Regulation of RANTES Promoter Activation in Gastric Epithelial Cells Infected with *Helicobacter pylori*

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Received 7 June 2005/Returned for modification 15 July 2005/Accepted 22 July 2005

RANTES, a CC chemokine, plays an important role in the inflammatory response associated with *Helicobacter pylori* infection. However, the mechanism by which *H. pylori* induces RANTES expression in the gastric mucosa is unknown. We cocultured gastric epithelial cells with wild-type *H. pylori*, isogenic *oipA* mutants, *cag* pathogenicity island (PAI) mutants, or double knockout mutants. Reverse transcriptase PCR showed that RANTES mRNA was induced by *H. pylori* and that the expression was both OipA and *cag* PAI dependent. Luciferase reporter gene assays and electrophoretic mobility shift assays showed that maximal *H. pylori*-induced RANTES gene transcription required the presence of the interferon-stimulated responsive element (ISRE), the cyclic AMP-responsive element (CRE), nuclear factor-interleukin 6 (NF–IL-6), and two NF-κB sites. OipA- and *cag* PAI-dependent pathways included NF-κB—NF-κB/NF-IL-6/ISRE pathways, and *cag* PAI-dependent pathways additionally included Jun N-terminal kinase—CRE/NF-κB pathways. The OipA-dependent pathways additionally included p38—CRE/ISRE pathways. We confirmed the in vitro effects in vivo by examining RANTES mRNA levels in biopsy specimens from human gastric antral mucosa. RANTES mRNA levels in the antral mucosa were significantly higher for patients infected with *cag* PAI/OipA-negative *H. pylori* or uninfected patients. The mucosal inflammatory response to *H. pylori* infection involves different signaling pathways for activation of the RANTES promoter, with both OipA and the *cag* PAI being required for full activation of the RANTES promoter.

Gastric epithelial damage in *Helicobacter pylori* infection is thought to be related to the cellular and humoral inflammatory response to the infection. The cellular immune response to the organism initially consists of the recruitment of neutrophils, followed by T and B lymphocytes, plasma cells, and macrophages. Members of the chemokine supergene family, particularly the CXC and CC chemokine subfamilies, are thought to be responsible for recruitment of these inflammatory cells into the gastric mucosa (7, 8, 14, 28, 31, 35–37). RANTES (regulated on activation normal T cell expressed and secreted) is a member of the CXC and CC chemokine subfamilies, are thought to be responsible for recruitment of these inflammatory cells into the gastric mucosa (7, 8, 14, 28, 31, 35–37). RANTES (regulated on activation normal T cell expressed and secreted) is a CC chemokine produced by epithelial cells, CD8+ T cells, fibroblasts, and platelets that mediates the trafficking and homing of classical lymphoid cells such as T cells and monocytes. RANTES also acts on a range of other cells, including basophils, eosinophils, natural killer cells, dendritic cells, and mast cells (2, 30).

Increased RANTES production is a feature of *H. pylori*-induced gastric inflammation (7, 14, 28, 31, 37). RANTES mRNA expression is also thought to play an important role in maintaining residual memory T lymphocytes and eosinophils in gastric mucosa following *H. pylori* eradication (14). The mechanisms responsible for inducible RANTES gene transcription associated with *H. pylori* infections have not been fully elucidated. Mori et al. (23) recently reported that the regulation of RANTES gene transcription by *H. pylori* was mediated primarily through the activation of nuclear factor-κB (NF-κB) and depended on the presence of the *cag* pathogenicity island (PAI). They provided information regarding NF-κB sites, but the RANTES promoter contains five important protein-binding sites for transcription factors, including the cyclic AMP-responsive element (CRE), the interferon-stimulated responsive element (ISRE), nuclear factor–interleukin 6 (NF–IL-6), and two NF-κB sites. The possible role of the proinflammatory virulence factor OipA, a member of one of the large outer membrane protein gene families (39), was also not examined, and both OipA and the *cag* PAI are necessary for full activation of the IL-8 promoter (38).

