INDUCIBLE REGULATION OF GASTRIN GENE EXPRESSION DURING *HELICOBACTER PYLORI* INFECTION

by

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DEDICATION

To Kenyatta, words alone cannot express what your love and support has meant to me. Thank you for always believing in me and always pushing me toward the finish line. I am because YOU are.

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ABSTRACT

INDUCIBLE REGULATION OF GASTRIN GENE EXPRESSION DURING *HELICOBACTER PYLORI* INFECTION

by

Tamara L. Tucker

Chair: Juanita L. Merchant

Gastrin is a peptide hormone expressed in the adult antral stomach. Gastrin is a potent regulator of acid secretion and is a growth factor required for the maintenance of gastrointestinal physiology. The expression of gastrin is regulated by fasting, re-feeding, and gastric pH.

Infection by the gut pathogen, *Helicobacter pylori* (*H. pylori*), increases the expression of gastrin. *H. pylori* is the most prevalent bacterial pathogen in man. In a percentage of infected subjects it generates a Th1-mediated chronic gastritis. Furthermore, *H. pylori* infection is a risk factor for the development of duodenal ulcers and gastric cancer. Elevated levels of gastrin have been observed in human cancers and contribute to the development of duodenal ulcers even in the absence of *H. pylori*. These findings implicate gastrin as a

contributing factor in the progression of *H. pylori*–mediated disease. Therefore, the regulatory elements mediating regulation of gastrin gene by *H. pylori* infection were defined and characterized.

In infected mice and AGS (human gastric cancer) cells, *H. pylori* induced a 7- to 8-fold increase in endogenous gastrin gene expression. I determined that the bacteria stimulated transcriptional initiation of gastrin by activating MAP kinase signaling. I identified GC rich promoter elements that mediated induction of the gastrin promoter by *H. pylori* and demonstrated that the bacteria induced binding of the transcription factors Sp1 and Sp3 to these elements.

Also, the effect of the pro-inflammatory cytokine IFNγ on gastrin gene expression was studied. An increase in the numbers of G cells and plasma gastrin following IFNγ infusion was observed in mice. However, changes in gastrin gene expression were not detected. Treatment of AGS cells with IFNγ had no effect on gastrin gene expression. Therefore, I concluded that IFNγ does not regulate gastrin gene expression directly.

In summary, I established that regulation of gastrin gene expression by *H. pylori* is mediated directly by the bacteria through GC rich elements that bind Sp1 and Sp3. These findings contribute to our knowledge of the regulatory mechanisms that activate gastrin gene expression.

Chapter I

INTRODUCTION

Significance of the Study

Over a century ago, gastrin was identified as the chemical regulator of gastric acid secretion. It was these initial studies that established a paradigm for chemical control of organ function that we now understand as hormonal regulation (Bayliss and Starling 1902; Edkins 1906). Despite years of technological and scientific advances these earliest observations form the basis for our understanding of the hormone, gastrin. Gastrin is a potent regulator of acid secretion in response to fasting and re-feeding. Additionally, gastrin is one of the most important growth factors for gastrointestinal cells and plays a critical role in maturation, proliferation, and organization of cells within the gastric mucosa. More recent studies reveal an increasingly complex role for gastrin as a regulator of expression for a wide variety of genes involved with essential cellular processes, including cell growth, differentiation, migration, cytoskeletal organization, epithelial maintenance, and signal transduction (Dimaline and Varro 2007). Additionally, the over-expression of gastrin is associated with various human diseases including chronic atrophic gastritis, peptic ulcer disease, gastric metaplasia, and cancer.

It is the broad objective of this thesis to contribute findings that will further our understanding of the factors that regulate the expression of gastrin in the context of normal physiology and disease. This introduction will present a brief overview of the gastrin gene, protein, distribution and roles in the gastric mucosa

as a regulator of acid secretion and as a gut growth factor. Subsequently, a review of the physiologic and transcriptional regulators of gastrin will be presented with a focus on the regulation of gastrin during infection by the gastrointestinal pathogen, *Helicobacter pylori (H. pylori)*.

Gene Structure, Protein Structure and Processing

The gastrin gene occupies a single gene locus of approximately 3600 bp on human chromosome 17 (Ito, Sato et al. 1984; Wiborg, Berglund et al. 1984). The gene structure includes three exons and two introns. The first and largest intron lies between the first exon, which is transcribed but not translated, and the second exon. The second, small intron falls between the coding exons and demarcates the C-terminal portion of the protein, which is cleaved from the N-terminal fragment during processing. The gene contains a canonical TATA box sequence 26 base pairs from the 5’ cap site and a poly A tail (Fig 1.1A) (Ito, Sato et al. 1984; Wiborg, Berglund et al. 1984).

The gastrin gene is translated into the preprogastrin protein consisting of 101 amino acids. The complete peptide contains a 21 amino acid signal peptide, a 37 amino acid spacer sequence, a 9 amino acid extension, and the 34 amino acid gastrin pro-fragment, which includes the residues for the secreted forms of gastrin (Fig 1.1B). The gastrin peptide is synthesized in the endoplasmic reticulum and is then modified in the Golgi. The peptide is cleaved in transport vesicles to generate the 34 and 17 amino acid fragments, gastrin-34 and gastrin-

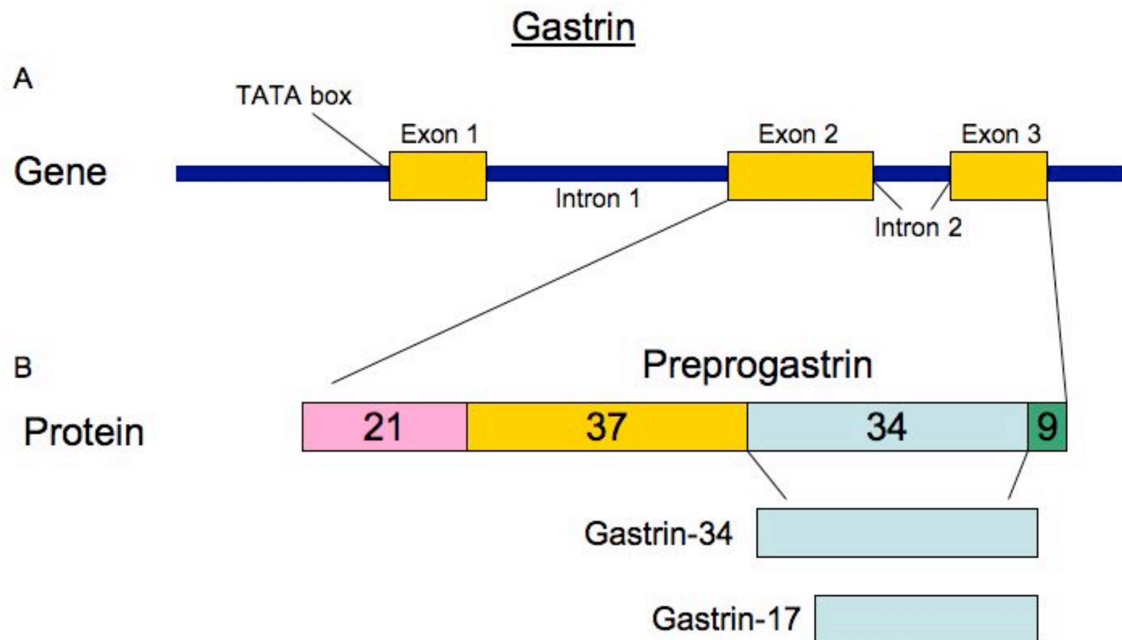

Figure 1.1. Gastrin Gene and Protein Structure. Schematic representation of the gastrin gene locus (A) and the gastrin protein (B). Exons and introns are labeled. The gastrin gene has three exons, however only exons 2 and 3 are transcribed and translated. The product of the gastrin gene is a101amino acid preprogastrin peptide. This initial peptide contains the following fragments indicated by the number of amino acids (aa); 21aa - signal peptide, 37aa - spacer sequence, 34aa - progastrin fragment, and 9 - aa extension. The major biologically active forms of gastrin (Gastrin-34 and Gastrin-17) are shown below and are comprised within the 34aa progastrin fragment.

17. Gastrin peptides are modified into the glycine-extended and amidated forms in secretory vesicles prior to release from the cell (Hilsted and Rehfeld 1987; Dickinson and Yamada 1991; Dockray, Varro et al. 2001).

The two major bioactive forms of the protein, gastrin-34 and gastrin-17, are amidated prior to being released from the cell (Dockray 1999). There are minor forms of gastrin, which were initially considered to be biologically inactive, but have since been shown to act as growth factors in the pancreas and in other gastrin-expressing tissues beyond the stomach. These peptides include the glycine extended forms of gastrin-34 and gastrin-17 as well as gastrin-71, gastrin-52, gastrin-14, and gastrin-6 (Dockray 1999).

Gastrin-Producing Cells and Tissue Distribution

Gastrin is predominantly expressed in the adult antrum with low-level expression in the duodenum, colon, pancreas, bronchial mucosa, brain, and reproductive organs (Rehfeld 1998). During fetal development gastrin is expressed transiently in the pancreas. Low levels of gastrin are also detected in the stomach, the small intestine, and the colon prior to birth (Track, Creutzfeldt et al. 1979). The expression of gastrin in the antral stomach is primarily associated with its regulation of acid secretion. However, gastrin also acts as a growth factor for the cells of the stomach, foregut-derived cell populations, and the lung (Walsh 1990; Rehfeld 1998). There are trace amounts of gastrin expressed in the brain

and reproductive organs, however, the biological role for gastrin in these tissues is unknown.

Gastrin peptide is produced in the antrum by neuroendocrine cells that are referred to as, G cells. The distribution of antral G cells varies between species with the G cell population appearing in the middle of the antral gland between the neck cells and the glandular base in both humans and canines. In mice and rats, G cells tend to be localized at the base of the gland. G cells are found in the human and rodent duodenum in very low numbers. However, observations indicate that there are differences between the function and regulation of antral versus intestinal G cells. In the antrum, G cells originate from a gastric progenitor population within the neck cell region of the gland (Inokuchi, Fujimoto et al. 1985). Upon acute stimulation of the G cell, gastrin protein content is secreted from these cells into the bloodstream.

Regulation of Acid Secretion

The primary biological role for gastrin is the regulation of acid secretion in the stomach. Gastrin gene expression, peptide production, and release are stimulated by components of a meal such as amino acids, proteins, and calcium. Additionally, low levels of acid trigger gastrin gene expression, followed by the production and secretion of the gastrin protein. The peptide is released from the G cells into the blood stream, and ultimately binds to the gastrin/CCK2 receptor that is found on parietal cells and enterochromaffin-like (ECL) cells of the corpus. Gastrin has a direct stimulatory effect on parietal cells that produce and secrete

acid. However, the primary stimulus for acid secretion is histamine, which is produced by ECL cells in response to gastrin. Histamine acts on the parietal cell to stimulate acid production. The decrease in the pH in the stomach stimulates, the release of somatostatin from the D cells. Somatostatin then inhibits the release of acid as well as gastrin gene expression and release of the gastrin protein, which completes a negative feedback loop(Walsh and Grossman 1975; Walsh 1994).

Gastrin as a Growth Factor

Gastrin is important for normal development and growth of the gastric mucosa. This is especially true for cells within the acid-secreting portion of the stomach including ECL and parietal cells. These cells have gastrin receptors that facilitate the regulation of acid secretion but also enable gastrin to affect the growth and maturation of these cells directly. Early studies into the role of gastrin revealed that removal of the antrum resulted in atrophy of the stomach (Neuburger, Lewin et al. 1972). Furthermore, patients with high levels of circulating gastrin show significant hyperplasia of the gastric mucosa (Walsh and Grossman 1975). Thus the role of gastrin as an important trophic factor is confirmed by changes in the growth of the gastric epithelium due to either the loss of gastrin or an excess of the hormone. Beyond the stomach, gastrin has been shown to have trophic effects in the small intestine and colon as well. Our understanding of the role of gastrin as a growth factor has been expanded through studies of transgenic mice, which either lack or over-express gastrin.

Studying mouse models that do not express gastrin has improved our understanding of the necessity for gastrin in normal growth and development of the stomach. Gastrin-deficient mice show significantly reduced levels of basal and stimulated acid (Friis-Hansen, Sundler et al. 1998). The major cell types including parietal, ECL, chief, and mucous cells were present in the stomachs of gastrin-null mice, however, there were significant decreases in the numbers of parietal and ECL-cells (Koh, Goldenring et al. 1997; Friis-Hansen, Sundler et al. 1998). Furthermore, there were functional defects in both parietal and ECL-cells. These findings indicate that while gastrin is not required for the development of these cell types it is clear that it is important for maintaining their normal function (Koh, Goldenring et al. 1997; Friis-Hansen, Sundler et al. 1998). Additionally, studies have shown that gastrin can indirectly influence growth of the gastric mucosa by regulating the expression of other growth factors, such as EGF ligands, that are produced in the stomach (Jain, Brunkan et al. 2006).

INS-GAS mice, which produce excess gastrin peptide, show increased proliferation of the gastric mucosa (Wang, Koh et al. 1996; Singh, Velasco et al. 2000; Jain and Samuelson 2006). Increased numbers of parietal and ECL-cells were also observed (Wang, Koh et al. 1996; Singh, Velasco et al. 2000; Jain and Samuelson 2006). Furthermore, these mice showed increased secretion of gastric acid as well. Sustained increases in the levels of gastrin ultimately resulted in the loss of parietal cells and increased the development of gastric metaplasia in these mice (Wang, Koh et al. 1996). Thus, the role of gastrin as a

growth factor in the stomach is complex but is clearly related to its ability to maintain gut homeostasis.

Physiologic Regulation

The molecular mechanisms for the regulation of gastrin by acid (pH), amino acids, or calcium are unknown although it is presumed that a direct regulatory effect of the G cell is involved. The peptide bombesin/ gastrin releasing peptide (GRP, in humans) stimulates gastrin protein release in response to vagal nerve stimulation (Dockray 1979). However, bombesin/GRP does not affect gastrin gene expression (Godley and Brand 1989; Ford, Valle et al. 1997). Additionally, gastrin inhibitory peptide (GIP), vasoactive intestinal peptide (VIP), secretin, glucagons, and calcitonin are also known to inhibit gastrin protein release. The inhibition of gastrin by VIP is indirect whereby VIP actually stimulates the release of somatostatin from the D cell and then somatostatin inhibits gastrin gene expression and protein release (Sawada and Dickinson 1997). GRP, VIP, and secretin are expressed in the pancreas as well as in the stomach and are presumed to regulate gastrin gene expression during fetal pancreatic development and neoplastic transformation (Brand and Stone 1988).

Transcriptional Regulation

It is known that physiologic stimuli trigger the production and release of the gastrin peptide, however, these stimuli also regulate expression of the gastrin gene. For this reason, a significant number of studies have been performed to

understand transcriptional regulation of the human gastrin gene. Early studies identified EGF growth factors as potent regulators of gastrin gene expression (Godley and Brand 1989). Specifically, a GC-rich gastrin EGF response (gERE) element located at – 68 bp was identified as the region that mediates responsiveness of the gastrin gene to EGF receptor ligands (Fig 1.2) (Merchant, Demediuk et al. 1991). This element was shown to bind the zinc finger binding proteins, Sp1 and Sp3. Sp1 was shown to be required for both basal and EGF-induced expression (Merchant, Shiotani et al. 1995; Merchant, Du et al. 1999). Whereas Sp3 minimally activated that gastrin promoter and might act as a repressor by competing with Sp1 binding (Merchant 2000).

Further studies by our group to understand EGF in the context of physiologic regulators of gastrin gene expression examined the role of cFos as an inducer of gastrin. Expression of the transcription factor cFos increases in response to growth factors such as EGF. Specifically, our group demonstrated that EGF stimulates cFos expression (Marks, Iyer et al. 1996). Furthermore, expression of cFos mRNA enhances EGF activation of the gastrin promoter (Marks, Iyer et al. 1996). Thus we have shown that cFos is required for induction of the gastrin promoter by EGF(Marks, Iyer et al. 1996).

While studies by our group have elucidated the transcriptional mechanism for the regulation of the gastrin gene by EGF, the physiological significance of EGF regulation of gastrin has remained elusive (Murayama, Miyagawa et al. 1995). EGF growth factors are produced in the gastric mucosa by parietal cells and also produced by immune cells during an inflammatory response (Madtes,

Malden et al. 1991; Murayama, Miyagawa et al. 1995; Calafat, Janssen et al. 1997). Thus, additional studies have examined transcriptional regulation of the gastrin gene by factors other than EGF receptor ligands with the hope of identifying additional mechanisms that are related to the physiologic regulation of the gene.

The second messenger, Cyclic AMP (cAMP), induces gastrin through an element between - 148 and - 40 bp of the human gastrin promoter (Shiotani and Merchant 1995). This mechanism of gastrin regulation is likely to be more applicable in the pancreas since bombesin and VIP are expressed predominantly within this tissue and are known to activate cAMP signaling (Shiotani and Merchant 1995).

Some colon cancer cell lines and colorectal tumors show increased levels of gastrin mRNA when compared to normal tissue. These particular tumors were shown to possess mutations in Ki-ras that resulted in increased gastrin gene expression (Nakata, Wang et al. 1998). This same study demonstrated that Ras over-expression could activate the gastrin promoter *in vitro* (Nakata, Wang et al. 1998). Ultimately, activation of the gastrin promoter by Ras was shown to be mediated by ERK signaling through the gERE element (Merchant, Du et al. 1999). Additional studies have shown that the human gastrin promoter can be induced by β-catenin through an element at – 103 bp (Fig 1.2) (Koh, Bulitta et al. 2000). The authors concluded that the APC-β catenin pathway can also regulate gastrin and might be relevant to the induction of gastrin in colon cancer.

Gastrin gene expression is inhibited as levels of acid in the stomach increase (Walsh and Grossman 1975). It is thought that the negative regulation of the gene occurs in response to the drop in gastric pH. Moreover, the affect appears to be related to the ability of acid to stimulate the D cell to produce somatostatin. Somatostatin is a potent inhibitor of gastrin release and exerts its paracrine effects directly on the G cell through the somatostatin receptor, SSTR2. Somatostatin has been shown to inhibit both basal and induced gastrin gene expression (Giraud, Soll et al. 1987). This regulatory effect was mapped to a helix-loop-helix element between - 82 to - 69 bp of the gastrin promoter (Fig 1.2) (Bachwich, Merchant et al. 1992).

ZBP-89 is zinc finger binding protein that was shown to bind the gERE element and decrease gastrin promoter activity (Merchant, Iyer et al. 1996). Interestingly, the gene for ZBP-99 at 1q32, a close relative to ZBP-89 that also binds the gastrin promoter, resides on chromosome at 1q32. Mutations at 1q21-23 are associated with increased growth and metastasis of sporadic gastrinomas (Chen, Vortmeyer et al. 2003). This finding suggests that ZBP -99 also exerts a negative regulatory effect on gastrin expression, however, it is not known if ZBP-99 is mutated in gastrinomas. Moreover, the regulation of the gastrin promoter by ZBP-99 has yet to be characterized (Chen, Merchant et al. 2003).

