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Helicobacter pylori Induction of the Gastrin Promoter Through GC-Rich DNA Elements

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Abstract

Background: *Helicobacter pylori* (*H. pylori*) infection has been linked to the development of chronic gastritis, duodenal ulcer disease, and gastric cancer. *Helicobacter pylori*- infected patients and animal models develop hypergastrinemia, chronic gastritis, and gastric atrophy. Since gastrin is an important regulator of gastric acid secretion and cell growth, *H. pylori* regulation of this hormone has been implicated in its pathogenesis.

Objectives: To investigate the effect of *H. pylori* on gastrin gene expression in mice and of human bacterial isolates on gastrin mRNA expressed in a human cell line.

Methods: Gastrin mRNA was measured by qRT-PCR in *H. pylori*-infected mice. *H. pylori* were co-cultured with AGS cells to study regulation of human gastrin gene expression. Various MAP kinases were implicated in signal transduction from the bacteria using specific inhibitors. Gastrin reporter constructs and gel shift assays were used to map DNA responsive elements.

Results: In addition to an increase in gastrin mRNA in *H. pylori*-infected mice, *H. pylori* induced the endogenous human gastrin gene through MAP kinase-dependent signaling but not NFκB-dependent signaling. Activation of gastrin through MAPK signaling did not require CagA or VacA virulence factors. Transfection studies demonstrated that a GC-rich motif mediated *H. pylori*-induction of the gastrin promoter and that the motif inducibly binds Sp1 and Sp3 transcription factors.

Conclusions: Direct contact of live *H. pylori* bacteria with human cells is sufficient to induce gastrin gene expression.

Helicobacter pylori (*H. pylori*) is a gastrointestinal pathogen that has been linked to the development of chronic gastritis, duodenal ulcer disease, and gastric cancer [1–4]. Although the global average rate of infection is near 60%, most individuals infected with *H. pylori* remain asymptomatic. Nevertheless, gastric carcinoma remains the second leading cause of cancer deaths worldwide and *H. pylori* infection increases the risk of developing this disease [3,5–7]. However, the mechanisms by which *H. pylori* contribute to the development and progression of gastrointestinal pathology are unclear.

There is significant genetic diversity among *H. pylori* strains although bacteria that express the cytotoxin-associated gene A pathogenicity island (cagPAI) and virulence factors such as CagA are typically associated with more severe disease manifestations [5,6,8,9]. The cagPAI encodes a type IV secretion system (T4SS), which translocates the CagA protein into the host cell where it is phosphorylated [8,9]. In comparison with cagPAI mutant or deficient strains, wild-type bacteria stimulate activation of transcription factors such as NFκB and AP1. CagPAI+ strains also show stronger activation of MAP kinase signaling pathways [6,10,11]. Therefore, host cell gene expression is modulated by components of the T4SS- and possibly cagPAI-encoded virulence factors such as CagA. Furthermore, genetic variations among *H. pylori* species might account for the highly variable consequences of *H. pylori* infection in humans [12].

In addition to gastritis and gastric atrophy, hypergastrinemia is observed in a subset of *H. pylori*-infected patients [13–15]. However, there are few studies examining *H. pylori* regulation of human gastrin gene expression in vivo or in primary human G cells. Calam and coworkers showed that patients infected with *H. pylori* increased gastrin mRNA levels [16], whereas Sumii et al. [17] reported no change in gastrin mRNA.

Buchan and coworkers reported that *H. pylori* increases basal levels of gastrin in primary G-cell cultures, but does not induce secretion, suggesting that *H. pylori* increases gastrin synthesis and possibly gene expression, but mRNA levels were not measured [18].

Similarly in several rodent models of *H. pylori* infection, the number of antral G cells increase coincident with elevated serum gastrin levels [19,20]. Antral gastrin is the primary hormonal regulator of gastric acid secretion. Furthermore, gastrin acts as a growth factor for gut-derived cell types. The essential role of gastrin in acid regulation and maintenance of gastric homeostasis suggests possible mechanisms by which *H. pylori* might alter gastrointestinal physiology by regulating the levels of gastrin. As *Helicobacter* infection inhibits acid secretion [21], the observed hypergastrinemia might be in response to the hypochlorhydria. Indeed, direct inhibition of acid secretion by omeprazole is sufficient to stimulate gastrin gene expression in vivo [22]. Prior studies suggested that *Helicobacter* colonizing the gastric antrum might create an alkaline pH sufficient to stimulate G cells through its production of urease and conversion of urea to ammonia [23]. However, this mechanism was subsequently disproven by studies showing that *H. pylori* products rather than the live organism can stimulate gastrin release from cultured G cells [24,25]. These results suggest that cellular components even from dead bacteria are sufficient to stimulate hormone release.

Recent studies by Kidd et al. [26] demonstrate that multiple extracellular signals, e.g., pH, lipopolysaccharide (LPS), serotonin, β-adrenergic receptors and intracellular pathways, e.g., cAMP, ERKs (extracellular signal-regulated kinase-1), NFκb, regulate gastrin release from primary G cells. However, the authors did not examine regulation of gastrin gene expression in response to these various conditions. Although suppression of somatostatin is the strongest inducer of gastrin release, we found that pro-inflammatory cytokines were able to stimulate gastrin release in the somatostatin null mouse demonstrating that extracellular signals other than somatostatin are capable of inducing gastrin secretion [27]. Taken together, it is unclear whether the hypergastrinemia that occurs in *H. pylori*-infected individuals is attributable to hypochlorhydria, suppression of somatostatin, chronic gastritis, gastric atrophy or the direct induction of gastrin gene expression by the bacteria itself. Certainly, several mechanisms are likely to contribute to the increase in plasma gastrin.