This report describes experiments designed to test the hypothesis that the *cag* PAI and OipA are involved in the regulation of RANTES gene transcription. The study design included investigating the role of OipA or OipA in conjunction with the *cag* PAI in the induction of RANTES gene transcription in gastric epithelial cells. To provide an in vitro-in vivo correlation, we also examined *H. pylori*-induced RANTES mRNA levels in human gastric antral mucosal tissues.

MATERIALS AND METHODS

*H. pylori* preparation. The clinical *H. pylori* isolate JK51 was used (38, 39). This strain is *cag* PAI positive and expresses OipA (16). Isogenic *oipA*-, *cag* PAI, and *cag* PAI *oipA* (representing a double knockout of the *cag* PAI and the *oipA* gene) mutants were also used. Isogenic *oipA* mutant strains were constructed as previously described (39). For the construction of isogenic mutants with a totally deleted *cag* PAI regions upstream (hp0518-hp0519; 545,254 to 547,164 bp [“hp” numbers and locations are from *H. pylori* strain 26695, available under GenBank accession number AE000511]) and downstream (hp0549-hp0550; 584,570 to 586,563 bp) of the *cag* PAI were amplified from the *H. pylori* strain 26695.
chromosome to delete the entire cag PAI from the H. pylori chromosome. These fragments, separated by a chloramphenicol resistance cassette (a gift from D. E. Taylor, University of Alberta, Edmonton, Canada), were cloned into the T7Blue vector (Novagen, Madison, WI). A kanamycin resistance gene cassette (a gift from R. Haas, Max von Pettenkofer Institut, Munich, Germany) was also inserted into the SspI site of insert DNA for the opel gene, and the resulting plasmid was used for dual inactivation of the cag PAI and opel by selection on a chloramphenicol- and kanamycin-containing plate. All plasmids (1 μg) were used for the inactivation of cosmosomal genes by natural transformation as previously described (11, 39). We picked at least eight clones of each mutant.

Inactivation of the genes was confirmed by PCR amplification followed by Southern blotting as well as by Western blotting for OipA (16) and CagA (Austral Biologicals, San Ramon, CA).

H. pylori cells were cultured on brain heart infusion agar plates containing 7% horse blood and incubated at 37°C under microaerophilic conditions for 24 to 36 h. The bacteria were then inoculated into phosphate-buffered saline, and the density was estimated by spectrophotometry (A600) and microscopic observation. For in vitro experiments, we used a multiplicity of infection (MOI) of 100, which eliminates possible effects of reduced adherence of oipA mutants (38).

Cell culture. The human gastric cell line MKN45 (Japanese Cancer Research Bank, Tsukuba, Japan) was maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA), penicillin G (100 U/ml), and streptomycin (100 μg/ml). In some experiments, cells were pretreated (30 min prior to H. pylori infection) with mitogen-activated protein kinase (MAPK) inhibitors (U0126, SB203580, and SP600125) (Calbiochem, San Diego, CA) or the proteasome inhibitor N-benz-L-leu-L-leu-L-leucinal MG-132 (MG-132) (Calbiochem, Cambridge, MA). To avoid the influence of serum, MKN45 cells were serum starved for 16 h and maintained throughout the period of infection in all in vitro experiments.

Quantifying RANTES mRNA levels in MKN45 cells. Subconfluent monolayers of MKN45 cells in 24-well microplates were stimulated with H. pylori for 0 to 24 h. The total RNA was extracted from the cells using TRIZOL reagent (Invitrogen). The levels of RANTES mRNA and β-actin mRNA as a housekeeping gene were determined using Quantikine mRNA colorimetric quantification kits (R&D Systems Inc., Minneapolis, MN). The levels of RANTES mRNA were expressed as ratios of RANTES mRNA to β-actin mRNA (β-actin mRNA as 100%).