Several elements have been identified that mediate tissue specific expression of the gastrin gene (Fig 1.2). Regulatory elements that mediate the expression of gastrin in pancreatic islet-cells were identified at -150, -109, and -103 bp within the promoter. A homeodomain element and a gastrin negative

Human Gastrin Promoter (-240bp to -40bp)

- 240 bp
AGCTGGAGAGCTGCCGCCACCCCGCTCCAGCCCCT

- 200 bp
CACCCACCATGAAGGTCAACTCCCCTATCCTTCCCCC

- 150 bp
ACATCCTGGATGACTGACTGACACTAAATGAAA
Homeodomain

GGGCGGGGCAGGGTGATGGGCTGTACCTGTGCC
Sp1

- 100 bp
CCACCCCATTCCTCTCGCCTGGACTCATATGGCAGG
CACC element βcat/ GASNE Somatostatin

- 50 bp
GTAGGGGCGGGGTGGGGGGACAGTTGGGAGGGA
gERE

Figure 1.2. Human Gastrin Promoter Elements. Shown is the sequence of the gastrin promoter from -240 bp to -40 from the start site of transcription. Regulator elements are indicated by underlines and are labeled. Tissue specific elements are labeled in red.

response element (GASNE) at – 150 and -103 respectively were show to bind nuclear proteins, however, the identities of these proteins were not determined (Wang and Brand 1990; Chung, Brand et al. 1995). The transcription factor, RinZF (also known as ZBTB 10), was shown to bind at a CACC element on the gastrin promoter at – 110 bp (Fig 1.2). RinZF was shown to mildly activate the gastrin promoter when over-expressed, however, it blocked Sp1 induction of gastrin (Tillotson, Wang et al. 1994; Tillotson 1999). This result indicated that in response to physiologic stimuli this transcription factor might function as a repressor by interfering with the binding of Sp1 (Tillotson 1999).

Beyond studying the regulation of gastrin *in vitro,* it is still not clear what role these elements and transcription factors have in the regulation of the gastrin gene *in vivo*. Recent studies from our lab have sought to address this issue by examining regulation of the gastrin gene in transgenic mice that express human gastrin. The hGasBAC mice model expresses a 250 Kb human BAC clone that contains the human gastrin gene on a gastrin-deficient mouse genetic background (Mensah-Osman, Labut et al. 2008). While G cell specific expression of human gastrin was observed in these mice, the protein was not processed or secreted until the endogenous levels of somatostatin were suppressed by infusing rodent gastrin (Mensah-Osman, Labut et al. 2008). Therefore, human gastrin is exquistely sensitive to the tissue levels of somatostatin.

Gastrin in Human Disease Etiology

From a clinical perspective, hypergastrinemia is defined as elevated levels of gastrin in the blood. However there are varying degrees to which this occurs in patients as well as different causes of this condition. Gastrinomas are tumors that produce and secrete high amounts of gastrin. These tumors are rare but can occur sporadically or in patients who have multiple endocrine neoplasia type I (MENI) syndrome. Gastrinoma patients have circulating levels of gastrin up to 10 times higher than normal, a condition which is called Zollinger-Ellison syndrome (ZE). Both sporadic and MENI gastrinomas typically occur in the pancreas and the duodenum. These tumors rarely occur in the antrum where gastrin expression is thought to be tightly regulated by fasting, re-feeding, and pH.

In developed nations, the prolonged use of proton pump inhibitors (PPIs) to treat digestive diseases such as acid-reflux increases the incidence of hypergastrinemia. Early restrictions on the long term use of PPIs were enacted by the FDA when animal studies showed increased development of fundic polyps and carcinoid tumors following long term suppression of acid (el-Zimaity, Jackson et al. 1997). It is thought that acid suppressive therapy leads to increased levels of gastrin by decreasing the acidity in the stomach (Brand and Stone 1988). However, compared to the levels observed in ZE patients, the increase in gastrin levels due to PPI use are modest (approximately 2 times normal). It has been shown that bacterial overgrowth occurs in the absence of acid, so it is likely that the chronically low levels of acid and the presence of bacteria in the stomach

contribute to elevated levels of gastrin during prolonged PPI usage (Zavros, Rieder et al. 2002).

Chronic atrophic gastritis is a condition that is usually caused by persistent infection with *H. pylori*. In the context of this condition, a subset of patients have elevated levels of circulating gastrin. Estimates place the average global rate of infection at 60% with a high degree of infection (80-90%) in the developing world, whereas, rates in developed nations are at about 25%. While the majority of infected individuals remain asymptomatic, significant rates of infection make *H. pylori*-induced hypergastrinemia a relatively common occurence. Significant discussion in this introduction will further explore the connection between *H. pylori* infection, elevated levels of gastrin, and human disease.

It remains controversial whether gastrin has a significant role in the growth and development of gastrointestinal tumors. Gastrin has been linked to colon cancer using epidemiological data and *in vitro* studies (Renga, Brandi et al. 1997). Gastrin has also been shown to promote the growth of colon tumors in mice (Stepan, Sawada et al. 1999). Gastrin receptors have been found in pancreatic tumor tissue, and studies have shown that gastrin promotes the growth of these tumors (de Weerth, van Geert et al. 1999). However, it is unknown whether gastrin actually promotes tumor growth or whether it is simply induced by other factors present within cancers. Several groups are pursuing the issue of whether gastrin has a causative or contributory effect on colon tumor growth.

Studies with Zollinger-Ellison (ZE) patients provide evidence for a role of gastrin in ulcer development. Ninety five percent of ZE patients, that have hypergastrinemia in the absence of *H. pylori* infection or gastritis, develop peptic ulcers suggesting that gastrin alone is driving ulcer development. This effect is presumably through its ability to induce acid secretion. Ulcers are a major cause of morbidity within human populations worldwide and the vast majority of ulcers are associated with *H. pylori*. Past or present infection with *H. pylori* is linked to 60% of gastric and almost 90% of duodenal ulcers. However, it is unclear whether the chronic inflammatory response by the host or overproduction of acid driven by high gastrin levels has the greatest impact on ulcer development. This interplay between bacterial infection, inflammation, and elevated levels of gastrin will be explored throughout the course of this thesis.

***H. pylori*, Bacterial Pathogen of the Stomach**

Until late in the 20th century, various factors including smoking, eating spicy food, stress, and excess acid in the stomach were thought to cause ulcers. The discovery of *Helicobacter* species within the gut of patients with gastritis was the first step in shifting our understanding of the causes of ulcers in man. The work of Marshall et al. demonstrated that colonization by *H. pylori* alone was sufficient to cause gastritis in patients and most dramatically in Marshall himself (Marshall, Armstrong et al. 1985).

H. pylori is a bacterial pathogen that infects the epithelium of the stomach. Past or current infection with *H. pylori* is a risk factor in 80% of gastric cancer

cases. For this reason, it is categorized as a class I carcinogen by the World Health Organization. Overall the incidence of gastric cancer is rare in the United States but is the fourth most frequent cancer worldwide and the second leading cause of cancer-related deaths (Parkin 2004). This high rate of mortality is most likely due to the fact that gastric cancer has a poor prognosis for treatment or cure, often metastasizes, and has a high rate of incidence.

Approximately 50-60% of gastric ulcers and 90% of duodenal ulcers are associated with *H. pylori* infection (Sawada and Dickinson 1997). Specifically, chronic inflammation due to the presence of *H. pylori* often precedes the development of peptic ulcers. Both *Helicobacter*-mediated gastritis and ulcer disease are thought to be precursor lesions that increase the risk for the development of gastric cancer. The strong correlation between *H. pylori* and human disease has prompted significant efforts by clinicians and scientists to understand the bacteria and its ability to cause disease in humans.

H. pylori is a gram negative bacteria that secretes a protective coating of urease that enables it to survive in the acidic environment of the stomach and ultimately colonize the gastric mucosa of humans and primates (Kusters, van Vliet et al. 2006). *H. pylori* is genetically heterogeneous and the variations in strain of the bacteria have been widely studied in the context of disease etiology and the effect on epithelial cells.

Within the *H. pylori* genome there is a cluster of genes known as the cag pathogenicity island (cagPAI). The majority of the genes contained within the cagPAI encode proteins that form a type IV secretion system (T4SS). This is a

syringe-like protein structure that perforates the membrane of epithelial cells and allows for the injection of bacterial proteins into the cell (Cascales and Christie 2003). In the case of *H. pylori*, the T4SS injects the primary gene product of the cagPAI locus, which is a 140 kDa protein known as cytotoxin associated gene A (CagA) (Kusters, van Vliet et al. 2006). From a clinical perspective, both cagPAI and CagA are associated with more virulent strains of the bacteria and are associated with more inflammation and an increased risk for the development of ulcers and gastric cancer (Kuipers, Perez-Perez et al. 1995; Blaser and Crabtree 1996). Additional virulence factors of *H. pylori* have also been studied. The vacuolating cytotoxin A (VacA) is a protein that is secreted by the bacteria and causes small vacuoles to form in epithelial cells (Kusters, van Vliet et al. 2006). The VacA protein is produced by approximately 50% of *H. pylori* strains and is associated with increased rates of peptic ulcer formation and gastric cancer as well (Atherton, Cao et al. 1995).

On a cellular level, *H. pylori* activates numerous signal transduction cascades within epithelial cells. Upon contact with gastric epithelial cells, *H. pylori* has been shown to increase the expression of growth factors such as EGF ligands and VEGF. The bacteria activates pathways associated with cell survival including PI3K, MAP kinase, and NFκB. Specifically, CagA is injected into epithelial cells and then is phosphorylated by Src family kinases (Selbach, Moese et al. 2002). Once phosphorylated, CagA interacts with signaling molecules including SHP-2 and Ras (Meyer-ter-Vehn, Covacci et al. 2000; Higashi, Tsutsumi et al. 2002). The presence of CagA is often associated with a

higher induction signaling by *H. pylori* including enhanced MAP kinase activation and increased induction of the AP1 transcription factors, cJun and cFos (Meyer-ter-Vehn, Covacci et al. 2000). VacA exerts its effects on epithelial cells primarily through its ability to create pores in the cell membrane. This results in the movement of ions across the membrane and the release of nutrients, events that are harmful to the cell (Atherton, Cao et al. 1995).

H. pylori causes a chronic inflammatory response in most infected patients (Dooley, Cohen et al. 1989). The inflammatory response begins with the recruitment of lymphocytes, neutrophils and macrophages to the infected epithelium. Binding of the bacteria to cell surface receptors, such as MHCII, primarily triggers this inflammatory response, since *H. pylori* rarely invades the mucosa (Fan, Gunasena et al. 2000; Suerbaum and Michetti 2002). In human patients and in animal models, *H. pylori* induces a predominantly T helper type 1(Th1) immune response (D'Elios, Manghetti et al. 1997; Bamford, Fan et al. 1998). Increased levels of pro-inflammatory cytokines such as IL-Iβ, IL-2, IL-8, TNFα, and IFNγ are observed (Crabtree, Shallcross et al. 1991; Yamaoka, Kita et al. 1996; Yamaoka, Kita et al. 1997). While many cytokines are produced, studies using mouse models suggest that IFNγ is the predominant cytokine required for the *H. pylori*-mediated inflammatory response. Specifically, mice that lack the expression of IFNγ did not develop inflammation in response to *H. pylori* (Sawai, Kita et al. 1999; Lohoff, Rollinghoff et al. 2000; Smythies, Waites et al. 2000; Yamamoto, Kita et al. 2004). Furthermore, IFNγ mediated the induction of MIP-2 and iNOS, two genes that enhance the inflammatory response, in

infected mice (Obonyo, Guiney et al. 2002). Thus, I have focused my studies on the role of IFNγ as a mediator of *H. pylori*-induced disease.

As a major product of the Th1 immune response, IFNγ further promotes the Th1 phenotype (Ellis and Beaman 2004). Specifically, IFNγ activates the transcription of many genes associated with Th1 inflammation (Boehm, Klamp et al. 1997; Carnaud, Lee et al. 1999; Schroder, Hertzog et al. 2004). IFNγ signals predominantly through the Jak-Stat pathway in a process that involves the activation of Janus kinases (Jaks) and ultimately Stat transcription factors (Schroder, Hertzog et al. 2004). Typically, Stat1 homodimers and to a lesser degree heterodimers between Stat1 and Stat2, Stat3, or other factors bind to the promoters of IFNγ-regulated genes (Matsumoto, Tanaka et al. 1999; Chatterjee-Kishore, van den Akker et al. 2000). However, there are studies that describe Stat1-independent IFNγ signaling pathways that demonstrate that IFNγ can regulate genes through diverse molecular mechanisms (Ramana, Gil et al. 2001; Ramana, Gil et al. 2002).

Changes in Gastrin During *H. pylori* Infection

Scientists and clinicians have sought to understand the causative mechanisms that drive *H. pylori*-mediated disease. Furthermore, the focus on acid as a causative agent in PUD led scientists to consider the impact of gastrin on the development of ulcers. In 1994, it was proposed that elevated levels of gastrin during *H. pylori* infection might contribute to enhanced proliferation of the gastric epithelium and ultimately contribute to the development of gastric cancer

(Blaser and Parsonnet 1994). It is unlikely that circulating gastrin plays a major role in cancer development, however, the regulation of acid by gastrin presents a more plausible role for this hormone in peptic disorders caused by *H. pylori.*

There is strong evidence that high levels of gastrin alone can contribute to the development of ulcers. In ZE patients there is a 95% incidence of ulcers attributed to high levels of gastrin, even in the absence of *H. pylori* infection (Pellicano, Peyre et al. 2006). This finding implicates gastrin in the development of ulcers but also contributes to our understanding of the impact of hypergastrinemia within the context of *H. pylori* infection and disease.

In the stomach, *H. pylori* infection causes both gastritis and elevated levels of gastrin. As gastrin increases, the parietal cells are stimulated to produce acid, which is the first line of defense against invasive pathogens in the gut. However, *H. pylori* is able to survive in the highly acidic environment of the stomach and can also evade the immune response mounted by the host. This results in a persistent infection, which leads to the development of chronic active gastritis and the overproduction of both gastrin and gastric acid. The excess acid erodes unprotected mucosal tissues, such as the duodenum, and the lining of the distal stomach and ultimately leads to the development of an ulcer.

Thesis Overview

While we understand that *H. pylori* infection leads to elevated levels of gastrin, the molecular mechanisms that mediate this effect are unknown. There is evidence to suggest that both the bacteria and the inflammation that occurs

due to the presence of the pathogen might cause elevated levels of gastrin (Go and Graham 1994). The overall goal of this dissertation is to understand how *H. pylori* infection leads to elevated levels of gastrin. Specifically, I explored the hypothesis that *H. pylori* infection induces the expression of the human gastrin gene and conducted studies designed to understand the molecular mechanism of this effect.

Regulation of the Gastrin Gene by *H. pylori*

In the study presented in Chapter II, I investigated the ability of *H. pylori* to regulate gastrin gene expression through a direct effect on gastrin-producing cells. I first examined the expression of the gastrin gene in *H. pylori*-infected mice. Secondly, I utilized *in vitro* studies in a human gastric cell line to establish the ability of the bacteria to regulate gastrin gene expression. I measured the expression of the endogenous gene as well as the activity of a gastrin reporter construct to further characterize the effect of the bacteria on gastrin gene expression. Lastly, I examined protein-DNA interactions to define potential mechanisms for the regulation of the gastrin gene by *H. pylori*.

Regulation of the Gastrin Gene by the Inflammatory Cytokine, IFNγ

In Chapter III, I examined the regulation of gastrin gene expression by the Th1 cytokine, IFNγ. To begin these studies, I utilized a model previously established in our lab where IFNγ infusion was used to mimic *H. pylori*-induced gastritis and elevated the levels of plasma gastrin in mice. I also injected mice

with the cytokine and investigated whether IFNγ altered the expression of gastrin *in vivo*. Lastly, I used mouse primary gastric cell cultures and human gastric cell lines to investigate the ability of IFNγ to regulate the endogenous gastrin gene as well as gastrin reporter constructs.

Conclusions and Future Directions

In the fourth and final chapter of this dissertation, I summarize the findings of our studies. The implications of these findings will be placed in the current context of the field and future directions for further study will be discussed.

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CHAPTER II

HELICOBACTER PYLORI ACTIVATES GASTRIN GENE TRANSCRIPTION THROUGH CAC PROMOTER ELEMENTS

Summary

Helicobacter pylori (*H. pylori*) infection has been linked to the development of chronic gastritis, duodenal ulcer disease, and gastric cancer. However, the mechanisms by which *H. pylori* contributes to the development and progression of gastrointestinal pathology are unclear. Chronic gastritis, gastric atrophy, and hypergastrinemia are observed in *H. pylori* -infected patients and animal models. Gastrin is essential for normal acid regulation and the maintenance of gut homeostasis. Therefore, *H. pylori* might alter gastrointestinal physiology by regulating the levels of gastrin. I investigated the effect of *H. pylori* infection on gastrin gene expression in mice and in a gastric epithelial cell line to explore possible mechanisms for the regulation of gastrin by the bacteria. I observed elevated levels of gastrin mRNA in *H. pylori*-infected mice. I found that the bacteria induced endogenous expression of gastrin in AGS cells by stimulating transcriptional initiation through the activation of MAPK signaling pathways. In transfection studies, I identified a CAC motif that contributes to *H. pylori*-induction of the gastrin promoter. Furthermore, inducible binding of Sp1 and Sp3 at these elements in response to *H. pylori* was observed.

Introduction

Helicobacter pylori (*H. pylori*) is a gastrointestinal pathogen that has been linked to the development of chronic gastritis, duodenal ulcer disease, and gastric cancer (Peterson 1991; Blaser and Parsonnet 1994; Parsonnet, Hansen et al. 1994; Peek and Blaser 2002; Parkin 2004). 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 (Parkin 2004; (Parsonnet, Hansen et al. 1994; Covacci, Telford et al. 1999; Naumann, Wessler et al. 1999). 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 and bacteria that express the cytotoxin-associated gene A pathogenicity island (cagPAI) and virulence factors including CagA are typically associated with more severe disease manifestations (Covacci, Telford et al. 1999; Naumann, Wessler et al. 1999; Odenbreit, Puls et al. 2000; Peek 2002). The cagPAI encodes a type IV secretion system (T4SS), which translocates the CagA protein into the host cell where it is phosphorylated (Odenbreit, Puls et al. 2000; Peek 2002). In comparison to 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, which demonstrates the ability of *H. pylori* to regulate cell signaling (Naumann, Wessler et al. 1999;

Wessler, Hocker et al. 2000; Keates, Sougioultzis et al. 2001). These studies allude to the ability of *H. pylori* to mediate cell signaling. Target gene expression is enhanced by the presence of the T4SS and possibly other cagPAI encoded virulence factors such as CagA. Furthermore, genetic variation among *H. pylori* strains may account for the highly variable consequences of *H. pylori* infection in humans (Blaser, Perez-Perez et al. 1995).

In addition to gastritis and gastric atrophy, hypergastrinemia is observed in a subset of *H. pylori*-infected patients (Levi, Beardshall et al. 1989; Chittajallu, Dorrian et al. 1991; Calam, Gibbons et al. 1997). Similarly, in several rodent models of *H. pylori* infection, elevated serum gastrin levels and increased numbers of antral G cells were observed (Zavros, Rieder et al. 2002; Rieder, Merchant et al. 2005). Gastrin is a peptide that is expressed in the adult antral stomach and is the primary hormonal regulator of gastric acid secretion. Furthermore, gastrin acts as a growth factor for gut-derived cell types. The integral role of gastrin in acid regulation and maintenance of gut homeostasis suggests that *H. pylori* can alter gastrointestinal physiology by regulating the levels of gastrin. However, it is unclear whether hypergastrinemia occurs as a consequence of the symptoms of *H. pylori* infection including chronic gastritis, gastric atrophy, and changes in the hormonal regulators of gastrin, or due to the presence of the bacteria itself.