It has been shown using cell lines that oncogenic K-ras and epidermal growth factor (EGF) receptor ligands induce gastrin gene expression [28–30]. Indeed, a gastrin EGF response element (gERE) was mapped to a GC-rich sequence within the first 240 bp of the gastrin promoter. This proximal promoter element binds the transcription factors Sp1, Sp3, and ZBP-89 [31]. Moreover, we showed previously that Mek1-dependent phosphorylation of Sp1 mediates EGF activation of the gastrin promoter through this element [32]. It has been established in numerous studies that *H. pylori* and possibly bacterial proteins such as CagA regulate gene expression through activation of signal transduction cascades that target specific transcription factors. Furthermore, a prior study established a MEK/ERK-dependant mechanism for the activation of both Sp1 and Sp3 in the *H. pylori*-mediated induction of the vascular endothelial growth factor-A (vegf-A) gene [33]. Therefore, we tested the hypothesis that *H. pylori* regulate gastrin gene expression through specific DNA promoter elements that bind Sp1.

Methods

Helicobacter Strains and Cultures

The 26695 and SS1 *H. pylori* WT and mutant strains were generated as previously described [34]. The J99 strain was obtained from ATCC. All *H. pylori* strains were grown on blood agar plates prior to inoculation of Brucella broth (Difco , Livonia, MI) supplemented with 10% heat-inactivated fetal bovine serum (FBS), Skirrow's antibiotic, and amphotericin B. *H. pylori* cultures were maintained by shaking in a gas exchange incubator under microaerophilc conditions at 37 °C. The broth used for infection was cultured overnight. The presence of *H. pylori* was verified biochemically by catalase and urease tests as well as microscopic analysis for size, shape, and motility.

In vivo *H. pylori* Infection in Mice

Six- to 8- week-old C57BL/6 mice were orally inoculated once with 1×10^8 CFU of broth-cultured *H. pylori*, strain SS1, in 0.1 mL of broth. Control mice were not given bacteria. Mice were killed 6 months after inoculation, and gastric tissue was collected for histologic examination, PCR, and qRT-PCR analysis. Gastric RNA was harvested from mucosal scrapings of the whole stomach using TrIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Purification of mRNA was accomplished using the Qiagen's RNeasy MiniKit (Qiagen, Valencia, CA) and stored at −80 °C. Generation of cDNAs was performed using the Invitrogen SuperScript First Strand kit and random hexamer primers.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was isolated from AGS cells [human gastric adenocarcinoma cell line (ATCC, Manassas, VA)] using

Trizol reagent (Invitrogen) and DNAse treated (Roche, Indianapolis, IN). RNA quantitation was performed using the Quant-iT RiboGreen RNA Assay Kit (Molecular Probes, Eugene, CA). Synthesis of cDNA was performed for 1 μg of mouse and human RNA using the I-script cDNA Synthesis Kit (Bio-Rad, Hercules, CA).

All qRT-PCR reactions were preformed in triplicate using the Bio-Rad I-cycler. The following primers were used to detect and quantify gene expression: mouse gastrin: (forward) ACACAACAGCCAACTATTC, (reverse) CAAAGTCCATCCATC CGTAG. Mouse GAPDH: (forward) TCAAGAAGGTGGTGAAGCAGG, (reverse) TATTATG GGGGTCTGGGATGG. Human gastrin (forward) CCCAGGCTCTCATCATCGAAGG, (reverse) GCCGAAGT CCATCCATCCATAGG. Human 18S rRNA: (forward) GATATGCTCAT GTGGTGTTG, (reverse) AATCTTCTTCAGTCGCTCCA [35]. Human IL-8: (forward) TAGCAAAA TTGAGGCCAAGG, (reverse) AAACCAAGGCACAGTGG AAC.

Reactions using mouse RNA and primers were performed at a volume of 20 μL and contained the following: 1× reaction buffer, 5.5 mmol/L $MgCl_2$, 100 nmol/L of forward and reverse primers, 10 nmol/L fluorescein, 200 μmol/L dNTPs, SYBR green, and 0.025 units of Platinum Taq polymerase (Invitrogen). Amplification was performed under the following conditions: 3 minute at 95 °C, 35–40 cycles of 9 seconds at 95 °C and 1 minutes at 60 °C, and 1 minutes at 55 °C. Expression of mouse target genes was normalized to GAPDH.

Reactions using human primers were performed at a volume of 20 μL and contained the following: 1× reaction buffer, 1.5 mmol/L $MgCl_2$, 100 nmol/L of forward and reverse primers, 10 nmol/L fluorescein, 200 μmol/L dNTPs, SYBR green, and 0.025 U of Amplitaq Gold polymerase (Applied Biosystems, Carlsbad, CA). Reactions were performed with the following conditions: 50 °C for 2 minutes and 95 °C for 10 minutes followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minutes. Gene expression was normalized to 18s rRNA. Expression of human target genes was normalized to 18s rRNA. The fold change for all reactions was calculated using the following equation $(C_t - C_{thi}) = n_{target\ gene}$, $2n_{target}/2n_{normalization}$.

