oligonucleotides incubated with 5 μ g Caco2-BBE nuclear extracts in the presence of a 200-fold excess of nonspecific competitor oligonucleotide; lane 6, biotin-labeled binding site-mutated oligonucleotides incubated with 5 μ g Caco2-BBE nuclear extracts; lane 7, biotin-labeled binding site-mutated oligonucleotides incubated with 5 μ g Caco2-BBE nuclear extracts in the presence of cold competitor oligonucleotide. In A, lanes 8-10 are the controls provided with the kit; lane 8 is biotin-EBNA control DNA; lane 9 is biotin-EBNA control DNA + EBNA extract; and lane 10 is biotin-EBNA control DNA + EBNA extract + unlabeled EBNA DNA.

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 -stim-

ulated promoter activities (Fig. 7), whereas Sp1(-187)Mut, Sp1(-177)Mut, and NF- κ B Mut showed significantly reduced basal and IFN- γ -stimulated promoter activities (by ~25%) (Fig. 7). Interestingly, when we examined the double-mutant

Fig. 6. Sp1 and NF- κ B may play a role in the IFN- γ induction of CD98 promoter activity. Chromatin immunoprecipitation (ChIP) assays were performed as described in MATERIALS AND METHODS. The antibodies indicated were incubated with cross-linked DNA isolated from Caco2-BBE cells that had been treated in the absence (-) or presence (+) of IFN- γ . Sp1 (I, II, and III/IV) and NF- κ B promoter elements in the immunoprecipitates were detected by PCR using the specific primers shown in Table 1.