Furthermore, correlations between hypergastrinemia and changes in gastrin gene expression have not been established within the context of *H. pylori* infection. Gastrin gene expression is physiologically regulated by gastric pH and

feeding.   Similarly the oncogenic factor K-ras, and EGF ligands have also been shown to regulate gastrin gene expression (Brand and Stone 1988; Godley and Brand 1989; Ford, Valle et al. 1997) .

An EGF response element was mapped to a GC-rich sequence within the first 240 bp of the human gastrin promoter. This site was shown to bind the transcription factors ZBP-89, Sp3, and Sp1 (Merchant, Du et al. 1999). EGF-activation of the gastrin promoter is mediated by Mek1-dependent phosphorylation of Sp1 (Chupreta, Du et al. 2000). 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 and transcription factors. Furthermore, a prior study established a MEK/ERK-dependent mechanism for the activation of both Sp1 and Sp3 in the *H. pylori* -mediated induction of vascular endothelial growth factor-A (vegf-A) (Strowski, Cramer et al. 2004). EGF regulation of the gastrin promoter occurs in a MEK1- and Sp1-dependent manner, therefore, it is possible that *H. pylori* might regulate gastrin gene expression through a similar mechanism (Merchant, Du et al. 1999; Chupreta, Du et al. 2000).

In the present study, we investigated the effect of *H. pylori* infection on gastrin gene expression in mice and examined the direct effect of the bacteria on a gastric epithelial cell line. We characterized the mechanism of this activation and analyzed virulence factors involved in *H. pylori* -mediated regulation. Lastly, we defined promoter elements of the gastrin promoter that are responsive to *H. pylori* and analyzed the nuclear factors that bind to these elements.

Materials and Methods

Helicobacter strains and cultures

The 26695 and SS1 *H. pylori* WT and mutant strains were provided by K. Eaton and were generated as previously described (Akopyants, Eaton et al. 1995). The J99 strain was obtained from ATCC. All *H. pylori* strains were maintained on blood agar plates and cultured for infection in Brucella broth (DIFCO) supplemented with 10% heat inactivated FBS, Skirrows antibiotic, and amphotericin B. Cultures were maintained by shaking in a gas exchange incubator under microaerophilic conditions at 37°C. Broths used for infection were cultured overnight and checked for the presence of *H. pylori* by catalase and urease tests as well as microscopic analysis for size, shape, and motility.

In vivo H. pylori infection in mice

Six to eight 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 sacrificed 6 months after inoculation, and gastric tissue was collected for histological examination, PCR, and qRT-PCR analysis. Gastric RNA was harvested from mucosal scrapings of the whole stomach using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Purification of mRNA was done using QIAGEN′s RNeasy MiniKit and stored at −80°C. Infection of mice and preparation of mouse RNA was done by B.Gray.

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was isolated from AGS cells using Trizol reagent (Invitrogen) and DNase treated (Roche). RNA quantification was performed using the Quant-iT RiboGreen RNA Assay Kit (Molecular Probes). Synthesis of cDNA was performed using 1 μg of mouse or human RNA using the Iscript cDNA Synthesis Kit (Bio-Rad).

All qRT-PCR reactions were preformed in triplicate using the Bio-Rad Icycler. The following primers were used to quantify gene expression: Mouse gastrin: (f) ACACAACAGCCAACTATTC, (r) CAAAGTCCATCCATC CGTAG. Mouse GAPDH: (f) TCAAGAAGGTGGTGAAGCAGG, (r) TATTATG GGGGTCTGGGATGG. Human gastrin (f) CCCAGGCTCTCATCATCGAAGG, (r) GCCGAAGTCCATCCATCCATAGG. Human 18S rRNA: (f) GATATGCTCAT GTGGTGTTG, (r) AATCTTCTTCAGTCGCTCCA (Neuvians, Gashaw et al. 2005). Human IL-8: (f) TAGCAAAA TTGAGGCCAAGG, (r) AAACCAAGGCACAGTGGAAC.

Reactions using mouse RNA and primers were performed in a volume of 20μl and contained the following: 1x reaction buffer, 5.5 mM $MgCl_2$, 100nM of forward and reverse primers, 10nM fluorescein, 200μM dNTPs, SYBR green, and 0.025U of Platinum Taq polymerase (Invitrogen). Amplification was performed under the following conditions;3 min at 95°C, 35-40 cycles of 9 s at 95°C and 1 min at 60°C, and 1 min at 55°C. Expression of mouse target genes was normalized to GAPDH.

Reactions using human primers were done in a volume of 20µl and contained the following: 1x reaction buffer, 1.5 mM $MgCl_2$, 100nM of forward and reverse primers, 10nM fluorescein, 200uM dNTPs, SYBR green, and 0.025 U of Amplitaq Gold polymerase (Applied Biosystems). Reactions were performed under the following conditions: 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Gene expression was normalized to 18s rRNA. Expression of human target genes was normalized to 18s rRNA. Fold change for all reactions was calculated as $(C_t\text{-}C_{thi}) = n_{\text{target gene}}$, $2n_{target}/2n_{normalization}$= fold change

Cell culture

AGS (human gastric adenocarcinoma) and HeLa cells were grown in DMEM (Gibco-BRL) containing 10% FCS, 100 µg/ml penicillin, and 100µg/ml streptomycin at 37°C with 5% CO_2. Cells were serum starved 48 h in HAMS/F12 media (Gibco) prior to any treatment or co-culture with *H. pylori*.

Plasmids and constructs

The series of pGL3 gastrin reporter constructs were generated by M. Smith, segments from -3.3 Kb to - 0.190 Kb of the human gastrin promoter were ligated to the pGL3 basic luciferase vector (Promega). Gastrin promoter fragments were generated by restriction digest from the 3.3 Kb-pGL3 gastrin construct. I introduced site-directed mutations 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 done to introduce mutations at multiple sites.

The Path Detect pAP1-Luc Cis-Reporter Plasmid (Stratagene) was used as a positive control for transcriptional activation by *H. pylori* in transfection experiments.

Transient transfections and luciferase reporter assays

AGS and HeLa cells were seeded into 12 or 24 well plates and grown to 50%-60% confluency prior to transfection. Between 0.5-1 μg/well plasmid DNA 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 and allowed for normalization of firefly luciferase activity. Cells were treated and harvested for analysis 48 h post transfection. Firefly and Renilla luciferase reagents were obtained and 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 48 h prior to *H. pylori* infection. *H. pylori* was quantified and resuspended in F12 media without antibiotics. Cells were washed with PBS and maintained in serum-free and antibiotic-free media for the duration of bacterial co-culture experiments.

Western blot analysis

Whole cell extracts were prepared from AGS cells that were cultured in serum free media 48h prior to treatment with chemical inhibitors and *H. pylori* co-culture. Cell pellets were lysed with M-PER Mammalian Protein Extraction Reagent (Pierce) containing complete protease inhibitor tablets (Roche) according to the manufacturer's instructions. The protein concentration for each sample was determined by bicinchoninic acid assay (Pierce Biochemicals). Protein lysates were separated on 4-20% SDS- polyacrylamide gels, and then transferred to Hybond-C Extra nitrocellulose membranes (Amersham). Membranes were blocked using KPL blocking solution (KPL). Primary antibodies for GAPDH (Millipore), MEK1/2 (Cell Signaling), p38 (Cell Signaling), Sp1 (Santa Cruz Biotechnology), and Sp3 (Santa Cruz Biotechnology) were used at a concentration of 1:1000. Phospho-antibodies against p38 (Cell Signaling), MEK1/2 (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).

Electophoretic mobility shift assays (EMSA)

Nuclear extracts were prepared using non-ionic detergent extraction from AGS cells following co-culture with *H. pylori* at designated times. Oligonucleotide probes were designed according to regions of the human gastrin promoter. An

AP1 binding oligo was designed using two consecutive AP1 binding sites (TGCATCA x 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, and labeled with $[^{32}P]$ γ-ATP using polynucleotide kinase (Roche). Gel shift assays were carried out in a final volume of 20μl and contained 10mM Tris-HCl, 1mM EDTA, 1mM DTT, 5mM $MgCl_2$, 1mM $ZnCl_2$, 150mM KCl, 10% glycerol, and 300ng polydIdC. One microgram of antibody against Sp1 or Sp3 (Santa Cruz Biotechnology) was 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 TBE.

Chemical inhibitors

Cell signaling inhibitors were added to cells 30 min prior to co-culture with *H. pylori*. The following inhibitors were used; RNA polymeraseII inhibitor α-amanitin (Sigma), MekI inhibitor PD98050 (Cell Signaling Technology), NFκB inhibitor Ammonium Pyrrolidinedithiocarbamate (APDT, Calbiochem), JNKII inhibitor SB203580 (Calbiochem), and p38 inhibitor SP600125 (Calbiochem).

Statistics

Data were compared by using the Mann-Whitney U test. Significance levels were set at a p value of <0.05. Error bars on graphs indicate standard errors of the means (SEM)

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 correlated with changes in gastrin gene expression. 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, an 8-fold increase in gastrin mRNA was observed in mice infected with *H. pylori* when compared to uninfected controls (Fig. 2.1). The observed increase in gastrin mRNA expression suggested that there might be a regulatory effect of *H. pylori* directly on the gastrin gene. Since a direct effect of bacteria on the G cell cannot be established *in vivo,* we utilized the human gastric cell line, AGS, to characterize the effect of *H. pylori* on gastrin gene expression.

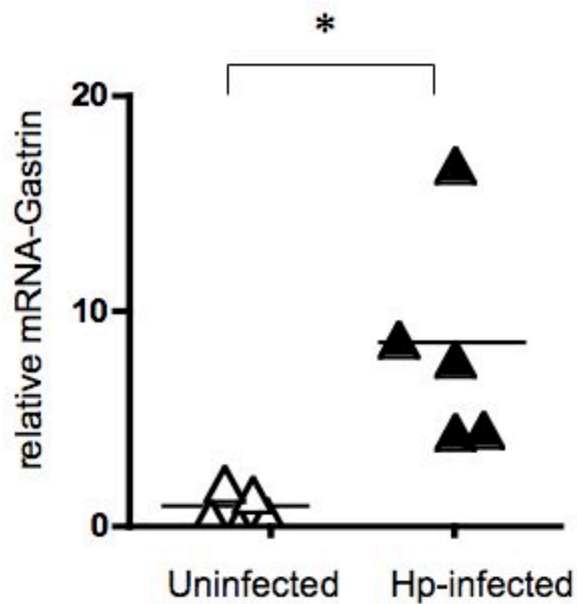


Figure 2.1. *H. pylori* Stimulates Gastrin Expression in Infected Mice. Mice were infected with SS1 *H. pylori* and analyzed six months after infection. qRT-PCR analysis of gastrin normalized to GAPDH was performed using RNA isolated from the stomachs of infected and uninfected (control) mice (n = 5). $* \ p < 0.05$. The results are expressed as fold change mRNA expression of infected mice compared to controls.

Gastrin mRNA Induction Requires Live Bacteria, But Not CagA

The ability of *H. pylori* to regulate the expression of endogenous gastrin in AGS cells was examined by qRT-PCR. Cells were co-cultured with the 26695 *H. pylori* strain, which is a wild type, human isolate (Tomb, White et al. 1997). Both time course and dose response experiments were performed to determine the optimal conditions required to induce gastrin gene expression. I found that endogenous gastrin was maximally induced by 9 h (Fig 2.2A). Additionally, 100 m.o.i. maximally induced gastrin mRNA 6- to 7-fold (Fig 2.2B).

After defining the optimal conditions for gastrin induction, I investigated whether live bacteria were required to induce gastrin gene expression. AGS cells were co-cultured with live *H. pylori* or heat-inactivated and formalin-fixed bacteria. Indeed, I found that inactive bacteria were unable to induce gastrin mRNA expression (Fig 2.2C). 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 the *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. I investigated whether differences among *H. pylori* strains influenced the ability of the bacteria to induce gastrin gene expression. AGS cells were co-cultured with two wild type strains 26695 and J99, as well as the SS1 strain, which lacks a functional T4SS injection apparatus and does not inject CagA into the host cell. Additionally, I

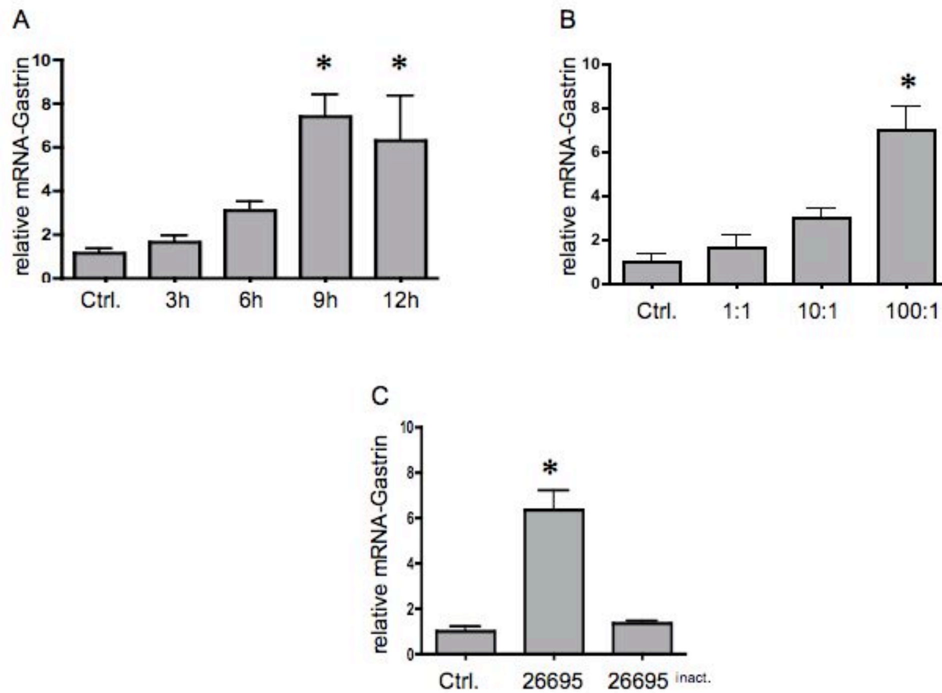


Figure 2.2. *H. pylori* Stimulates Expression of Endogenous Gastrin in Human Gastric Cells. AGS Cells were infected with 26695 *H. pylori* 48h after serum starvation. Cells were cultured with bacteria at a 100:1 m.o.i. for time course experiments (A). Cells were treated with increasing m.o.i. doses and harvested at 9h (B). All subsequent *H. pylori* co-culture experiments were done with 100:1 m.o.i. and harvested 9h post infection. AGS cells were co-cultured with live and inactive (formalin fixed, heat inactivated) bacteria (C). qRT-PCR analysis of gastrin and 18s rRNA transcripts was performed from total RNA. For all experiments, the mean $\pm$ SEM is shown for the fold changes of three experiments. $*$, $p < 0.05$ compared to untreated (control) cells.

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 strains of *H. pylori* induced gastrin mRNA expression and higher levels were observed with the CagA- and VacA-deficient strains as well as the SS1 strain (Figure 2.3). Since 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 was not influenced directly by either CagA or VacA, but instead might be the result of other factors produced by the bacteria.

Induction of Gastrin Requires Activation of MAPK Signaling

It has been shown previously that *H. pylori* activates multiple signaling pathways, including MAP kinase, NFκB, and PI3K (Meyer-ter-Vehn, Covacci et al. 2000) . To assess which signal transduction pathways were involved in the activation of the gastrin gene by *H. pylori,* I blocked the induction of gastrin with chemical inhibitors of the MAP kinase pathways MEK-ERK, JNK, and p38. I also treated cells with a chemical inhibitor of NFκB signaling proteins. AGS cells were pre-treated for 30 min with inhibitors prior to co-culturing with *H. pylori* for 9 h (Fig. 2.4A). Mek1 and p38 inhibitors completely blocked gastrin induction by *H. pylori* while the NFκB inhibitor did not block induction. The JNK inhibitor decreased *H. pylori* induction of gastrin by 50%. To confirm the role of MAPK signaling, western blot analysis was performed. I observed phosphorylation of

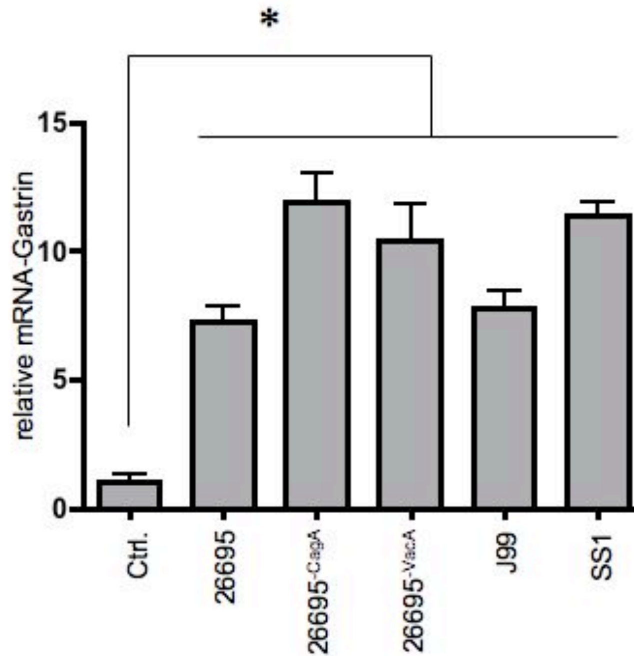


Figure 2.3. Gastrin Expression is Induced By Wild Type and Mutant Strains. AGS Cells were infected with WT 26695, or 26695 mutant isotypes that were CagA- or VacA-null. A second wild type strain, J99 and the mouse adapted SS1 strain were used as well. qRT-PCR analysis of gastrin and 18s rRNA transcripts was performed using total RNA. Data shown represents the mean $\pm$ SEM of the fold changes from three experiments. $*$, $p < 0.05$ compared to untreated (control) cells.