Cell Culture

AGS cells (human gastric adenocarcinoma) were grown in DMEM (Gibco-BRL) containing 10% FCS, 100 μg/mL penicillin, and 100 μg/mL streptomycin at 37 °C with 5% CO_2. The cells were serum starved for 48 hour in serum-free HAMS/F12 media [Gibco (Invitrogen) Carlsbad, CA] prior to any treatment or co-culture with *H. pylori*.

Plasmids and Constructs

To generate the pGL3 gastrin reporter constructs, segments from −3.3 to −0.190 kb of the human gastrin promoter were ligated into the pGL3 basic luciferase vector (Promega, Madison, WI). Gastrin promoter fragments from −2.3 to −0.19 kb were isolated by restriction digest from the −3.3 kb gastrin proximal promoter fragment. Site-directed mutations were introduced into the −0.240- kb construct using the QuickChange II Kit (Stratagene). Primers designed to introduce mutations were generated using the primer design application on the Stratagene website. All constructs were confirmed by sequence analysis. Several rounds of single mutation reactions were performed to generate constructs with mutations in multiple sites. The activity of the Path Detect pAP1-Luc Cis-Reporter Plasmid (Stratagene) was used as a positive control for activation by *H. pylori* observed in the transfection experiments.

Transient Transfections and Luciferase Reporter Assays

AGS cells were seeded onto 12- or 24-well plates and grown to 50–60% confluency prior to transfection. Plasmid DNA at concentrations of 0.5–1 μg/well was transfected using Fugene 6 (Roche) according to the manufacturer's protocol. The promoterless phRG-B Renilla luciferase reporter (Promega) served as a transfection control to normalize firefly luciferase activity. The cells were treated then harvested at 48 hour after transfection. Firefly and Renilla luciferase were measured using the Dual Luciferase Assay Kit (Promega).

H. pylori Co-culture with AGS Cells

AGS cells were seeded at 50–75% confluency and cultured in serum-free media containing antibiotics for 48 hour prior to *H. pylori* inoculation. *H. pylori* were quantified then resuspended in F12 media without antibiotics. The AGS cells were washed with PBS and maintained in serum-free and antibiotic-free media for the duration of the co-culture experiments with *H. pylori*.

Western Blot Analysis

Whole cell extracts were generated from AGS cells that were cultured in serum-free media 48 hour prior to treatment with chemical inhibitors and *H. pylori* co-culture. Cell pellets were lysed with M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL) containing complete protease inhibitor tablets (Roche) according to the manufacturer's instructions.

The protein concentration for each sample was determined using the bicinchoninic acid assay (Pierce Biochemicals). Protein lysates were resolved on 4–20% SDS–polyacrylamide gradient gels then transferred to Hybond-C Extra nitrocellulose membranes (Amersham, Piscataway, NJ). Membranes were blocked using KPL blocking solution (KPL, Inc, Gaithersburg, MD, USA). Primary antibodies for GAPDH (Millipore, Bedford, MA), ERK1/2 (Cell Signaling, Beverly, MA), p38 (Cell Signaling), Sp1 (Santa Cruz Biotechnology, Santa Cruz, CA), and Sp3 (Santa Cruz Biotechnology) were used at a concentration of 1 : 1000. Phospho-antibodies against p38 (Cell Signaling), ERK1/2 (Cell Signaling), and cJun (Cell Signaling) were used at a 1 : 500 concentration. Proteins were visualized using HRP-conjugated secondary antibodies and ECL reagent from the SuperSignal West Pico Chemiluminescent Kit (Thermo Fischer Scientific, Pittsburgh, PA).

Electrophoretic Mobility Shift Assays (EMSA)

Nuclear extracts were prepared using non-ionic detergent extraction from AGS cells following co-culture with *H. pylori* for various times. Oligonucleotide probes were designed according to regions of the human gastrin promoter. An AP1 binding oligo was designed using two consecutive API binding sites (TGCATCA × 2) and used as a positive control for *H. pylori*-induced DNA binding. All oligos were flanked by BamHI and Bgl II sequences at the 5′ and 3′ ends, respectively, annealed then labeled with [^{32}P] γ-ATP using polynucleotide kinase (Roche). Gel shift assays were carried out at a final volume of 20 μL and contained 10 mmol/L Tris–HCl, 1 mmol/L EDTA, 1 mmol/L DTT, 5 mmol/L $MgCl_2$, 1 mmol/L $ZnCl_2$, 150 mmol/L KCl, 10% glycerol, and 300 ng polydI-dC. One microgram of antibodies against Sp1 or Sp3 antibodies (Santa Cruz Biotechnology) were used for gel shift assays to identify DNA binding proteins. One microliter of labeled probe at 30,000 cpm/μL was added to each reaction. DNA complexes were resolved on a 6% nondenaturing polyacrylamide gel containing the 45 mmol/L Tris base, 45 mmol/L boric acid, 1 mmol/L EDTA (TBE) buffer.