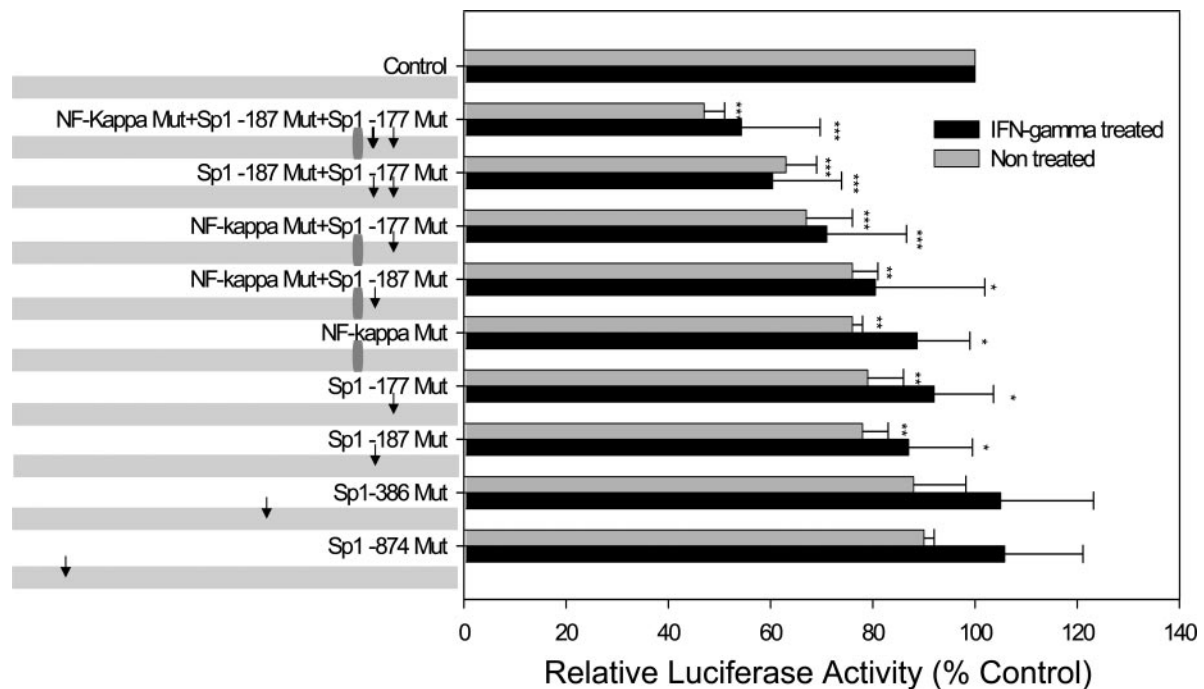


Fig. 7. Effects of GC box mutations on CD98 promoter activity. Arrows depict mutations introduced into the CD98 promoter constructs. The various GC boxes were mutated singly or in combination, and the expression constructs were transiently transfected into Caco2-BBE cells. A: basal or IFN- γ -stimulated CD98 promoter activities of the full-length wild-type construct were set to 100% (control). Presented values represent means \pm SD of at least 3 independent sets of transfection experiments performed in triplicate. * P < 0.05, ** P < 0.01, and *** P < 0.001 vs. controls.

constructs NF- κ B Mut/Sp1(-187)Mut, NF- κ B Mut/Sp1(-177)Mut, Sp1(-187)Mut/Sp1(-177)Mut and the triple mutant NF- κ B Mut/Sp1(-187)Mut/Sp1(-177)Mut, basal and IFN- γ -stimulated promoter activities were further reduced by \sim 40% (Fig. 7). Together, these results demonstrate that the Sp1(-187), Sp1(-177), and NF- κ B sites, but not the Sp1(-874) and Sp1(-386) sites, play a role 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- κ B sites or their binding partners in determining basal and IFN- γ -stimulated CD98 promoter activities.

Reduction of Sp1 or NF- κ B expression reduced CD98 protein expression in unstimulated and stimulated Caco2-BBE cells by IFN- γ . One approach to study the functional role of a specific protein is to knock down its expression. As shown in Fig. 8, we found that Caco2-BBE cells transfected with siRNA against Sp1 and NF- κ B showed lower expression of the targeted proteins Sp1 (Sp1 lane 1) and NF- κ B (NF- κ B lane 5) compared with Caco2-BBE cells transfected with scrambled control Sp1 siRNA (Sp1 lane 4) and NF- κ B (NF- κ B lane 8). Interestingly, basal CD98 protein expression was reduced in Caco2-BBE cells transfected with siRNA Sp1 (CD98 lane 1 vs. CD98 lane 4) or NF- κ B (CD98 lane 5 vs. CD98 lane 8). Thus, siRNA to Sp1 and NF- κ B 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- κ B. It was of interest to investigate whether siRNA against Sp1 and/or against NF- κ B reduced the IFN- γ induction of CD98 protein expression in Caco2-BBE cells. As shown in Fig. 8, IFN- γ -stimulated Caco2-BBE cells

transfected with Sp1 siRNA or NF- κ B siRNA displayed lower CD98 protein expression (CD98 lane 2 and CD98 lane 6, respectively) compared with IFN- γ stimulated Caco2-BBE cells transfected with scrambled control Sp1 siRNA (CD98 lane 3) and NF- κ B (CD98 lane 7).