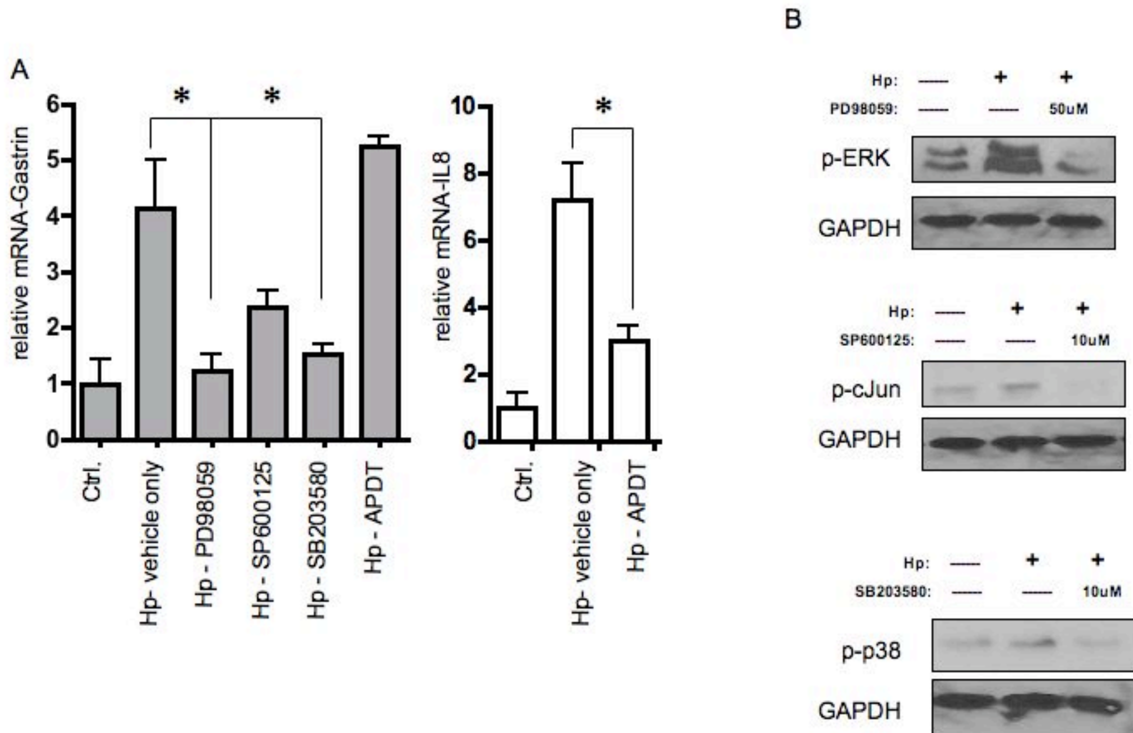


Figure 2.4. *H. pylori*-induced Gastrin Expression is Blocked by MAP Kinase Inhibitors. AGS Cells were treated with either vehicle alone (control) and MAPK (PD98059: 50μM, SP600125: 10μM, SB203580: 10μM) or NFκB (APDT: 10μM) chemical inhibitors 30min prior to co-culture with 26695 *H. pylori* (Hp). qRT-PCR analysis of gastrin and 18s rRNA transcripts was performed using total RNA (A). Data shows the mean ± SEM for the fold changes from three experiments. ∗, $p < 0.05$ compared to untreated (control) cells (A). For western blot analysis, whole cell extracts were prepared from cells cultured without (-) or with (+) *H. pylori* following treatment with the indicated inhibitors and doses (B).

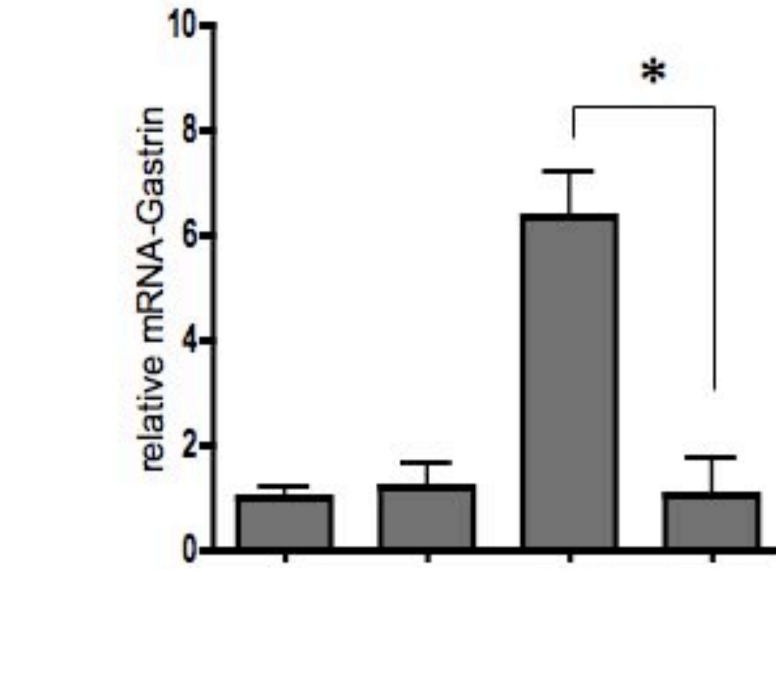
ERK, cJun, and p38 by *H. pylori* (Fig 2.4B). We also confirmed that inhibitor doses were sufficient to block the activation of these pathways by *H. pylori* (Fig 2.4B) and demonstrated that APDT inhibited NFκB- mediated activation of IL-8 by qRT-PCR (Figure 2.4A) (Bhattacharyya, Pathak et al. 2002).

H. pylori **Stimulates Gastrin Expression Through Regulation of Transcription**

To determine if the induction of gastrin by *H. pylori* due to enhanced transcription, AGS cells were pre-treated with α-amanitin for 30 min prior to co-culture with bacteria. The toxin alone did not affect basal expression of gastrin at 0.1μM, 0.5μM, or 1μM (data not shown). However, treatment with α-amanitin blocked *H. pylori* -induced gastrin gene expression when the cells were co-cultured with the 26695 strain (Fig 2.5). Similar results were obtained using the SS1 strain (data not shown). These results demonstrated that *H. pylori* induced gastrin by increasing transcription of the gastrin gene.

H. pylori **Stimulates Gastrin Expression Through C_2AC_4 DNA Elements**

To identify an *H. pylori* -responsive element within the gastrin promoter, a series of luciferase constructs representing the sequences between - 3.3 Kb to -190 bp of the gastrin promoter were generated (Fig 2.6A). The basal activity of each reporter construct is shown as relative light units and indicated that the highest activity was within the first 240 bp of the promoter as previously reported



relative mRNA-Gastrin
10
8
6
4
2
0
*

Hp: ----- ------ Hp Hp

α-amanitin: ----- 1μM ------ 1μM

(Fig 2.6B)(Merchant, Demediuk et al. 1991) . Transient transformants of AGS cells expressing the various gastrin reporters were co-cultured with *H. pylori.* i observed a significant induction of promoter activity for all gastrin promoter constructs from -3.3 to -0.240 Kb (Fig 2.6C). However, when the 43 bp region between - 240 bp and -190 bp was deleted, I observed a 30% decrease in promoter activation suggesting that an *H. pylori* responsive element was contained within this region. AGS cells expressing an AP1 luciferase reporter was significantly induced when co-cultured with *H. pylori.* This induction is consistent with previous studies that demonstrated the ability of *H. pylori* to activate target genes through AP1 DNA elements (Naumann, Wessler et al. 1999). To validate that the decrease in gastrin promoter activation was due to an *H. pylori* -specific effect, transfected cells were also treated with PMA (Fig. 2.6D). There was no difference in PMA-induced promoter activity between the - 240 bp and – 190 bp promoter constructs suggesting that the region contained an *H. pylori* -specific response element. I observed a significant reduction in PMA-induced activity of the -2.3 Kb reporter. However, this construct did not show a difference in Hp- induced activity. I concluded that this segment was lacking a regulatory region that was associated with induction by PMA and not *H. pylori*.

Upon examination of the region between -240 bp and -190 bp, I identified two cytosine-rich sites as the putative *H. pylori* responsive elements (C_2AC_4) (Fig 2.7A, Site1). However, since the -190 bp construct retained the ability to be induced by *H. pylori* (4-fold), I also analyzed the remaining promoter sequences

and identified two other loci containing this C_2AC_4 motif at -120 bp and the reverse complement at -74 bp (Fig 2.7A, Site 2 and 3).

To determine whether the C_2AC_4 elements were required for *H. pylori* induction, I introduced mutations to disrupt binding at these elements (Fig 2.7A). The results showed that mutation of the C_2AC_4 elements did not alter basal activity of the reporters with the exception of mutations within site 1 alone or sites 2 and 3 together (Fig 2.7B). The other combinations of mutations did not alter basal activity. When cells that were transfected with mutant reporters were co-cultured with *H. pylori,* I observed a significant decrease in the activity of promoter constructs with mutations of the site 1 element (Fig 2.7C). This result was comparable to the decrease in activity observed between the - 240 bp and the -190 bp reporter (~ 30%). I observed decreased activation with a mutation of site 3 alone and a mutation of site 1 with either mutations of sites 2 or 3. There was no effect on the activation of 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 largest decrease in *H. pylori* -activation (down from 6.5 to 2-fold, Fig 2.7C). I therefore concluded that C_2AC_4 elements were required for maximal activation of the gastrin promoter by *H. pylori*.

H. pylori **Influences Binding of Nuclear Proteins at C_2AC_4 Gastrin Elements**

To gain further insight into the mechanisms of *H. pylori* regulation of the gastrin promoter, I characterized the binding of nuclear proteins within gastrin

promoter regions that contained the C^2AC^4 elements by electrophoretic mobility shift assay. I observed two major complexes, which were identified as Sp1 and Sp3 by supershift analysis (Fig 2.8). I observed increased protein binding activity in extracts from *H. pylori*-treated cells on the -228 and -120 probes (Fig 2.8, lanes 1, 2, 5 and 6). Sp1 binding to the -74 element was unchanged, but I observed a decrease in the intensity of the Sp3 complex in extracts treated with bacteria (Fig 2.8, lanes 9 and 10). I observed an increase in AP1 protein binding, which was consistent with prior studies demonstrating that *H. pylori* can increase AP1 binding (Fig 2.8, lanes 13 and 14) (Ashktorab, Daremipouran et al. 2007; Ding, Olekhnovich et al. 2008).

I performed competition assays using unlabeled wild type and mutant versions of these sequences. All unlabeled wild type oligos were able to disrupt protein binding, however, point mutations that reduced the activation of gastrin reporters by *H. pylori* also inhibited the competitive binding of nuclear proteins to the -228 and -120 unlabeled oligos (Fig 2.9, lanes 1-6). The mutant competitor for the -74 region was still able to disrupt protein binding. This finding was consistent with previous studies indicating that the Sp1 and Sp3 can bind to the first half of this sequence, which was not mutated (Merchant, Demediuk et al. 1991).

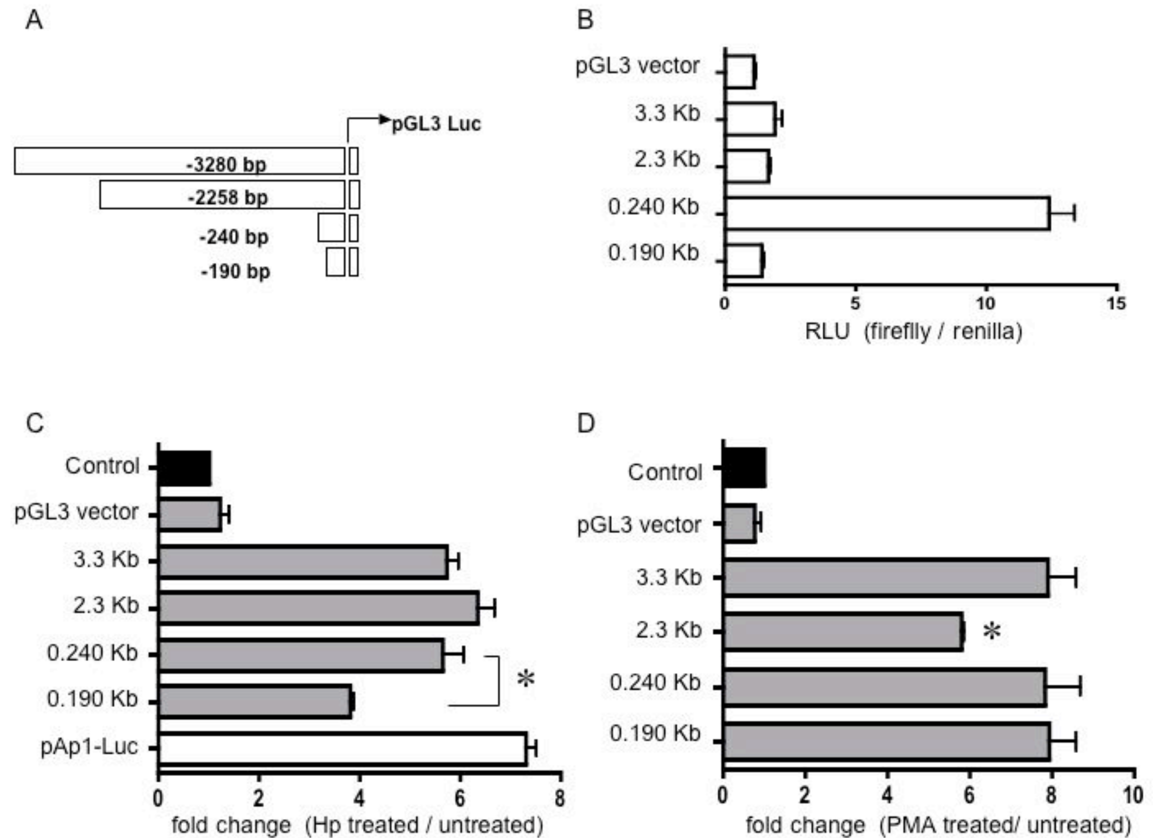


Figure 2.6. *H. pylori* Stimulates the Proximal Gastrin Promoter. AGS cells were transiently transfected with a series of gastrin luciferase deletion constructs, empty vector (pGL3), or an Ap1 luciferase reporter (pAp1-Luc). A schematic representation of the gastrin luciferase constructs is shown (A). The basal activity of gastrin constructs was presented as relative light units (RLU) of firefly compared to Renilla activity (B). Cells were co-cultured with *H. pylori* (C) or PMA (D) 48h post-transfection. The data are shown as mean ± SEM for three separate experiments performed in triplicate. Firefly luciferase activity was normalized to Renillla luciferase. Fold change is the ratio of stimulated reporter activity over basal expression. Black bars represent the baseline expression for each construct which was set to 1.

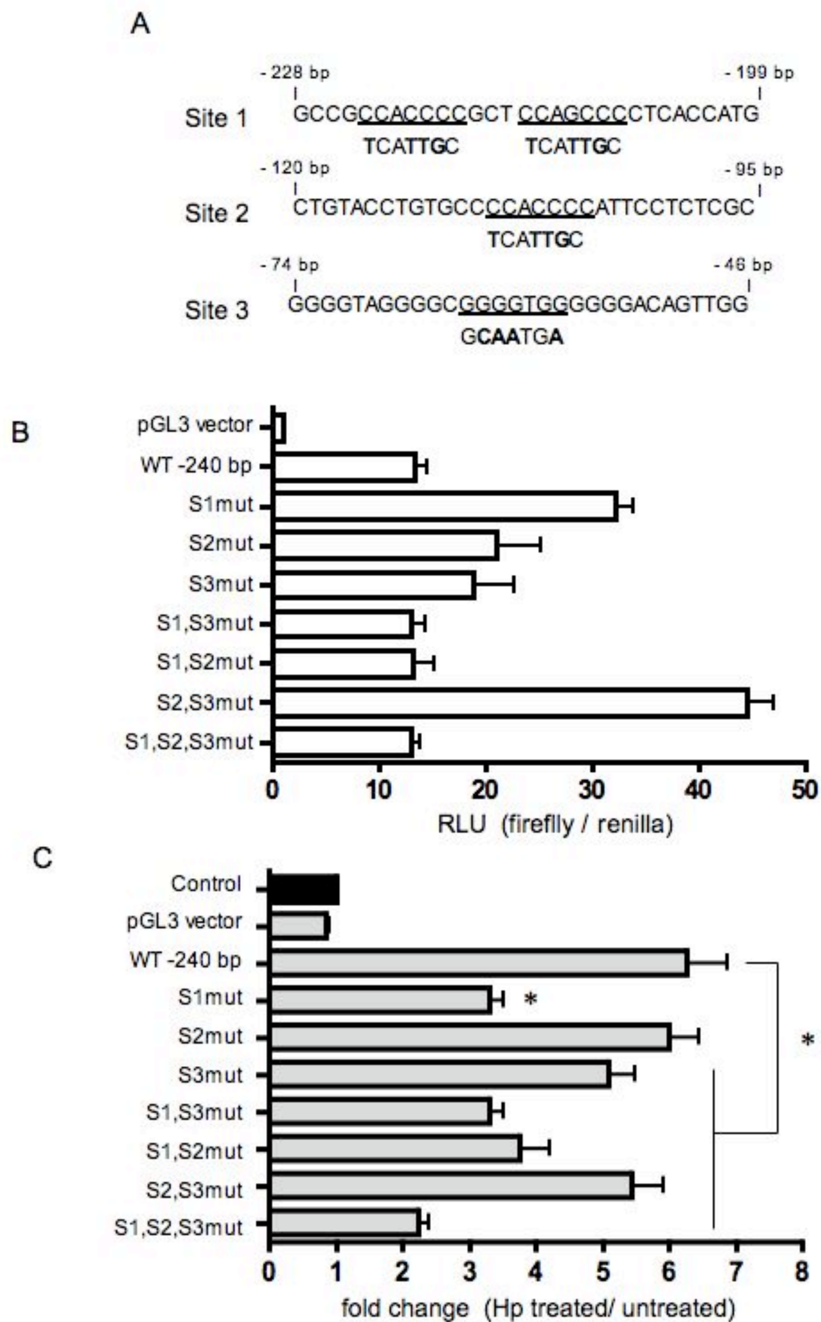


Figure 2.7. *H. pylori* Stimulates the Gastrin Promoter Through C_2AC_4 Elements. AGS cells were transiently transfected with the wild type (WT) 240 bp gastrin luciferase reporter or the reporter containing point mutations. Altered nucleotides are shown in bold (A). The basal activity of gastrin constructs was presented as relative light units (RLU) of firefly compared to Renilla activity (B). Cells were co-cultured with *H. pylori* 48 h post-transfection (C). The data are shown as mean $\pm$ SEM for three separate experiments performed in triplicate. Firefly luciferase activity was normalized to Renilla luciferase. Fold change is the ratio of stimulated reporter activity over basal expression. The black bar represents the baseline expression for each construct was set at 1.