Chemical Inhibitors

Inhibitors were added to cells 30 minutes prior to co-culture with *H. pylori*. The following inhibitors were used: α-amanitin (Sigma), PD98050 (Cell Signaling Technology), ammonium pyrrolidinedithiocarbamate (APDT, Calbiochem), SB203580 (Calbiochem), and SP600125 (Calbiochem).

Statistics

Data were compared using the Mann–Whitney U test. The mean ± standard error of the mean (SEM) is shown. A p value of <0.05 (*) was considered significant.

Results

H. pylori Infection Induces Gastrin Gene Expression in Mice

In human subjects and animal models, *H. pylori* infection is associated with chronic gastritis and hypergastrinemia. However, increases in plasma gastrin and the population of G cells have not been consistently correlated with changes in gastrin gene expression [16,17]. Therefore, we studied gastrin gene expression in a mouse model of *H. pylori* infection. C57BL/6 mice were infected with the mouse-adapted SS1 strain of *H. pylori* and harvested after 6 months. Persistence of the bacterial infection was confirmed by PCR amplification of the bacterial 16S gene. Using quantitative PCR, a 10-fold increase in gastrin mRNA was observed in mice infected with *H. pylori* when compared to uninfected controls (Fig. 1). The observed increase in gastrin mRNA expression suggested that infection by *H. pylori* correlated with an increase in gastrin gene expression. Because a direct effect of the bacteria on the G cell cannot be established in vivo, we utilized a human AGS gastric cell line to characterize the effect of *H. pylori* on gastrin gene expression.

Gastrin mRNA Induction Requires Live Bacteria, but not CagA

We examined the ability of *H. pylori* to regulate endogenous gastrin gene expression in AGS cells by

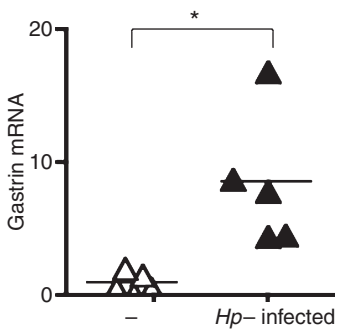

Figure 1 *Helicobacter pylori* stimulate gastrin gene expression in infected mice. Mice were infected with *H. pylori* SS1and analyzed 6 months after infection. qRT-PCR analysis of gastrin normalized to GAPDH (Gastrin mRNA) was performed using RNA isolated from the stomachs of infected and uninfected (—) mice (N = 5 mice). The mean is indicated by a bar. *p < .05.

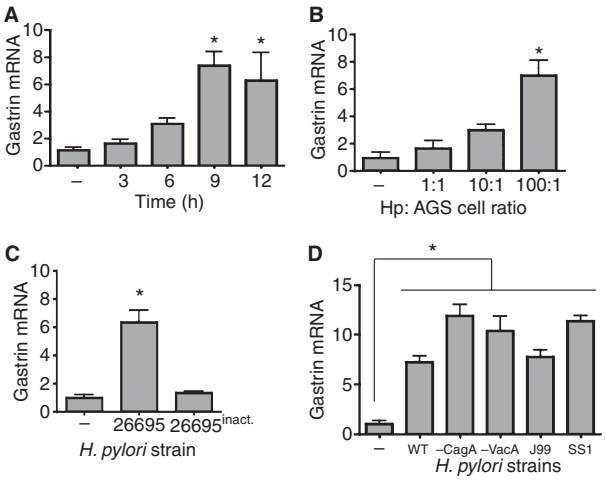

Figure 2 *Helicobacter pylori* stimulate expression of gastrin in AGS Cells. AGS cells were infected with *H. pylori* strain 26695 (a human isolate) 48 hour after serum starvation. The cells were cultured with bacteria at a m.o.i of 100–1 at various times (*A*) or with increasing amounts of bacteria (m.o.i) for 9 hour (*B*). All subsequent *H. pylori* co-culture experiments were performed at 100:1 m.o.i. for 9 hour. AGS cells were co-cultured with live or inactive (formalin fixed, heat-treated) bacteria (*C*). qRT-PCR analyses of gastrin and 18S rRNA transcripts were performed on total RNA, and the amount of gastrin mRNA was normalized to 18S rRNA (Gastrin mRNA). All samples were compared to the untreated sample that was set to 1. The mean fold change ± SEM is shown for three independent experiments. $*p < .05$ compared to untreated cells was considered significant. (*D*) AGS cells were co-cultured with the wild-type *H. pylori* strain 26695 (WT), or isotypes mutants of 26695 that were null for CagA (-CagA) or VacA (-VacA). Two additional human Hp isolates were also used, the wild-type J99 strain and the mouse-adapted SS1 strain. qRT-PCR analyses of gastrin and 18S rRNA transcripts were performed. Gastrin mRNA was normalized to the 18S transcript. The mean fold change ± SEM three experiments is shown. $*p < .05$ compared to untreated cells was considered significant.

qRT-PCR. Cells were co-cultured with the 26695 *H. pylori* strain, which is a wild-type human isolate [36]. The endogenous human gastrin gene was maximally induced 6- to 7-fold by 9 hour (Fig. 2A) with the maximal 100 m.o.i. (Fig. 2B).

After establishing the optimal conditions for gastrin induction, we investigated whether live bacteria were required for the effect. AGS cells were co-cultured with live *H. pylori* or bacteria that was heat-inactivated and formalin-fixed. Indeed, we found that killed bacteria were unable to induce gastrin mRNA expression (Fig. 2C). Similar results were observed using the SS1 strain under the same conditions (data not shown).