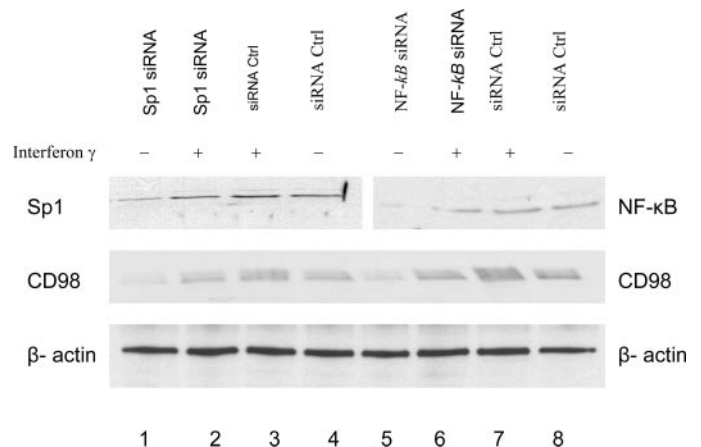


Fig. 8. Reduction of Sp1 or NF- κ B expression reduced CD98 protein expression in unstimulated Caco2-BBE cells and in Caco2-BBE cells stimulated by IFN- γ . Caco2-BBE cells were transfected with the indicated small inhibitory (si)RNA, and cells were then unstimulated or stimulated by IFN- γ for 24 h as described in MATERIALS AND METHODS. Cells were harvested and subjected to Western blot analysis using Sp1, NF- κ B (p65), and CD98 antibodies as described in MATERIALS AND METHODS. As a loading control, β -actin was also detected in the same membrane using anti- β -actin antibody. siRNA-Ctrl was an siRNA scrambled sequence from Ambion.

DISCUSSION

CD98 is constitutively expressed by a variety of tissues, including intestinal epithelial cells (25, 30, 31). We (25) have previously demonstrated that DSS-induced colitis is a useful model for examining the role of CD98 in the colonic mucosa and found that the effect of DSS on epithelial CD98 expression is mediated via IFN- γ . IFN- γ is present at high levels in IBD tissues, where it prepares enterocytes to function in host defense (16). Antibody-based inhibition of endogenous IFN- γ has been shown to ameliorate the chronic stage of colitis, indicating that IFN- γ 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 IFN- γ -treated intestinal epithelial cells and examined the mechanisms responsible for the IFN- γ -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 transcription factor Sp1 along with a transcription factor NF- κ B binding site. The latter result is in agreement with the cloned CD98DNA sequence analysis from the HPB-MLT human T cell tumor line, which revealed that the 5' flanking region of the gene encoding CD98 contains a hypomethylated CpG island and four potential binding sites for transcription factor Sp1 but did not contain TATA or CCAAT boxes (24). Both competition EMSA and antibody supershift experiments revealed that Sp1 and NF- κ B both interacted with the promoter region. We found that different Sp1 binding sites generated different DNA-protein interaction profiles, which suggested that each of these binding sites had distinct functional properties. Furthermore, ChIP analysis showed *in vivo* DNA-Sp1 and DNA-NF- κ B interactions under basal and IFN- γ -stimulated conditions. The 5' flanking region does not contain TATA or CCAAT boxes, and primer extension and 5'-RACE assays revealed that the major transcriptional initiation site was located 129-bp upstream from the first ATG codon. This is consistent with previous identifications of single start sites for TATA-less promoters in other genes, including 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 TSS. This region contains the NF- κ B site at -213 along with Sp1(-187) and Sp1(-177), suggesting that these three sites are important for basal and IFN- γ -stimulated transcriptional activities of CD98 in the tested intestinal cell line. These findings are consistent with previous studies indicating that IFN- γ treatment can enhance NF- κ B binding (32) and activity (4, 33) and that IFN- γ treatment can induce the formation of a Sp1-like complex in HT-29 cells (8). However, this is the first report showing that IFN- γ increases 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 has been shown to be involved in inducible gene transcription in response to stimu-

lators 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- κ B, a heterodimeric protein complex consisting of 50- 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- κ B, Sp1(-187), and Sp1(-177) sites may cooperate in mediating basal and IFN- γ -stimulated CD98 transcription. The interaction between NF- κ B 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 the reduction of Sp1 or NF- κ B expression reduced CD98 protein expression in unstimulated and IFN- γ -stimulated Caco2-BBE cells.

It has been demonstrated that the increased epithelial expression of cell surface proteins in patients suffering from IBD occurs through a number of different pathways, including the production of cytokines by activated mucosal lymphocytes. IFN- γ is present at high levels in IBD tissues. Here, we showed that IFN- γ transcriptionally enhances CD98 glycoprotein expression via Sp1 and NF- κ B transcription factors in intestinal epithelial cells. We suggest that during intestinal inflammation, CD98 expression is upregulated in immune cells but also in intestinal epithelial cells. The upregulation of CD98 in intestinal epithelial cells could affect functions such as integrin- β 1-mediated events (14, 20), which have been implicated in the etiology of various pathological conditions, including inflammatory disorders such as IBS.

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