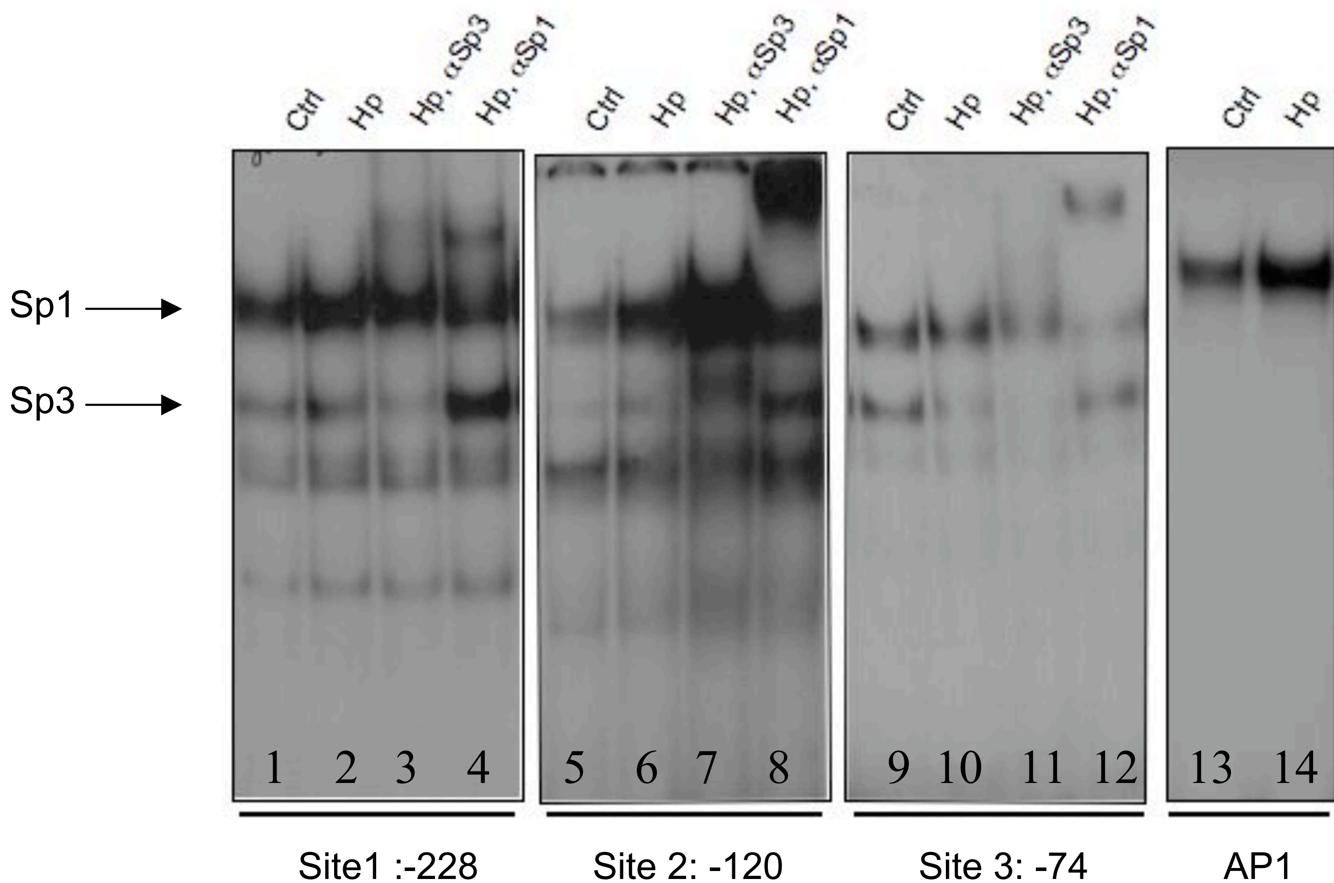


Figure 2.8. ***H. pylori*** **Stimulates Binding of Sp1 and Sp3 at C_2AC_4 Elements.** EMSA reactions were carried out with 4µg of nuclear extracts from AGS cells that were untreated (Ctrl) or co-cultured with 26695 *H. pylori* for 3 h (Hp). Labeled probes represent regions of the gastrin promoter and an AP1 binding sequence. Samples were incubated with antibodies against Sp3 or Sp1 as indicated. Arrows indicate bands representing Sp1 and Sp3 complexes.

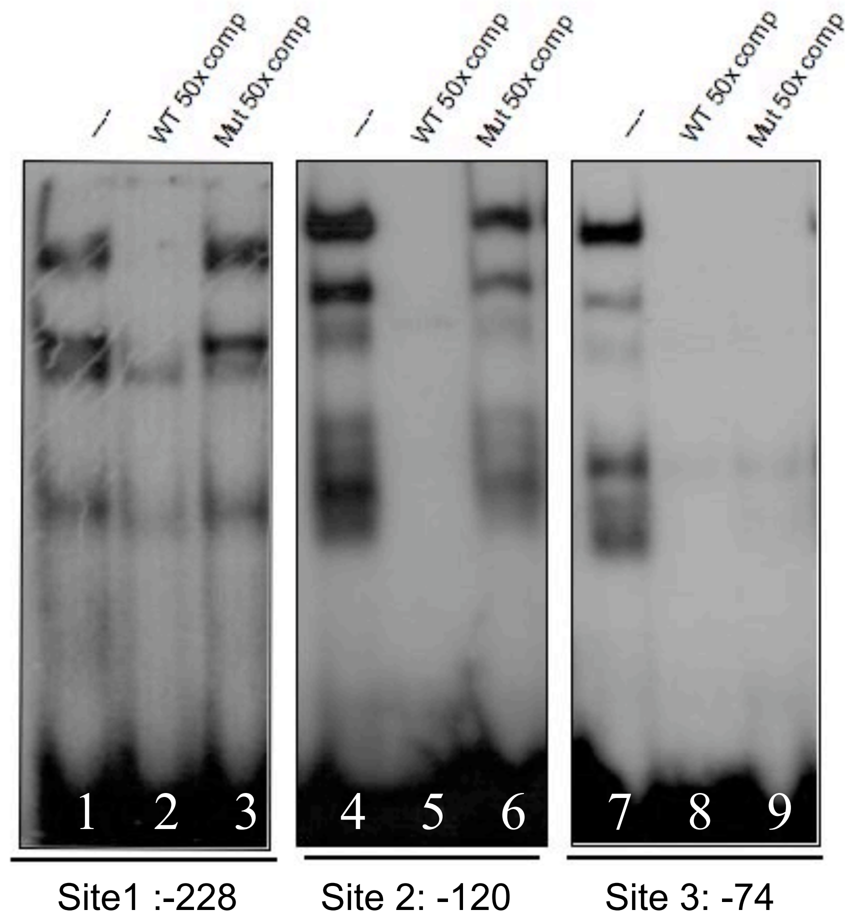


Figure 2.9. Mutations Disrupt Protein Binding at C_2AC_4 Elements. EMSA reactions were carried out with 4μg of AGS cell nuclear extracts. Labeled probes represent regions of the gastrin promoter as indicated (see also, Fig 2.7A). Lanes represent samples that contain extract alone (---) or extract with either WT or the mutant versions of the binding site at a 50x molar concentration.

Discussion

In summary, our findings demonstrate that gastrin expression is increased in the mouse stomach as a result of *H. pylori* infection. Using the AGS cell line, I established that the bacteria alone induces endogenous gastrin and the bacterial virulence factors, CagA and VacA were not required for this effect. The results indicate that live bacteria are required for the induction of gastrin and that MAPK signaling is an important mediator this effect. I determined that *H. pylori* stimulated transcription of gastrin and identified multiple CCACCC (C_2AC_4) binding motifs that contribute to activation of the promoter. Lastly, I demonstrated that *H. pylori*-induced binding of Sp1 and Sp3 to the first two C_2AC_4 elements and that the third element was involved in promoter activation but was not a site for inducible binding of Sp1 or Sp3.

My initial observation was that gastrin gene expression was higher in *H. pylori*-infected mice when compared to uninfected mice. It has been widely reported that the number of G cells and the release of gastrin is increased during *H. pylori* infection in human subjects and various animal models (Konturek, Konturek et al. 2001; Rieder, Merchant et al. 2005; Kudo, Lu et al. 2007). Few studies have reported on modulations in the expression of the gene itself thus, I began my report with this observation. I expected to observe an increase in gastrin mRNA since there were several factors that could have contributed to this finding such as an increase in the total number of G cells. However, I found an 8-fold increase in gastrin gene expression, which exceeded the expected increase due solely to the presence of more G cells. This result suggested that

each G cell increased the amount of gastrin mRNA, thus forming the basis for the current studies. However, a limitation of *in vivo* studies is that the effects of the bacteria alone cannot be distinguished from the effects of chronic inflammation and the progressive epithelial damage due to chronic gastritis. Therefore, I proceeded to determine whether the bacteria exert a direct effect on gastrin gene expression in the AGS cell line.

I found that *H. pylori* alone was sufficient to induce endogenous gastrin. Furthermore, live bacteria were required for this response since I observed no induction following co-culture with dead bacteria. I then considered the impact of different bacterial strains and the presence of virulence factors on the induction of gastrin. *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 impact on cells as well (Keates, Keates et al. 1999; Keates, Sougioultzis et al. 2001; Pillinger, Marjanovic et al. 2007). Specifically, *H. pylori* strains that lack CagA tend to induce less inflammation and are less commonly associated with the development of ulcers and adenocarcinoma (Blaser, Perez-Perez et al. 1995; Nomura, Perez-Perez et al. 2002). Although the mechanisms by which *H. pylori* contributes to these diseases are not well understood, there is a correlation between elevated levels of gastrin, even in the absence of *H. pylori*, and ulcers. I found that the proteins CagA and VacA were not required for induction of the gastrin gene and therefore probably do not contribute to increases in serum gastrin. Since the CagA- and VacA-null strains, as well as the SS1 strain, did induce gastrin these results

suggest that another factor produced by the bacteria might be involved in this effect.

Using chemical inhibitors of three MAPK signaling pathways, I determined that *H. pylori* activates the MEK-ERK, p38, and JNK signaling pathways to induce gastrin gene expression. I was able to abolish the effect of *H. pylori* on gastrin using both Mek1 and JNK inhibitors while p38 inhibition reduced gastrin induction levels by half. Since blockade of either prohibited maximal induction, I predict that that the bacteria activates all three signaling pathways. MAPK regulation of the gastrin gene has been previously observed in a study detailing regulation of the gastrin promoter by EGF through the activation of the MAPK kinase, Mek1 (Chupreta, Du et al. 2000). Activation of the EGF receptor by *H. pylori* has also been reported (Ashktorab, Daremipouran et al. 2007). Furthermore, in some colorectal cancers, mutations in Ki-ras, which signals through ERK kinase, were found in tumors with elevated levels of gastrin (Nakata, Wang et al. 1998). These findings reiterate the idea that activation of MAPK signaling is a key regulatory mechanism for the inducible regulation of gastrin and potentially on tumor growth.

I identified multiple copies of a binding motif within the gastrin promoter that inducibly bind Sp1 and Sp3 at Sites 1 and 2. Inducible binding at Site 3 was not observed. However, mutation of this site in conjunction with Sites 1 and 2 was necessary to obtain the greatest reduction in *H. pylori*-responsiveness. Interestingly, EGF-responsiveness was previously mapped to a GC-rich element that also contains the third *H. pylori*-responsive site (Site 3) (Merchant, Demediuk

et al. 1991). While this site was required for EGF activation of gastrin, inducible binding of proteins at this site was not observed. The phosphorylation of Sp1 in response to EGF was necessary for regulation of the gastrin promoter via the gastrin EGF response element (gERE). Neither Sp1 or Sp3 phosphorylation was examined in the current study. However, I would predict that an increase in Sp1 and Sp3 binding might be due to phosphorylation of these proteins in response to the activation of signaling pathways by *H. pylori*.

In conclusion, the current study demonstrates that *H. pylori* induces gastrin gene transcription and confirms the significance of MAPK signaling in the regulation of this gene. Through the findings of this study, I have defined a mechanism for induction of the gastrin gene by *H. pylori*. Subsequent studies are needed to ultimately understand the clinical significance and pathophysiological impact of elevated gastrin gene expression but this current study proposes a molecular mechanism through which the impact of the bacteria on gastrin might be abrogated.

References

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Chapter III

REGULATION OF GASTRIN GENE EXPRESSION BY THE INFLAMMATORY CYTOKINE INTERFERON-GAMMA (IFNγ)

Summary

In a subset of patients, *Helicobacter pylori (H. pylori)* infection leads to gastritis and elevated levels of gastrin. These changes are associated with an increased risk of duodenal ulcers and gastric cancer. *In vivo* and *in vitro* studies have shown that *H. pylori* infection elicits a Th1 immune response from the host. Previous studies established that IFNγ is a major component of the *H. pylori*-mediated inflammatory response. Furthermore, gastritis and increased levels of plasma gastrin were observed when the pro-inflammatory cytokine IFNγ was infused into mice. The molecular mechanism of how IFNγ might regulate gastrin gene expression has yet to be elucidated. Therefore, I set out to determine whether the effect of IFNγ on gastrin gene expression is direct or indirect. I did not observe changes in gastrin mRNA levels following IFNγ treatment in mice and in mouse primary gastric cultures. Furthermore, I did not observe significant changes in gastrin expression using a human gastric cancer cell line, AGS. Lastly, I did not observe regulation of the gastrin promoter by IFNγ. These results suggest that IFNγ is not sufficient to induce the gastrin gene.

Introduction

Helicobacter pylori (*H. pylori*) infection is a common cause of gastric inflammation and is associated with an increased risk for the development of duodenal ulcers and gastric cancer (Blaser and Parsonnet 1994; Bamford, Fan et al. 1998; Bhattacharyya, Pathak et al. 2002; Watanabe, Kiriya et al. 2008). *H. pylori* is able to survive in the highly acid environment of the stomach and evade the immune response of the host, resulting in a persistent infection and the development of chronic gastritis (Suerbaum and Michetti 2002). The presence of the bacteria stimulates a Th1 immune response, which includes the recruitment of predominantly IFNγ-expressing lymphocytes to the stomach (Mohammadi, Nedrud et al. 1997; Bamford, Fan et al. 1998; Eaton, Kersulyte et al. 2001). Furthermore, IFNγ-deficient mice do not mount an inflammatory response during chronic infection with the bacteria (Sawai, Kita et al. 1999; Smythies, Waites et al. 2000). This result suggests that IFNγ significantly impacts the progression of disease during *H. pylori*-induced gastritis.

H. pylori infection affects the number and function of the many cell types that regulate normal acid secretion and stomach function (Sumii, Sumii et al. 1994; Fox, Dewhirst et al. 1998). Specifically, *H. pylori* infection causes an increase in the number of gastrin-expressing G cells and a decrease in the number of somatostatin-secreting D cells (Konturek, Konturek et al. 2001; Kudo, Lu et al. 2007). These alterations contribute to elevated levels of gastrin in human subjects and animal studies (Graham, Go et al. 1993; Graham, Lew et al. 1993; Calam 1995; Verhulst, Hopman et al. 1995; Liu, Vosmaer et al. 2005;

Sokic-Milutinovic, Todorovic et al. 2005). Ultimately, elevated levels of gastrin prime the stomach for maximal secretion of acid. The highly acidic environment in the stomach is the first line of defense against harmful bacteria. However, during *H. pylori* infection, the overproduction of acid is thought to have a deleterious effect on the unprotected epithelial lining of the duodenum.

Based on the findings of previous studies, we hypothesized that Th1 cytokines contribute to hypergastrinemia by stimulating gastrin gene expression. A study by Suzuki et al. demonstrated that the Th1 cytokines TNFα and IL-1 regulate gastrin gene expression through a direct effect on enriched cultures of G cell (Suzuki, Grand et al. 2001). Likewise, we have shown that the infusion of IFNγ stimulates gastrin secretion in mice (Zavros, Rathinavelu et al. 2003) Furthermore, this effect on gastrin occurred in the absence of somatostatin. This result suggests a direct effect of IFNγ on the G cell. However, the impact of IFNγ on gastrin gene expression is not known. Thus, we investigated the ability of IFNγ to regulate gastrin gene expression in mice and human gastric cell lines.

Materials and Methods

Animal Treatments

C57BL/6 mice between eight and twelve weeks of age were infused with 15 IU/day of recombinant mouse IFNγ (rmIFNγ) (R&D Systems). Alzet micro-osmotic pumps (model 1007D, Durect Corporation) were surgically implanted into the peritoneal cavity of mice. Mice were maintained for 7 days following pump implantation and were then sacrificed for analysis. Comparable volumes of

PBS were infused for control treatment groups. A 12 h time course was performed in mice that were administered a 15 IU/day equivalent dose of rmIFNγ by interperitoneal injection. Mice in the control group were injected with PBS (vehicle). The mice were fasted 12 h prior to injections.

Immunohistochemstry

Gastric sections were prepared from IFNγ-treated and control mice for histological analysis. Tissue from the fundus to the duodenum was removed and fixed in 4% paraformaldehyde. Fixed tissues were paraffin embedded for sectioning (3μm). Slides were deparaffinized and then boiled in sodium citrate buffer for antigen retrieval. Hematoxylin and eosin (H&E) staining was done for histological analysis. Non-specific antigen binding was blocked with a 20% serum/PBS solution. Slides were incubated with primary antibody against gastrin (DAKO) at a concentration of 1:800 and washed using with a PBS/ 0.01% TritonX-100 buffer. Diaminobenzidine (DAB) staining was done using the Vectastain Elite ABC Kit (Vector Laboratories) according to the manufacturer's protocol.

Gastrin Radioimmunoassay

Blood was collected by cardiac puncture from mice and then centrifuged to allow separation of the plasma. Mice were fasted 12 h prior to sacrifice. Plasma samples were assayed for gastrin content using I^{125} labeled antibody according to a previously described procedure (Zavros, Rieder et al. 2002).

Isolation and Culture of Mouse Gastric Primary Cells

Gastric cells were isolated from the stomach mucosa (fundus and antrum) of 12-week old C57BL/6 mice according to a previously reported method (Zavros, Rieder et al. 2002). Mice were fasted for 12 h prior to sacrifice and harvesting of stomach tissue. Individual cells were dissociated by dispase digestion and mechanical separation of glands from the mucosa. Cells were maintained in RPMI media with 5% serum and 1% penicillin/ streptomycin and cultured on Matrigel-coated plates (BD Biosciences). Cells were treated with rmIFNγ (R&D Systems) 24 h after collection.

RNA Isolation and Quantitative Real-Time PCR (qRT-PCR)

RNA was isolated from mouse antral tissue and mouse primary gastric cells using Trizol reagent (Invitrogen) and then DNase-treated and purified using the RNeasy RNA Isolation Kit (Qiagen). Total RNA was isolated from AGS cells using Trizol reagent (Invitrogen) and DNase-treated (Roche). RNA quantification was performed using the Quant-iT RiboGreen RNA Assay Kit (Molecular Probes). Synthesis of cDNA was performed with 1 μg of mouse and human RNA using the Iscript cDNA Synthesis Kit (Bio-Rad). The following primers were used to detect and quantify gene expression using SYBR green dye (Molecular Probes); for mouse gastrin: (f) ACACAACAGCCAACTATTC, (r) CAAAGTC CATCCATCCGTAG. Mouse CXCL10: (f) GAAATCATCCCTGCGAGCCTATCC, (r) GCAATTAGGACTAGCCATCCACTGGG (Gasper, Petty et al. 2002). Mouse

GAPDH: (f) TCAAGAAGGTGG TGAAGCAGG, (r) TATTATGGGGGTCTGGGATGG. Human gastrin (f) CCCAGGCTCTCATCATC GAAGG, (r) GCCGAAGTCCATCC ATCCATAGG. Human 18S rRNA: (f) GATA TGCTCATGTGGTGTTG, (r) AATCTTCTTCAGTCGCTCCA. All reactions were carried out in triplicate using a Bio-Rad Icycler.

Reactions using mouse RNA and primers were done in a volume of 20μl and contained the following: 1x reaction buffer, 5.5 mM $MgCl_2$, 100nM of forward and reverse primers, 10nM fluorescein, 200uM dNTPs, SYBR green, and 0.025 U of Platinum Taq polymerase (invitrogen). Amplification was performed under the following conditions; 3min at 95°C, 35-40 cycles of 9s at 95°C and 1 min at 60°C, and 1 min at 55°C. Gene expression was normalized to GAPDH.

Reactions using human primers were done at a volume of 20μl and contained the following:- 1x reaction buffer, 1.5 mM $MgCl_2$, 100nM of forward and reverse primers, 10nM fluorescein, 200μM dNTPs, SYBR green, and 0.025 U of Amplitaq Gold polymerase (Applied Biosystems). Reactions were performed with the following conditions; 50°C for 2 min. and 95°C for 10 min. followed by 40 cycles of 95°C for 15 sec. and 60°C for 1 min. Gene expression was normalized to 18s rRNA. Fold change was calculated as $(C_t\text{-}C_{thi}) = n_{\text{target gene}}$, $2n_{target}/2n_{normalization}$=fold change

Cell Culture

Human gastrointestinal cell lines (AGS, HGT, KATOIII, HCT116, and MKN45) and HeLa cells were obtained from American Type Culture Collection

(ATCC). All cells (except HCT116) were cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 100 μg/ml penicillin, 100μg/ml streptomycin, and 10% fetal calf serum. HCT116 cells were cultured in McCoy's 5A medium with 100 μg/ml penicillin, 100 μg/ml streptomycin, and 10% fetal calf serum. Cultures were incubated at 37°C with 5% CO_2. Cells were serum starved 24-48 h prior to treatment with IFNγ in HAMS/F12 media (GibcoBRL).