In *H. pylori*-infected patients, there is significant variability in the clinical outcome that is often associated with the genetic variations between *H. pylori* strains. Similarly, laboratory studies have indicated that cagPAI+ and CagA+ strains influence the ability of *H. pylori* to regulate gene expression and activate specific signal transduction pathways. Therefore, we investigated whether there were differences among *H. pylori* strains that might influence their ability to induce the gastrin gene. AGS cells were co-cultured with three wild-type strains 26695, J99 as well as the SS1 strain, all of which contain complete cagPAI [37], despite the prevailing view that the SS1 cagPAI is dysfunctional. Moreover, we examined gastrin gene expression following co-culture with 26695 isogenic mutants that lacked the CagA and vacuolating toxin A (VacA) gene with AGS cells. All the *H. pylori* induced gastrin mRNA expression. Higher levels were observed with the CagA- and VacA-deficient strains as well as the SS1 strain (Fig. 2D). As the VacA-deficient strain showed levels of gastrin induction comparable to those of the CagA-deficient and SS1 strains, we concluded that the increased activation of gastrin mRNA is not influenced directly by either CagA or VacA but may be the result of other bacterial proteins.

Induction of Gastrin Requires Activation of MAPK Signaling

It has been shown previously that *H. pylori* activate multiple signaling pathways including MAP kinase, NFκB, and PI3K [38]. Therefore, AGS cells were treated with chemical inhibitors of the ERK, JNK and p38 MAP kinases (MAPK) to identify the signal transduction pathways activated by *H. pylori*. AGS cells were pretreated for 30 minutes with inhibitors and then co-cultured with *H. pylori* for 9 hour (Fig. 3A). ERK and p38 inhibitors completely blocked gastrin induction by *H. pylori* whereas the JNK inhibitor blocked *H. pylori* induction by 50%. Although *H. pylori* clearly activate NFκB signaling [39,40], inhibiting this pathway with APDT did not block induction of the gastrin gene. To confirm that the inhibitors blocked the activation of ERK, cJun, and p38 MAPKs, Western blots were performed and demonstrated that the doses of the inhibitors were sufficient to block activation of these signaling pathways (Fig. 3C). Moreover, the dose of APDT inhibited NFκB-mediated induction of IL-8 gene expression (Fig. 3B) [41]. Thus, we concluded that *H. pylori* induction of the human gastrin gene is mediated by MAPK rather than through NFκB signaling.

H. pylori Stimulates Transcriptional Initiation of Gastrin mRNA

To determine whether the induction of gastrin by *H. pylori* was because of the transcriptional initiation,