Immunofluorescence

AGS cells were grown on Matrigel-coated coverslips and treated with recombinant human IFNγ (R&D Systems) for various times. Following treatment, the cells were washed with PBS and fixed with 4% formaldehyde for 30 min. The cells were incubated with methanol then blocked against non-specific antigen with a 20% serum/PBS solution. The slides were incubated with primary antibody against the IFNγ receptor α and β chains (1: 500, PBL Biomedical Laboratories), and pStat1 (1:500, Cell Signaling). Slides were washed using a buffer of PBS/ 0.01% TritonX-100. Fluorescent secondary antibodies (Molecular Probes) were used at a concentration of 1:200.

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Western Blot Analysis

Whole cell extracts were made from AGS cells that were cultured in serum free media 24 h prior to treatment with 100 ng/ml IFNγ. Cell pellets were lysed with M-PER Mammalian Protein Extraction Reagent (Pierce) containing

Complete protease inhibitor tablets (Roche) according to the manufacturers instructions. The protein concentration for each sample was determined using bicinchoninic acid assay (Pierce Biochemicals). Protein lysates were separated on 4-20% SDS- polyacrylamide gels, then transferred to Hybond-C Extra nitrocellulose membranes (Amersham). Membranes were blocked using the KPL blocking solution (KPL). Primary antibodies for IFNγRα and IFNγRβ were purchased from PBL Biomedical Laboratories and used at a concentration of 1:1000. The pStat1 antibody was purchased from Cell Signaling and used at 1:750. GAPDH (Millipore) and Stat1 (Santa Cruz Biotechnology) antibodies were used at a 1:1000 dilution. Alexa Fluor (Molecular Probes) antibodies were used to visualize proteins on the Odyssey Infrared Imaging System (Li-Cor Biosciences).

Plasmids and Constructs

Gastrin reporter constructs were made by M. Smith using a 3.3kb fragment of the human gastrin promoter ligated into the pGL3 basic luciferase vector (Promega). The 240 bp pGL3 reporter was generated using the gastrin promoter fragment from the 240GasLuc construct, as previously described (Shiotani and Merchant 1995).

Transient Transfections and Luciferase Assays

AGS and HeLa cells were seeded into 12 or 24 well plates and grown to 50%-60% confluency prior to transfection. Between 0.5-1 μg/well plasmid DNA

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 and permitted normalization of firefly luciferase readings. The Path Detect pGAS (IFN gamma-activating sequence)-Luc Cis-Reporter Plasmid (Stratagene) was used as a positive control for IFNγ signaling. The cells were treated with 100 ng/ml of rhIFNγ or 60 ng/ml of EGF (R&D Systems) and harvested for analysis 48 h post transfection. Firefly and Renilla luciferase activites were measured using the Dual Luciferase Assay Kit (Promega).

Statistics

Groups were compared by using the Mann-Whitney U test. Significance level was set at a p value of <0.05. Error bars on graphs indicate standard errors of the means (SEM).

Results

Effect of IFNγ on Gastrin Expression *in vivo*

I have previously published a model where IFNγ infusion was used to recapitulate *H. pylori* infection in mice, including the induction of gastritis and hypergastrinemia (Zavros, Rathinavelu et al. 2003). Following a 7-day infusion of IFNγ, I observed inflammation in the fundus and an increase in the number of G cells in the antrum of mice compared to the PBS-treated control group (Fig 3.1A and B). Furthermore, I observed a 1.8- fold increase in plasma gastrin levels, demonstrating that I was able to induce gastritis and hypergastrinemia in IFNγ-infused mice (Fig 3.1C). I isolated mRNA from the antrum to determine whether an increase in gastrin gene expression contributed to elevated gastrin levels in these mice. I detected no difference in the gastrin mRNA levels between IFNγ and PBS infused groups after 7 days (Fig 3.1D).

Given the possibility that the effect of IFNγ on gastrin gene expression might be an early event, I injected mice with the cytokine to investigate gastrin mRNA levels within a period of 12h. Again, I observed no significant difference in gastrin mRNA expression between the treated and control groups (Fig 3.2A). However in the same samples, I detected a significant increase in the expression of the cytokine receptor gene, CXCL10, which is known to be transcriptionally regulated by IFNγ (Fig 3.2B) (Kaur, Lal et al. 2007).

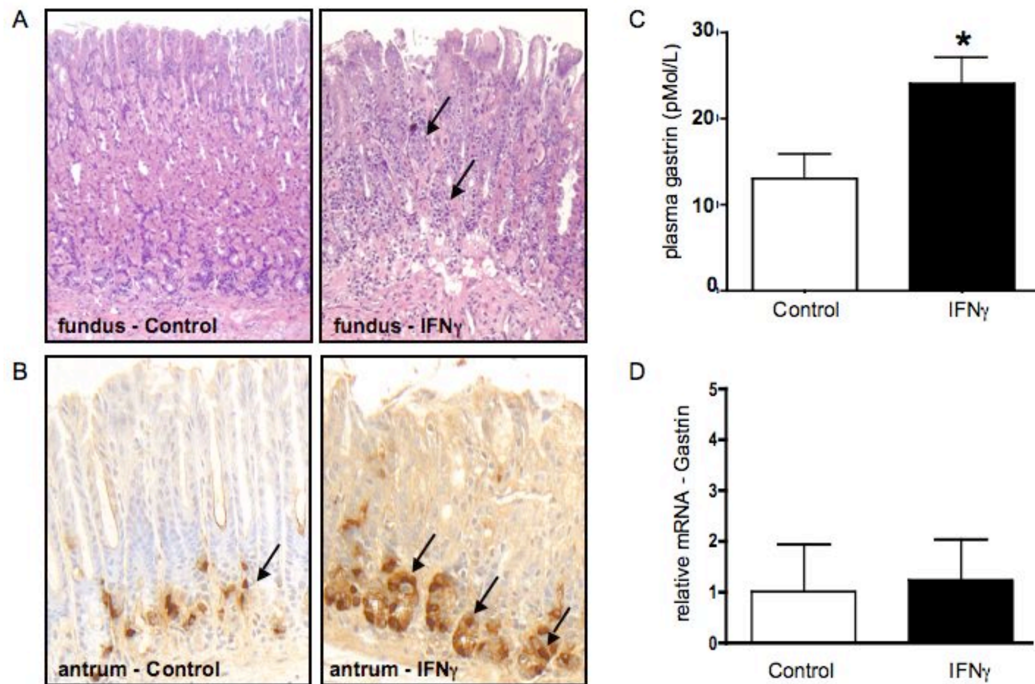


Figure 3.1. Inflammation and Hypergastrinemia Caused by IFNγ Infusion in Mice. Mice were infused for 7 days with rmIFNγ or PBS (control). Two separate experiments were performed with 4 mice per group. (A, B) Representative sections are shown of paraffin sections of mice from treated and control groups. (A) H&E staining was done to visualize gland morphology and inflammation. Arrows indicate inflammatory cells within the mucosa. (B) G cells were observed by immunohistochemical staining with arrows indicating positively stained cells. (C) Plasma gastrin levels were determined by RIA. Shown is the SEM, n = 8 mice per group, *p < 0.05. (D) Gastrin mRNA expression was measured by qRT-PCR and reported as relative mRNA expression. Shown is the mean $\pm$ SEM, n = 7-8 mice per group.

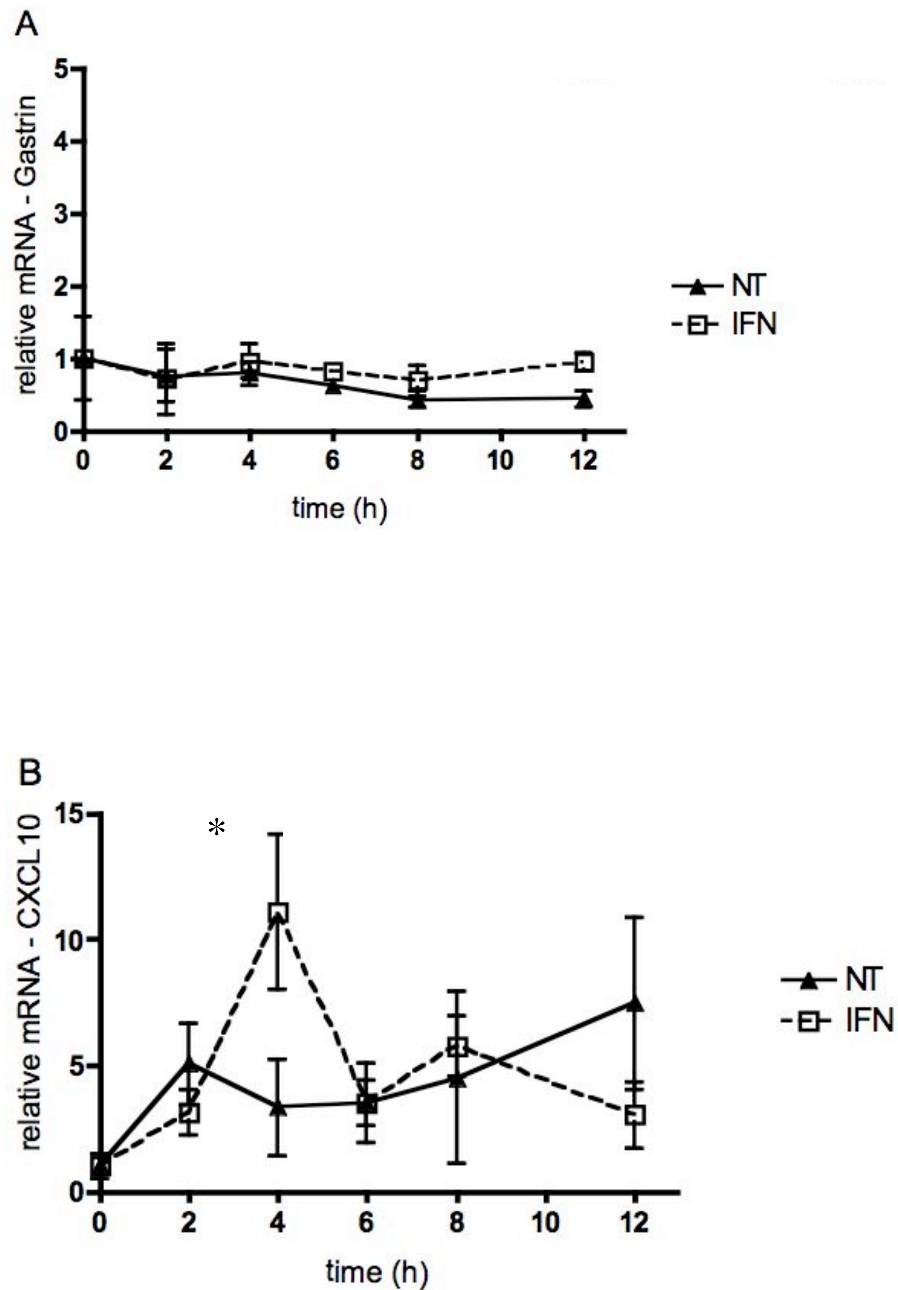


Figure 3.2. IFNγ Increases CXCL10 Expression but Not Gastrin Within 12h. Mice were injected with rmIFNγ or PBS (control) and harvested at times between 0 and 12h. qRT-PCR was used to determine levels of gastrin (A) and CXCL10 (B). Expression was normalized to GAPDH and reported as relative mRNA. n = 2-3 mice per time point, *$p < 0.05$ (A, B). Insets show scatter plots for the line graphs below.

Effect of IFNγ on Mouse Primary Cells

In the Suzuki et al. study, regulation of gastrin gene expression was observed using an enriched population of G cells (Suzuki, Grand et al. 2001). Therefore, I took a reductionist approach and used a primary culture to study cytokine regulation of gastrin. Mouse primary cells were incubated with IFNγ for up to 24 h and harvested at various times for qRT-PCR analysis.

A significant increase in the expression of gastrin within the first 12h of incubation was not detected (Fig 3.3). However, I did observe an average 2-fold increase in gastrin expression after 24 h however, the change was not statistically significant (Fig 3.3).

Gastrin Expression in Human Gastric Cell Lines

There are inherent limitations to using a heterogeneous cell population to investigate whether there is a direct regulatory effect. Thus, we sought to establish a suitable *in vitro* system for our studies using gastrin-expressing human cell lines. We compared the basal expression of gastrin in several lines, which were previously reported to express the gene. Using qRT-PCR analysis, we compared the relative levels of human gastrin expression between several cell lines of gastric or intestinal origins and HeLa cells, which are derived from a non-gastrointestinal tissue. We found that gastrin expression in AGS cells was significantly greater than in the other cell lines. Gastrin mRNA levels in AGS cells were 7-fold higher than the second highest expressing line, KATOIII, which was followed by HGT, HCT116, and MKN45 (Fig 3.4). Subsequent experiments

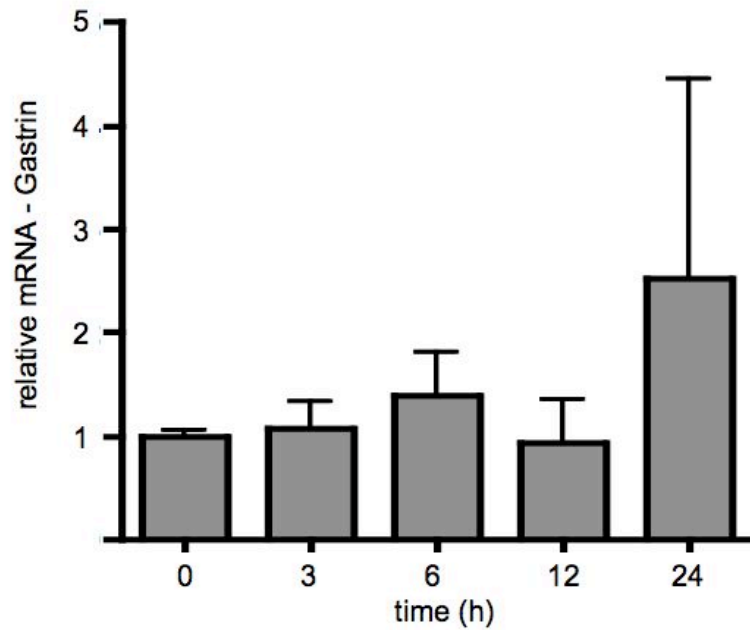


Figure 3.3. Gastrin Expression in Primary Gastric Cultures Is Unchanged Following IFNγ Treatment. Isolated primary gastric cultures were treated with 100ng/ml rmIFNγ from 0 to 24h and harvested for RNA isolation. Data shown are from three separate experiments performed in triplicate. qRT-PCR was used to determine expression of gastrin, which was normalized to GAPDH. Values were expressed as fold change of treated over untreated cells. Shown is the mean ± SEM, n = 3.

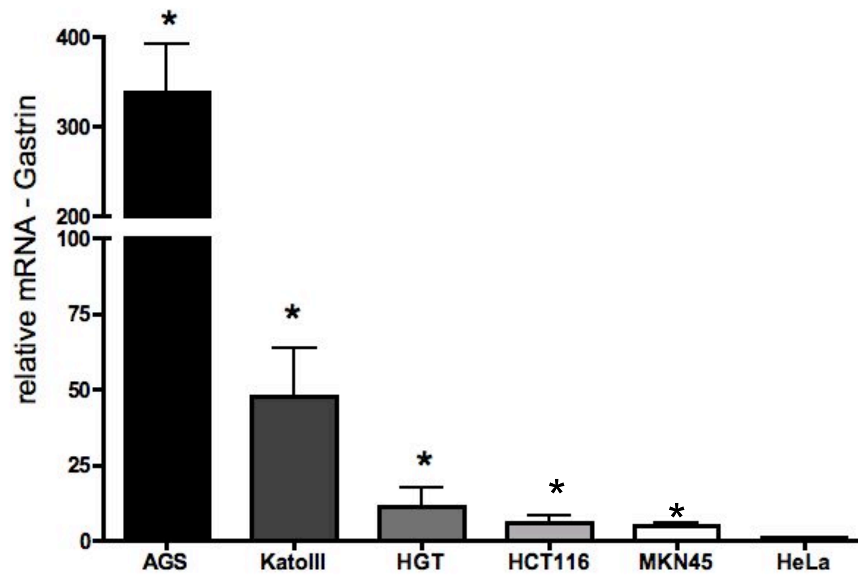


Figure 3.4. Endogenous Gastrin Expression in Gastrointestinal Cell Lines. Various human gastrointestinal-derived cell lines were cultured under similar conditions and harvested for qRT-PCR analysis. Expression of gastrin was normalized to 18s rRNA expression. Data represent three experiments, assayed in triplicate. Shown is the mean $\pm$ SEM expressed as fold change over the lowest expressing cell line (HeLa), n=3, *$p<0.05$.

were carried out using the AGS cell line since they expressed the highest levels of gastrin and might be amenable to observing small modulations.

Evaluation of IFNγ-Stat1 Signaling in AGS Cells

Previous reports in the literature have suggested that IFN-Stat1 signaling in AGS cells might be altered due to low or absent expression of the IFNγ receptor and Stat1 as well. I therefore, assessed IFNγ-Stat1 signaling in AGS cells. Initially I examined expression of the IFNγ receptor α and β chains (IFNγRα, IFNγRβ) in this cell line. I detected basal levels of expression for both receptor chains in AGS cells by immunofluorescence and western blot analysis (Fig 3.5A). Following treatment with IFNγ, there was a rapid increase in the levels of IFNγRα while there was a more gradual increase of IFNγRβ up to 30 min followed by a gradual decrease in protein expression (Fig 3.5B). Cellular localization of either receptor chain was not altered by IFNγ treatment (data not shown).

To investigate signaling downstream of the receptor I measured levels of Stat1 and pStat1, which are part of the canonical IFNγ signaling pathway. I detected low levels of pStat1 in unstimulated AGS cells as a diffuse and punctate staining pattern (Fig 3.6A, untreated). Following treatment with IFNγ, I observed a rapid increase in the levels of pStat1 and nuclear localization of the protein (Fig 3.6A, IFNγ). By western blot analysis, I determined that the protein levels of

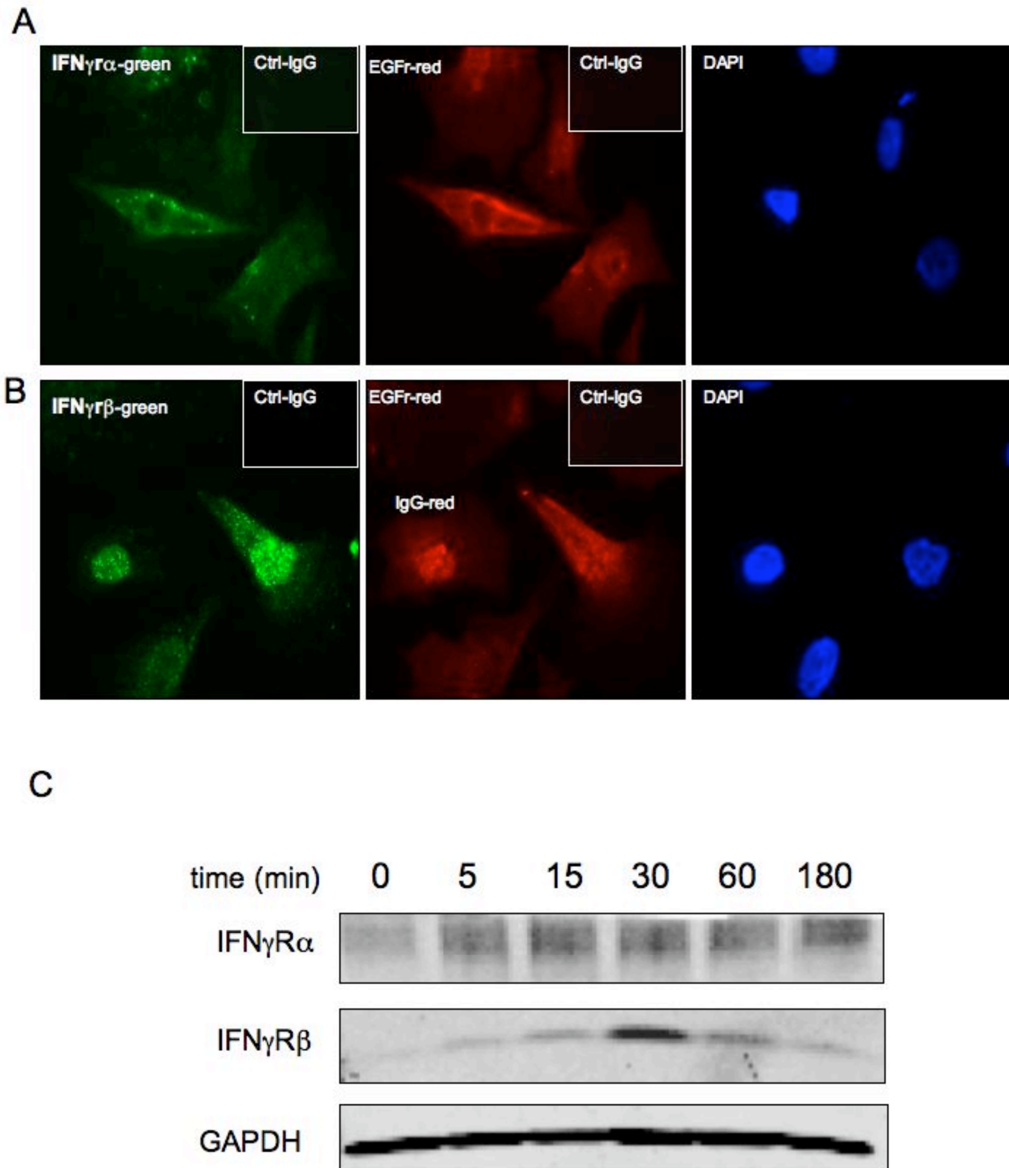
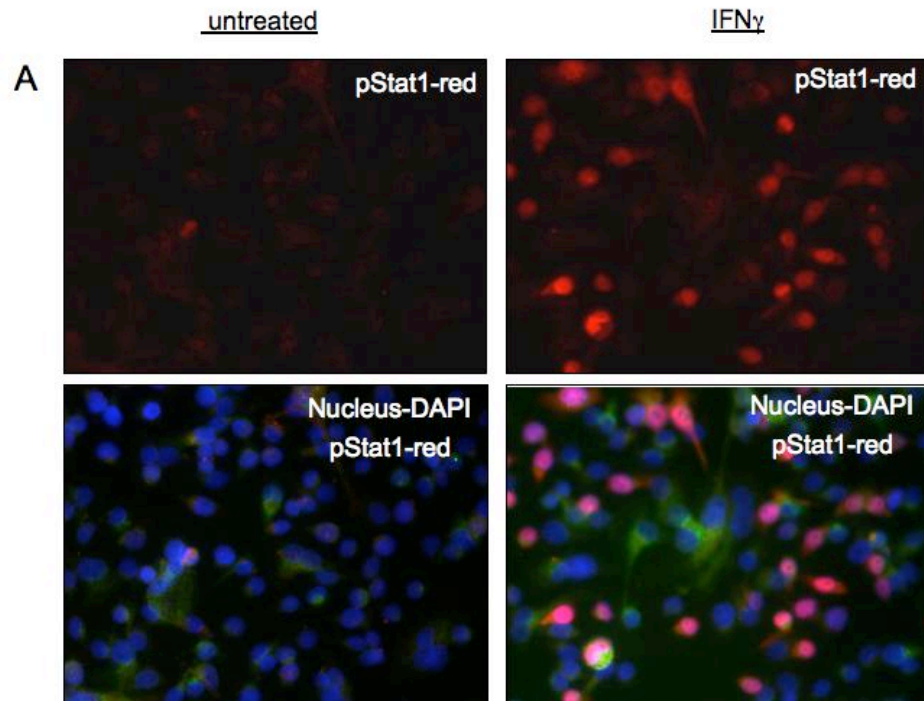


Figure 3.5. IFNγ Receptor Levels Were Increased by IFNγ in AGS Cells. AGS were cultured on coverslips and stained for IFNγRα (A) and β chains (B). Insets show negative controls for primary antibody staining (A, B). For western blot analysis, cells were serum starved for 24h prior to treatment with 100ng/ml rhIFNγ from 0-180min. Whole cell extracts were prepared for immunoblot analysis.



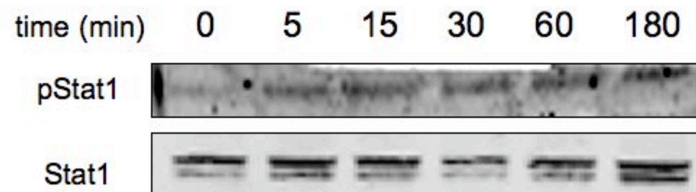


Figure 3.6. **IFNγ Activates Stat1 in AGS Cells**. For immunofloursence staining AGS were cultured on coverslips and serum starved for 24h prior to incubation with 100ng/ml of rhIFNγ for 30min (A). For western blot analysis, cells were serum starved for 24h prior to treatment with 100ng/ml rhIFNγ from 0-180min. Whole cell extracts were prepared for immunoblot analysis.

Stat1 remained unchanged while there was increased phosphorylation of Stat1 following IFNγ treatment that persisted for up to 3 h (Fig 3.6B). These results together suggested that IFNγ-Stat1 signaling pathways were intact and functional in AGS cells.

IFNγ Regulation of Endogenous Gastrin in AGS Cells

I continued the study of IFNγ regulation of the gastrin gene using the AGS cell line. I first observed regulation of endogenous gastrin gene expression by IFNγ using qRT-PCR. A time-course experiment from 0 to 12 h revealed no significant difference in the levels of gastrin expression following IFNγ treatment (Fig 3.7A). A significant regulatory effect of IFNγ on gastrin gene expression in HGT or HCT16 cells was also not observed (data not shown). Based on previous results, I measured EGF induction of gastrin as a positive control for induction of the gastrin gene. The expression of gastrin was significantly increased in EGF-treated AGS cells with increased expression by 3 h and maximal induction at 6 h (Fig 3.7B) (Merchant, Shiotani et al. 1995). Furthermore, the expression of CXCL10 was significantly increased following treatment with IFNγ in AGS cells with maximal induction by 3 h followed by a time dependent decrease in expression (Fig 3.7C).

IFNγ Regulation of Gastrin Reporters in AGS Cells

Lastly, I investigated the ability of IFNγ to regulate gastrin reporter constructs in AGS cells. Cells were transiently transfected with a 3.3 Kb and a 0.240 Kb gastrin promoter construct, then treated with IFNγ. The cytokine failed to activate the 3.3 Kb gastrin reporter at 3 h or 18 h after treatment (Fig 3.8A). Similar results were observed using the 0.240 Kb gastrin reporter (Fig 3.8B). IFNγ treatment also failed to increase gastrin reporter activity in HeLa cells (data not shown). However, the IFNγ-Stat1 responsive reporter, pGAS (GAS: interferon-γ activation sequence) showed a 3- and a 6-fold increase in expression following IFNγ treatment at 3 and 18 h respectively (Fig 3.8D). This same pGAS reporter was not activated by IFNγ in AGS cells which suggests that despite the presence of both the IFNγR and Stat1, activation of this pathway does not affect the gastrin promoter (Fig 3.8C).

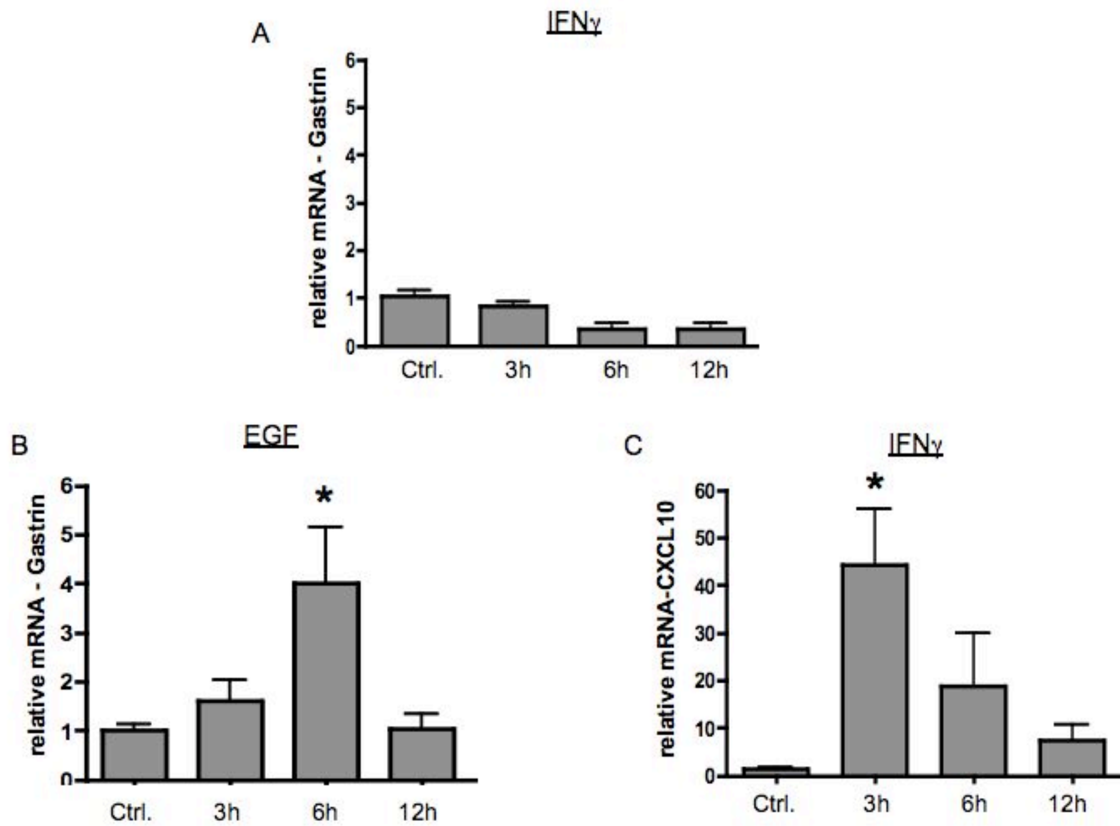


Figure 3.7. **Endogenous Gastrin Expression was Regulated by EGF but Not IFNγ.** AGS cells were serum starved for 24h prior to treatment with 100 ng/ml of rhIFNγ or 60 ng/ml of EGF from 0-12h. qRT-PCR analysis was performed on samples run in triplicate for three separate experiments. Gastrin (A, B) and CXCL10 (C) expression was normalized to h18s rRNA and calculated as fold change over untreated cells for each timepoint. Shown is mean ± SEM, n = 3-5, *$p<0.05$.

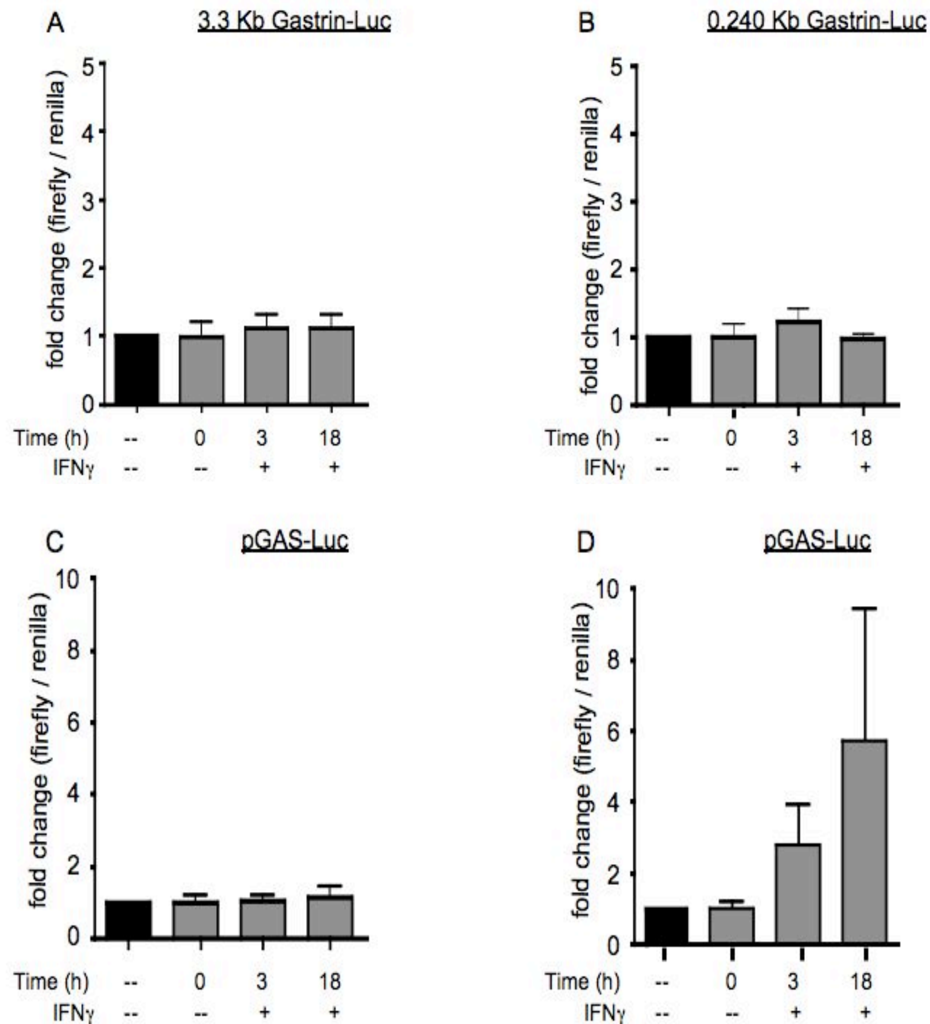


Figure 3.8. **Gastrin Reporter Expression was Unchanged by IFNγ Treatment**. AGS cells (A, B, and C) and HeLa cells (D) were transfected with gastrin luciferase reporter (A, B) and the IFNγ-responsive pGAS-Luc reporter (C, D). Luciferase data are shown as mean $\pm$ SEM for three separate experiments performed in triplicate. The data shows firefly luciferase activity normalized to Renilla luciferase. Fold change is the ratio of stimulated reporter activity over basal expression. Black bars represent the baseline expression for each construct which was set to 1.

Discussion

In this study, I sought to understand the regulatory effect of IFNγ on gastrin gene expression. I was able to induce gastritis and elevate levels of plasma gastrin in mice following a 7-day infusion with IFNγ although, I did not observe an effect of IFNγ on gastrin gene expression. Neither did I detect changes in gastrin gene expression within 12 h of injecting mice with the cytokine. I compared the endogenous expression of gastrin in several gastrointestinal-derived cell lines. I determined that gastrin expression was highest in the AGS cell line. Therefore, I used the AGS cells to carry out further studies. I confirmed the expression of the IFNγ receptor and Stat1 in AGS cells by immunofluorescence and western blot analysis. I also observed the activation of Stat1 following treatment with IFNγ. Lastly, I examined the expression of endogenous gastrin and the activity of gastrin promoter constructs in AGS cells. Treatment with IFNγ did not induce endogenous gastrin nor activate the gastrin promoter. I concluded from these studies that IFNγ alone is not sufficient to induce gastrin gene expression even though it stimulated gastrin release in vivo.

In Zavros et al., our group previously established the ability of IFNγ alone to mimic *H. pylori* infection in mice (Zavros, Rathinavelu et al. 2003). However, changes in gastrin mRNA were not previously investigated. In the current study, I utilized that same model system and did not detect changes in gastrin mRNA despite the fact that I observed increased numbers of G cells and increased

levels of plasma gastrin. I expected to observe increased levels of plasma gastrin even in the absence of a direct effect on the gene due to the increased number of G cells. I considered the possibility that changes in gastrin gene expression might occur early after a systemic increase of IFNγ, either experimentally or during bacterial infection, and might have returned to normal levels within 7 days. Thus, I conducted subsequent experiments with a shorter time course.

Following the injection of mice with IFNγ, I did not detect an increase in gastrin mRNA levels. While the expression of gastrin was approximately unchanged from 0 to 12 h, I did observe a subtle but not statistically significant decrease in the expression levels of gastrin in the PBS-treated mice. This was an unexpected finding and suggests that other unknown experimental factors (i.e. stress on the animals) might be blocking an increase in gastrin expression in the IFNγ and control groups. In this scenario, a slight increase in gastrin expression due to IFNγ would be negated by this unknown variable. Decreases in basal gastrin expression during fasting have been well established and might be skewing results in this short term study since both treatment groups were fasted prior to injection and analysis (Takeuchi, Speir et al. 1980; (Walsh and Grossman 1975). I did observe increases in a gene known to be regulated by IFNγ (CXCL10) that is not known to be impacted by fasting. I observed an increase the expression of CXCL10 in the control group over time. There might be an unknown experimental variable that altered the expression of both gastrin (decreased) and CXCL10 (increased) since I observed changes in the

expression of both genes following PBS treatment. An alternative interpretation is that that the sample groups (n=3) were too small to determine statistically significant changes in gene expression.

Following IFNγ treatment of mouse primary cells, I failed to detect significant induction of gastrin expression from 0 to12 h and detected variable amounts of gastrin mRNA 24 h post treatment. These results were unexpected since previous studies have shown that the gastrin promoter can be regulated by Th1 cytokines (Suzuki, Grand et al. 2001). Several studies have shown that IFNγ can regulate release of gastrin peptide from mouse and canine primary gastric cultures. However, the induction of the endogenous gastrin gene was not tested (Lehmann, Golodner et al. 1996; Suzuki, Grand et al. 2001; Zavros, Rathinavelu et al. 2003). I considered the possibility that the IFNγ dose was not sufficient to induce gastrin either *in vivo* or in primary gastric cultures. A survey of published studies revealed that the physiological concentration of IFNγ following *H. pylori* infection in mice is not known. Studies which address this issue reported levels for IFNγ as relative mRNA and did not define actual tissue levels of the cytokine (D'Elios, Manghetti et al. 1997; Avitzur, Galindo-Mata et al. 2005; Fukui, Nishio et al. 2006). We attempted to define a physiologically accurate doses of IFNγ that might be similar to what is produced in the tissue following *H. pylori* infection. We obtained tissue from *H. pylori*-infected mice but we were unable to detect the cytokine in tissue samples by ELISA possibly due to a rapid degradation of the peptide(Waghray et al., unpublished observations).