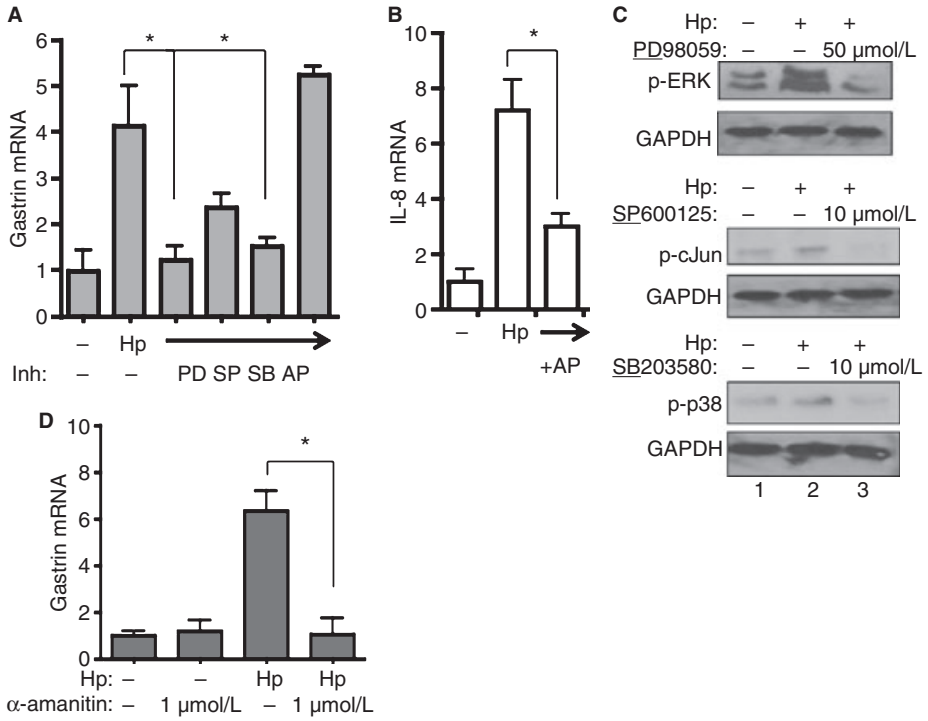

Figure 3 MAP kinase inhibitors block *Helicobacter pylori*-induced gastrin expression. AGS cells were treated with vehicle alone (—) or with chemical inhibitors of ERK, JNK, and p38 MAP kinases, respectively (PD98059: 50 μmol/L, SP600125: 10 μmol/L, SB203580: 10 μmol/L) or NFκB (APDT: 10 μmol/L) signaling 30 minutes prior to co-culture with the *H. pylori* 26695 strain (Hp). qRT-PCR analysis of gastrin transcripts (*A*) or IL-8 transcripts (*B*) were normalized to 18S rRNA. The mean fold change ± SEM three experiments is shown *$p < .05$ compared to untreated (—) cells. Western blots were performed on whole cell extracts prepared from cells cultured without (—) or with (+) *H. pylori* ± the indicated inhibitors (*C*). (*D*) AGS cells were treated without (—) or with 1 μmol/L α-amanitin prior to co-culture with the *H. pylori* 26695 strain. Gastrin mRNA normalized to 18S rRNA transcripts was determined by qRT-PCR of total RNA. The mean fold change ± SEM for three experiments is shown. *$p < .05$ compared to untreated cells. APDT, ammonium pyrrolidinedithiocarbamate.

AGS cells were pretreated with the RNA polymerase inhibitor α-amanitin for 30 minutes prior to co-culturing with the bacteria. The basal expression of gastrin was not affected by 0.1, 0.5, or 1 μmol/L of α-amanitin (data not shown). However, treatment with 1 μmol/L α-amanitin completely blocked *H. pylori* -induced gastrin gene expression when the cells were co-cultured with the 26695 strain (Fig. 3D). Similar results were obtained using the SS1 strain (data not shown). Therefore, we concluded that *H. pylori* induce gastrin by stimulating transcriptional initiation rather than by increasing mRNA stability.

H. pylori Stimulates Gastrin Expression through GC-rich DNA Elements

To identify the *H. pylori*-responsive element within the human gastrin promoter, we generated a series of luciferase constructs representing the sequences between −3.3 and −190 bp of the gastrin promoter (Fig. 4A). The basal activity of each reporter construct is shown as relative light units and indicated that the highest basal activity was within the first 240 bp of the promoter as previously reported (Fig. 4B) [42]. Transient transformants of AGS cells expressing the various gastrin reporters were co-cultured with *H. pylori*. We observed a significant induction of promoter activity for all gastrin promoter constructs from −3.3 to −0.24 kb (Fig. 4C). However, when the 43 bp region between −240 and −190 bp was deleted, there was a 30% decrease in promoter activation suggesting the presence of an *H. pylori* responsive element contained within this region. AGS cells expressing an AP1 luciferase reporter were significantly induced when co-cultured with *H. pylori*. This induction is consistent with prior studies demonstrating the ability of *H. pylori* to activate target genes through AP1 DNA elements. To validate that the decrease in gastrin promoter activation was attributable to an *H. pylori*-specific effect, transfected cells were also treated with PMA (Fig. 4D).

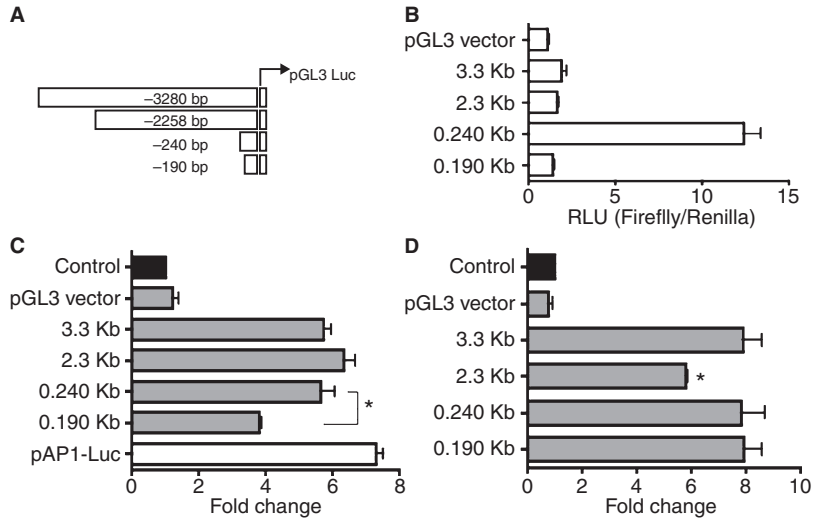

Figure 4 *Helicobacter pylori* stimulate the proximal gastrin promoter. AGS cells were transiently transfected with the empty vector (pGL3), a series of gastrin luciferase constructs, or an AP1 luciferase reporter (pAP1-Luc). A schematic representation of the gastrin luciferase constructs is shown (*A*). The basal activity of the gastrin constructs is shown as relative light units (RLU) of the firefly normalized to the Renilla luciferase activity (*B*). Cells were co-cultured with *H. pylori* (*C*) or 100 nmol/L PMA (*D*) 48 hour post-transfection. The mean fold change ± SEM for three separate experiments was performed in triplicate. The filled bar represents the baseline expression in RLU for each construct set to 1, and fold changes were the ratio of the stimulated activity compared to basal expression of each construct.

There was no difference in PMA-induced promoter activity between the −240- and −190- bp promoter constructs suggesting that the region contained an *H. pylori* -specific response element. A significant reduction in the PMA-induced activity of the −2.3- kb reporter did not show a difference in Hp-induced activity. Therefore, we concluded that this DNA segment contained a regulatory region associated with induction by PMA and not *H. pylori*.

Upon examination of the region between −240 and −190 bp, we identified two cytosine-rich sites as putative *H. pylori* responsive elements (C^2AC^4 = GC-rich) (Fig. 5A, Site 1). However, because the −190- bp construct retained the ability to be induced by *H. pylori* (4-fold), additional downstream promoter sequences containing a GC-rich motif at −120 bp and the reverse complement at −74 bp were also evaluated (Fig. 5A, sites 2 and 3).

To determine whether the GC-rich elements were required for *H. pylori* induction, point mutations were introduced to disrupt protein binding at these elements (Fig. 5A). The results showed that mutating the GC-rich elements within site 1 or sites 2 and 3 together increased the basal activity compared to that of the wild-type reporter (Fig. 5B). The introduction of other point mutations did not alter basal activity. Mutations of site 1 reduced gastrin promoter activation when *H. pylori* were co-cultured with cells transfected with WT or mutant reporters (Fig. 5C). This result was comparable to the decrease in activity observed with a deletion between −240 and −190 bp (∼30%). In addition, decreased activation was also observed with mutation of site 3 alone and mutation of site 1 with either site 2 or 3. There was no effect on the activation gastrin reporters when site 2 alone was mutated. The mutation of sites 2 and 3 together caused a slight decrease in *H. pylori*-induced activity. However, the mutation of sites 1, 2, and 3 together resulted in the greatest suppression in *H. pylori* activation (from 6.5 to 2-fold, Fig. 5C). Therefore, we concluded that the GC-rich elements between −240 and −190 were required for maximal activation of the gastrin promoter by *H. pylori*.

H. pylori Induce Sp1/Sp3 Protein Binding to GC-rich DNA Elements

Next, we identified the nuclear proteins binding to the specific GC-rich sites by electrophoretic mobility shift assay. We determined that the two major complexes binding these elements were transcription factors Sp1 and Sp3 (Fig. 6). We observed increased protein binding activity in extracts from *H. pylori*-treated cells to the −228 and −120 probes (Fig. 6, lanes 1, 2, 5 and 6). However, Sp1 binding to the −74 element remained unchanged while Sp3 binding decreased with *H. pylori*

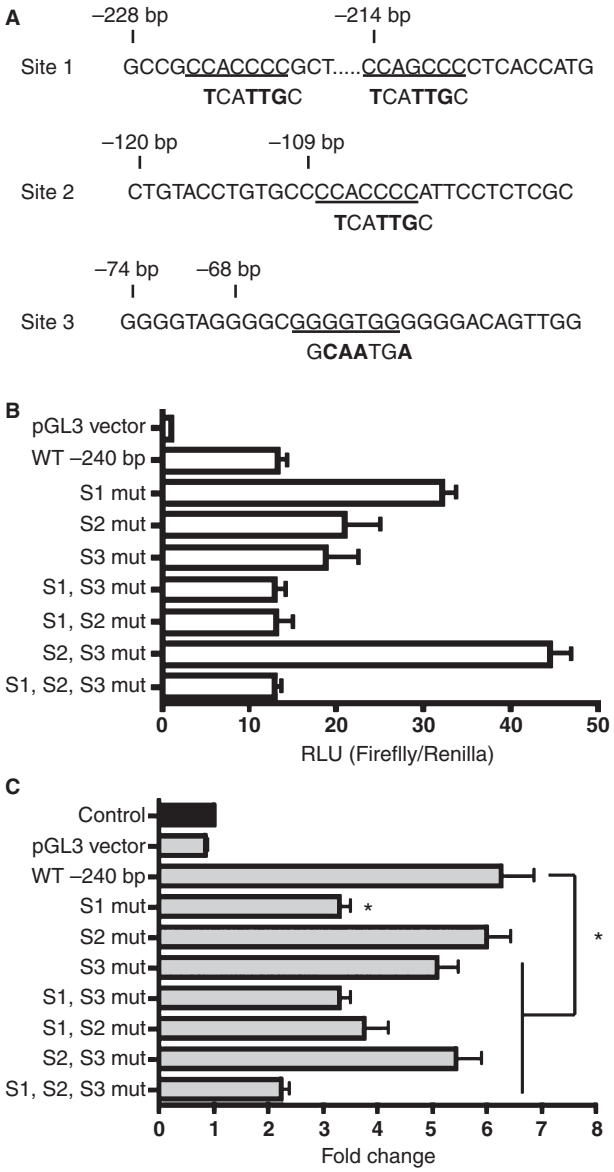

Figure 5 *Helicobacter pylori* stimulate the gastrin promoter through GC-rich DNA elements. AGS cells were transiently transfected with the wild-type (WT) 240- bp gastrin luciferase reporter or the reporter containing point mutations at the indicated sites (S1, 2, 3). Altered nucleotides are shown in bold font (A). The basal activity of gastrin constructs is shown as relative light units (RLU) of firefly to Renilla activity (*B*). The cells were co-cultured with *H. pylori* 48- hour post-transfection (*C*). The mean fold change ± SEM for three separate experiments was performed in triplicate. The filled bar represents the baseline expression in RLU for each construct set to 1 and fold changes were the ratio of the stimulated activity compared to basal expression.

treatment (Fig. 6, lanes 9 and 10). By contrast, AP1 protein binding increased consistent with prior studies (Fig. 6, lanes 13 and 14) [43,44].