Because there are no cell lines that are specifically derived from G cells, I sought to establish a model system for further experiments in a cell type that expressed endogenous gastrin. Upon comparison by qRT-PCR, we found that the AGS cell line expresses high levels of gastrin when compared to a battery of other gastrointestinal-derived cell lines. There were reports in the literature that Stat1 levels in AGS cells were sufficiently low enough to inhibit signaling by IFNγ (Abril, Real et al. 1998; Young, Carlos et al. 2007). In contrast, several studies report the regulation of gene expression by IFNγ specifically in AGS cells (Yasumoto, Okamoto et al. 1992; Nyhlen, Linden et al. 2000; Kraft, Riedel et al. 2001; Mitchell, Huynh et al. 2004). I addressed this issue by demonstrating that AGS cells did indeed express Stat1 and that the protein was phosphorylated in response to IFNγ.

I was unable to regulate endogenous gastrin, gastrin promoter constructs, or the IFNγ-responsive pGAS-Luc control reporter in AGS cells. I was able to regulate gastrin by EGF, which is a known regulator of the gene (Godley and Brand 1989). I also stimulated endogenous CXCL10 expression in AGS cells using IFNγ. The induction of CXCL10 would suggest that IFNγ signaling is not impaired in AGS cells. Furthermore, it validates that our system is technically sufficient to detect regulation of gene expression by IFNγ in AGS cells. I was also unable to activate the pGAS-Luc construct by IFNγ in the AGS cells but observed the activation of this reporter using HeLa cells as expected. This result would again argue that the gastrin promoter is not influenced by IFNγ. In fact, there is

no obvious Stat1 binding site in the gastrin promoter supporting the observation that IFNγ has no direct effect on the promoter.

My hypothesis is that there are indeed alterations in the signaling response to IFNγ in AGS cells. I tested protein levels and activation of Stat1 in the AGS cells, but did not assess the levels or activation of other Stat proteins. Specifically, the pGAS construct is responsive to not only Stat1 but preferentially responds to Stat2:Stat1 heterodimers (Ghislain, Wong et al. 2001). An interpretation of my results must take into account that the signal transduction pathway between IFNγ to CXCL10 might be different from the putative signaling path for the activation of gastrin by IFNγ. Secondly, activation through the GAS sequence might require other Stat proteins that are not present or functional in AGS cells. Lastly, IFNγ utilizes both Stat1–dependent and –independent mechanisms to regulate the expression of target genes (Ramana, Gil et al. 2001; Ramana, Gil et al. 2002). I did not test the possibility that IFNγ regulation of gastrin might be impaired in AGS cells due to a defect in a Stat1-independent pathway. My overall conclusion based on the results of the current study is that IFNγ does not directly regulate gastrin gene expression.

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CHAPTER IV

CONCLUSION

Conclusion of Results

The broad objective of this dissertation was to describe how *H. pylori* infection contributes to the development of hypergastrinemia. Specifically, I explored the idea that induction of the human gastrin gene was a key step in the etiology of *H. pylori*-induced hypergastrinemia. The studies presented in this work investigated the ability of the bacteria and the pro-inflammatory cytokine IFNγ to regulate the gastrin gene at the level of transcription.

Our investigation into the mechanisms that mediate the inducible regulation of gastrin by *H. pylori* was presented in Chapter II. We observed an 8-fold increase in gastrin mRNA levels in mice that were infected with *H. pylori* compared to uninfected controls. Many studies in the literature report changes in plasma gastrin levels, both in patients and in a wide variety of animal models, but there are significantly fewer studies that actually report mRNA levels for gastrin (Sumii, Sumii et al. 1994; Rieder, Merchant et al. 2005). Furthermore, there are conflicting reports as to whether gastrin mRNA increases or remains unchanged as a result of *H. pylori* infection (Sankey, Helliwell et al. 1990). It should be noted that most studies that reported gastrin mRNA levels were performed in human subjects. Therefore, while my observation was not completely novel, it is one of

the few studies done in mice that reports gastrin mRNA levels and furthermore, detected a significant change in gene expression.

Likewise, I detected a 6- to 7-fold increase in gastrin expression when AGS cells were cultured with *H. pylori*. I determined that live bacteria were required for this effect but the bacterial proteins CagA and VacA were not required. The SS1 strain, which lacks a segment of the cag pathogenicity island that encodes for T4SS injection proteins, was also able to activate gastrin expression (Crabtree, Farmery et al. 1994). This finding was somewhat unexpected since several of the pathways that are known to regulate gastrin are negatively impacted by the loss of the cagPAI and CagA (Crabtree, Farmery et al. 1994; Blaser and Crabtree 1996; Meyer-ter-Vehn, Covacci et al. 2000; Bhattacharyya, Pathak et al. 2002; Kim, Kim et al. 2005). Specifically, cag- strains show decreased activation of MAPK signaling compared to cag+ strains. Given this, our findings are even more surprising since I determined that activation of gastrin by *H. pylori* requires MAPK-associated pathways. Based on reports in the literature, I would have expected less activation or even an inability of the CagA-null and SS1 strains to activate gastrin, however my results did not support this prediction.

The signal transduction pathways that are activated by *H. pylori* and specific virulence factors are well characterized in the literature (Romano, Ricci et al. 2006). I focused my study primarily on MAPK-associated pathways (p38,

MEK, and JNK) based on previous reports that implicated this pathway in the transcriptional regulation of the gene (Merchant, Du et al. 1999; Chupreta, Du et al. 2000). As was expected, inhibition of Mek1 blocked activation of gastrin by *H. pylori,* but I also observed blockade with p38 and JNK inhibition as well. This result indicated that all three pathways were necessary for optimal induction by *H. pylori*. On a molecular level, this could indicate that there is a single protein that is activated by the bacteria, which goes on to signal via p38, JNK, and MEK to transcriptionally activate gastrin. Ras lies upstream off these three signaling pathways and is also known to activate the gastrin promoter (Nakata, Wang et al. 1998; Merchant, Du et al. 1999; Meyer-ter-Vehn, Covacci et al. 2000). Furthermore, Ras can be activated by *H. pylori* and its related virulence factors (Romano, Ricci et al. 2006). Thus activation of Ras may be central to *H. pylori*-mediated regulation of the gastrin promoter. Alternatively, this result may indicate that there are several proteins that are required to induce the gastrin promoter thus blocking any of the three signaling pathways would prohibit the activation of a required factor and ultimately, induction of the gastrin gene.

Studies using human gastrin promoter constructs enabled us to identify three sites that mediated activation of the promoter by *H. pylori*. These sites contained a CCACCCC (C_2AC_4) binding motif, which was required for optimal induction of the gastrin promoter by *H. pylori*. Gel shift analysis confirmed that the first two of these sites mediated inducible binding of Sp1 and Sp3 following *H. pylori* treatment. Interestingly, the third element did not show inducible binding of Sp1

and Sp3 in response to *H. pylori*. This result is supported by previous studies of this particular element in the context of EGF induction of gastrin.

A unique finding of this study was that Sp1 and Sp3 were involved in activation of the promoter. Sp1 is often associated with constitutive activation of genes and not necessarily involved in inducible regulation (Suske 1999). Furthermore, the activity of Sp1 is often mediated by an increase in phosphorylation, but not enhanced binding of the protein to DNA elements (Jackson, MacDonald et al. 1990; Alroy, Soussan et al. 1999). This was certainly the case in our studies of EGF regulation of the gastrin promoter where Sp1 did not inducibly bind but was, in fact, activated by phosphorylation of Sp1 (Chupreta, Du et al. 2000; Merchant 2000). A 2003 study that examined the activation of the vegf-A gene showed findings similar to the results presented in my study where Sp1 binding was induced in response to *H. pylori* (Strowski, Cramer et al. 2004).

The role of Sp3 in the current study also presents an unusual role for the protein in gene regulation. Sp3 typically lacks an intrinsic ability to stimulate promoter activity despite having the capacity to bind DNA (Hagen, Müller et al. 1994; Suske 1999). Furthermore, Sp3 often functions as a transcriptional repressor by completing with Sp1 for binding at the promoter (Hagen, Müller et al. 1994; Kennett, Udvadia et al. 1997; Majello, De Luca et al. 1997). In our previous studies, Sp3 minimally activated the gastrin promoter suggesting that it might behave as a repressor in this context (Merchant, Du et al. 1999; Merchant 2000). Once more, there appears to be a different paradigm for Sp3 activity

within the context of activation by *H. pylori*. In the Strowski et al. study, Sp3 activated the vegf-A promoter and did not appear to mitigate Sp1 activation (Strowski, Cramer et al. 2004). In unpublished observations, over-expression of Sp3 potentiated induction of the gastrin promoter by *H. pylori*. (Tucker, unpublished observations, 2008). These results again suggest that Sp3 functions as an activator and is a key mediator for transcriptional activation by *H. pylori*.

To be noted, the C_2AC_4 elements would be expected to bind ZBP-89 based on previous studies (Law, Du et al. 1999). ZBP-89 is a known repressor of the gastrin promoter. However, additional studies might reveal that this protein is a part of the regulatory complex at this site. We did not confirm the presence of this protein in gel shift assays in our studies, however, a western blot of ZBP-89 in *H. pylori* induced extracts showed increased levels of the protein in a dose dependant manner (Tucker, unpublished observations, 2008). This may ultimately indicate that the regulation of gastrin in response to *H. pylori* involves a combinatorial effect of transcription factors that mediate positive and negative regulation of the promoter.

Further experiments are needed to clarify whether both Sp1 and Sp3 are required for activation of the gastrin promoter by *H. pylori*. A siRNA “knock-down” of Sp1, Sp3, or both proteins would help to differentiate the specific contribution of each transcription factor. The role of ZBP-89 could also be defined using a similar approach although the first steps would be to look for the

presence of the protein in binding complexes that are induced by *H. pylori* using gel shift analysis.

The emerging theme from these recent studies and earlier findings is that the Sp1 family proteins play an essential role in gastrin promoter regulation. The role of ZBP-89 in this regulation requires further study, however, it is plausible that this protein might also play an essential role. Ultimately, these proteins and their associated regulatory elements might be involved in the inducible regulation of gastrin in response to a variety of physiological stimuli.

In Chapter III, a study of the regulatory effect of IFNγ on the gastrin gene was presented. Based on previous studies from our lab, I initiated this line of investigation using a well-established model for IFNγ-induced hypergastrinemia. I did not observe changes in gastrin mRNA levels following IFNγ treatment in mice despite observing increased numbers of G cells and higher levels of plasma gastrin in the 7-day infusion study. This result was unexpected since I was able to demonstrate that there were more gastrin-producing cells in mice following IFNγ treatment. It is possible that the time frame for this experiment did not allow for detection of changes in gastrin mRNA levels that might have occurred either before or after the 7-day time point. The latter of the two possibilities seems unlikely since mRNA synthesis typically precedes the production of protein. In the case of gastrin, it might be that the trigger for increased mRNA synthesis does not occur until there is a significant reduction in the gastrin peptide content within the cell. This scenario would mean that changes in serum gastrin would precede any observed increases in gastrin gene expression. A more plausible

explanation is that the changes in gastrin gene expression occurred prior to the observed increases in gastrin peptide and then returned back to basal levels by the time of my analysis.

The next series of studies were conducted using mouse gastric primary cultures. Again, I did not detect a significant increase in the levels of gastrin mRNA in response to IFNγ. The approach for this work was taken largely from studies done using canine primary gastric cultures (Lehmann, Golodner et al. 1996; Beales, Blaser et al. 1997; Suzuki, Grand et al. 2001). I expected to find changes in gastrin mRNA expression since previous studies reported that the release of gastrin peptide from both canine and mouse primary cultures could be stimulated by IFNγ treatment (Lehmann, Golodner et al. 1996; Beales, Blaser et al. 1997; Zavros, Rathinavelu et al. 2003). Additionally, the induction of the endogenous gastrin gene by other cytokines was reported in a study using canine primary cells (Suzuki, Grand et al. 2001). It is possible that my procedure for the isolation of cultures impaired the ability of the cells to respond to the cytokine. This hypothesis was not thoroughly tested in my system and limited the certainty of our observations.

The final studies in this chapter utilized an *in vitro* approach to investigate the regulation of the gastrin gene by IFNγ. I focused my studies on the AGS cell line which expressed high levels of gastrin mRNA. Furthermore, I did validate the status of IFNγ -Stat1 signaling in these cells. One caveat to this approach is that there are multiple proteins involved in IFNγ signaling that were not tested. This reveals an inherent limitation with using cell lines. By definition they are

transformed, and typically have mutations in key proteins that confer this phenotype. However, these mutations might also alter the signaling pathways that are required to activate a target gene of interest. I did not observe the induction of endogenous gastrin or the activation of the gastrin promoter when AGS cells were treated with IFNγ. These results suggest that IFNγ alone is not sufficient to induce the gastrin gene but more specifically may indicate that there is an alteration in a component of the IFNγ signaling pathway in AGS cells.

Future Directions

The results presented in this dissertation have contributed to our understanding of the molecular mechanisms that mediate the inducible regulation of gastrin during *H. pylori* infection. Most significantly, I have defined a mechanism whereby the bacteria regulates the expression of the gastrin gene through Sp1/Sp3 binding elements. The results of this study have also raised questions that challenge our hypotheses about regulation of the gastrin gene by inflammation. In either case, there are pertinent questions that can be raised and investigated to move beyond the conclusions of this body of work.

From a broad perspective, I sought to understand the etiology of *H. pylori*-induced disease. The established paradigm states that colonization of the stomach initiates a Th1 response in the host. I established that expression of the gastrin gene is up-regulated by a direct effect of the bacteria. While my studies were unable to establish a direct effect of IFNγ in regulation of the gastrin gene, I

cannot rule out that inflammation in general, or even specifically, IFNγ might contribute to the development of hypergastrinemia.

A common feature of *H. pylori*-induced hypergastrinemia is that an increased number of G cells are observed. A previous study in our lab measured the proliferative index for G cells and determined that inflammation induced proliferation in this cell population (Zavros, Rieder et al. 2002). However, the impact of the bacteria on the proliferation of G cells should be considered. It is plausible that *H. pylori* influences the expansion of this population by initiating gastrin gene expression in non-G cells.

Lastly, while this study has expanded our understanding of the relationship between *H. pylori* infection and hypergastrinemia, we do not clearly understand the factors that contribute to the diverse clinical outcomes of infection. Ultimately, the disease outcome in patients is influenced by the interplay of bacterial, environmental, and host factors (Kusters, van Vliet et al. 2006). Specifically, there might be a correlation between the severity of hypergastrinemia and the disease outcome. Furthermore, since we know most infected patients do not develop ulcers due to *H. pylori*, perhaps it is in patients that tend to develop hypergastrinemia that the propensity for ulcer development is increased. These are questions which can be addressed by further epidemiological studies.

Implications of the Study

The work presented in this dissertation has broad relevance within the field of cellular and molecular biology. The novelty of the work presented is that a molecular mechanism has been defined for the regulation of gastrin gene expression by *H. pylori*. Specifically, this work has unified what was previously known about the transcriptional regulation of gastrin and the transcriptional regulation of host genes by *H. pylori*. The current study has largely built upon previous work and fits with what has been generally understood about the regulation of gastrin and *H. pylori*-activation of gene expression with few results that challenge previous paradigms.

In this dissertation, a detailed analysis of the molecular mechanisms by which *H. pylori* induces gastrin gene expression was presented. Given the multitude of prior studies that demonstrated the regulation of host genes by *H. pylori*, I was not surprised to find that the bacteria had a direct regulatory effect on the gastrin gene. Interestingly, previous work in our lab investigated the ability of another bacteria, *Acinetobacter lwoffii* (*A. lwoffii*) to cause gastritis and hypergastrinemia (Zavros, Rieder et al. 2002). This work demonstrated that not only could A. lwoffii cause increases in the numbers of G cells and plasma gastrin levels but could also directly stimulate the gastrin promoter (Ofori-Darko, Zavros et al. 2000; Zavros, Rieder et al. 2002). In the 2002 study, specific bacterial proteins were able to activate the promoter in the absence of live bacteria (Ofori-Darko, Zavros et al. 2000). This work formed the basis for my hypothesis that *H. pylori* might directly regulate gastrin gene expression. Indeed, my findings supported

this hypothesis. My studies showed that the loss of *H. pylori* virulence factors did not inhibit the bacteria from regulating the gastrin gene. Since the induction of the gastrin gene occurs in a MAPK-dependent manner, it does challenge the idea that virulence factors, specifically CagA, are required for full activation of MAPK signaling. Since *H. pylori* can activate a broad range of signaling cascades and transcription factors, it was not surprising that the bacteria stimulated the gastrin promoter through Sp1/Sp3 binding elements. Furthermore, this result reinforces our previous studies on EGF-mediated induction of gastrin and suggests that Sp1 regulation is a mechanistic theme for the regulation of the gene.   The combined observations of the work presented in Chapter II sheds light on how *H. pylori* causes disease by characterizing a molecular mechanism for the regulation of a gastrointestinal peptide that is critical for gut homeostasis by the bacteria.

Perhaps the most surprising finding of this study was that IFNγ did not regulate gastrin gene expression. The regulation of gene expression by cytokines is firmly established in the literature and is often associated with mucosal damage and tumor development (Schroder, Hertzog et al. 2004; Yoshimura 2006). However, regulation by cytokines and specifically IFNγ can occur at various levels within the cell including regulating protein expression or release. Previous studies demonstrated that the release of gastrin from G cells is stimulated by pro-inflammatory cytokines including IFNγ, IL-8, and TNFα (Lehmann, Golodner et al. 1996; Beales, Blaser et al. 1997). Furthermore, in vivo studies done by our group established that IFNγ could enhance the

expression and release of gastrin protein in vivo (Zavros, Rathinavelu et al. 2003). My hypothesis that gastrin gene expression may also be regulated by IFNγ was largely based on a report by Suzuki et. al that showed the regulation of gastrin gene expression by both TNFα and IL-1 (Suzuki, Grand et al. 2001). While my results did not support this hypothesis, they are consistent with the body of work that places the regulatory impact of IFNγ on gastrin at the protein level.

In summary, this current study fits within the paradigms that have been well established by the previous research and findings. My overall synthesis of the data is that both the *H. pylori* bacterium and pro-inflammatory cytokines contribute to the development of hypergastrinemia. To extrapolate from my findings, a degree of nuance can be added to our understating of the mechanism for this phenomenon. I hypothesize that the induction of gastrin gene expression is mediated primarily by the, bacteria whereas, the release of gastrin is stimulated by inflammatory cytokines that are produced during the Th1 immune response. The finding of this study are consistent with this hypothesis although further studies would enable us to refine and verify this idea.

In the context of the physiological regulators of gastrin (i.e. feeding, gastric pH) changes in both gastrin gene expression and gastrin peptide release occur (Walsh and Grossman 1975). While significantly more studies have focused on the regulation of gastrin release as the main paradigm for the regulation of this hormone, my findings suggest that inducible regulation of gastrin gene expression might be an early step in the development of *H. pylori*-mediated

hypergastrinemia and ultimately, a key step in the progression of gastric pathophysiology.

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