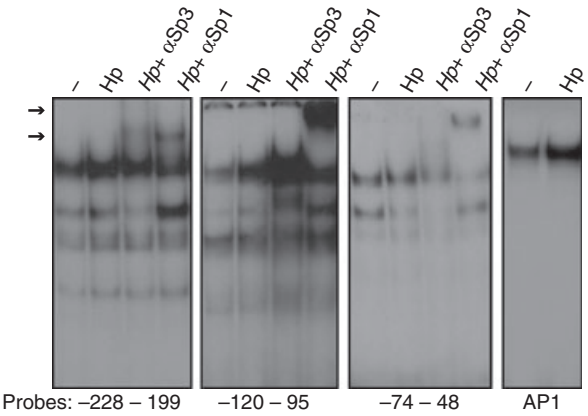

Figure 6 *Helicobacter pylori* stimulate binding of Sp1 and Sp3 to the GC-rich DNA elements. EMSA reactions were carried out with 4 μg of nuclear extracts from AGS cells that were untreated (—) or co-cultured with *H. pylori* 26695 strain for 3 hour (Hp). Labeled probes represent GC-rich DNA elements of the human gastrin promoter and a consensus AP1 binding sequence. The samples were incubated with antibodies against Sp3 or Sp1 as indicated. Arrows indicate the supershifted Sp1 and Sp3 DNA complexes.

Discussion

In this study, we demonstrated that the increase in gastrin mRNA initiated by an *H. pylori* infection is because of the transcriptional initiation. We used the AGS cell line to show that gastric cells can respond directly to the bacteria alone. It has been widely reported that the number of G cells and the release of gastrin increases during *H. pylori* infection in human subjects and various animal models [19,45,46]. Indeed, we also observed that gastrin gene expression was higher in *H. pylori*-infected mice when compared to uninfected mice. Yet, few studies have actually examined whether the increase is related to induction of gastrin gene expression as opposed to an increase in secretion of the hormone. Moreover, the cell line experiments were important to perform to distinguish between a direct induction of human gastrin gene expression by bacteria versus receptor-mediated activation by pro-inflammatory cytokines. Separating these two events is impossible to achieve in vivo in human subjects or mouse models prompting our use of a human cell line and human isolates of *H. pylori*. Although nearly pure rat G-cell cultures have been used to study gastrin release [26], transfecting these primary cells with various constructs still remains problematic.

We found that *H. pylori* alone effectively induced the endogenous human gastrin gene. Furthermore, no induction was observed when the cells were co-cultured with dead bacteria, which underscores the requirement for viable bacteria. *H. pylori* virulence factors have been associated with more severe clinical

outcomes for infected patients, and the presence or absence of these factors can alter the regulatory effect on cells as well [10,47,48]. Specifically, *H. pylori* strains that lack CagA tend to induce less inflammation and are less commonly associated with the development of ulcers and adenocarcinoma [49,50].

The bacterial virulence factors, CagA and VacA, were not required for an increase in gastrin mRNA, but rather other yet to be determined components of the live bacteria were required to induce gastrin through activation of MAPK signaling. Indeed, *Helicobacter* lipopolysaccharides have recently been shown to activate the ERK MAPK [51] whereas CagA and VacA activate host genes through the NFκB pathway [40,52]. Further Cag3 has recently been shown to be a non-CagA, non-VacA protein component of the T4S system that possibly activates a distinctive signal transduction pathway [53]. Therefore, we concluded that *Helicobacter* stimulates gastrin through a general bacterial factor, such as an outer membrane protein, or lipopolysaccharide as opposed to a virulence factor expressed from a pathogenicity island not present in all bacteria. *Helicobacter* LPS stimulation of gastrin release from primary G cells was partially inhibited by the ERK inhibitor PD98059 supporting LPS as one candidate [26]. Documenting that *H. pylori* stimulated transcriptional initiation permitted mapping of the DNA elements mediating the induction. We found that a CCACCC (GC-rich) binding motif, mediated gastrin promoter activation. More importantly, we showed that *H. pylori*-induced MAPK signaling induced binding of transcription factors Sp1 and Sp3 to the GC-rich element at −109 bp upstream from the transcription start site of the human gastrin gene, but not at the −68 GC-rich element previously shown to mediate EGF responsiveness [54]. Although the −68 GC-rich element binds Sp1, the induction of gastrin gene transcription by EGF through ERK signaling is achieved by phosphorylation of Sp1 [31] rather than inducible binding of Sp1 to the −68 GC-rich site (gERE) [32]. Activation of the EGF receptor by *H. pylori* has also been reported for immortalized human gastric cells [44]. *H. pylori* induction of gastrin gene expression and secretion might retrospectively explain the observations from the 1960s of duodenal ulcer patients exhibiting parietal cell hyperplasia and enhanced acid secretion [55,56]. Furthermore, in some colorectal cancers, mutations in Ki-ras, which signals through ERK kinase, were found in tumors with elevated levels of gastrin [30]. Thus, collectively, our findings demonstrate that *H. pylori* can directly induce gastrin gene expression and reiterates the idea that activation of MAPK signaling is the primary proximal signaling mechanism for induction of gastrin gene expression, an event that might also impact the ability of the bacteria to promote transformation.

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