RANTES protein levels from MKN45 cells cocultured with H. pylori. In vitro RANTES levels from MKN45 cells were examined as previously described (37). Briefly, H. pylori was added to exponentially growing epithelial cells in 24-well plates for 4 to 30 h. RANTES levels in the supernatants were determined by an enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN). In our laboratory, the ELISA sensitivity to RANTES was approximately 3.2 pg/ml.

siRNA for knockdown of NF-κB expression. To investigate the effect of NF-κB on RANTES mRNA expression, we used small interfering RNA (siRNA) to interfere with NF-κB p65 mRNA. NF-κB p65 siRNA expression plasmids were obtained from Panomics, Inc. (Redwood City, CA). For siRNA knockdown, NF-κB p65 siRNA plasmids or empty plasmids were transfected 72 h prior to H. pylori infection, using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer’s instructions.

Plasmid construction. 5′ deletion constructs of the human RANTES promoter (pGL2-400, pGL2-300, pGL2-220, pGL2-195, pGL2-150, pGL2-120, and pGL2-90) were produced by PCR using the full-length human RANTES promoter from nucleotides −974 to +55 (pGL2-974) as a template (4). Site-directed mutations in the RANTES promoter were introduced by using pGL2-220 as a template (4).

Cell transfection. Exponentially growing MKN45 cells in 24-well plates were transfected with the reporter gene plasmid of interest (2 μg) using the Lipofectamine 2000 reagent (Invitrogen). Luciferase assays were performed with a dual-luciferase reporter assay system (Promega, Madison, WI). In this system, the pHRL-TK plasmid, a Renilla reniformis luciferase vector DNA (10 ng), was cotransfected as an internal control and for the normalization of transfection efficiencies. Thirty hours after transfection, the medium was changed and the cells were stimulated with H. pylori, left unstimulated (negative control), or stimulated with tumor necrosis factor alpha (10 ng/ml) (positive control). The cells were lysed at intervals from 2 to 18 h, and the lysates were assayed for luciferase activity. Luciferase activity was normalized to that of Renilla luciferase vector DNA (normalized luciferase activity). We also assessed the luciferase activity as the increase in luciferase activity in H. pylori-infected cells relative to that of uninfected controls (fold increase). We confirmed that the expression of Renilla was not induced by H. pylori infection (data not shown).

EMSA. Nuclear extracts of uninfected and infected MKN45 cells (approximately 5 × 106 cells/ml) were prepared using hypotonic/nonionic detergent lysis (3). After extraction, equal amounts of nuclear proteins were used to bind to duplex oligonucleotides corresponding to the RANTES-specific CRE, ISRE, NF-IL-6, NF-κB1, and NF-κB2 wild-type and mutated binding sites. Sequences of the wild-type and mutated oligonucleotides and DNA-binding reactions used for electrophoretic mobility shift assays (EMSAs) were as previously described (4). The nuclear proteins were incubated with the probe for 15 min at room temperature and then fractionated by 6% nondenaturing polyacrylamide gel electrophoresis (PAGE) in Tris-borate-EDTA buffer (22 mM Tris-HCl, 22 mM boric acid, 0.25 mM EDTA, pH 8). After electrophoretic separation, gels were dried and exposed for autoradiography using Kodak XAR film and intensifying screens at −80°C.

For competition assays, 5 pmol of unlabeled competitors was added at the time of probe addition. In gel mobility supershift assays, commercial antibodies against specific transcriptional factors (anti-p50 [sc-1191], anti-p65 [sc-109], anti-c-Fos [sc-413], anti-c-Jun [sc-44], anti-C/EBPβ [sc-186], anti-ATF-1 [sc-241], and anti-CCAAT/enhancer binding proteins [anti-C/EBPα [sc-60], anti-C/EBPβ [sc-150], and anti-C/EBP-δ [sc-151]]) (Santa Cruz Bio-technology, Inc., Beverly, MA). MKN45 cells (approximately 5 × 105 cells/ml) were either left uninfected or infected with H. pylori (MOI of 100) for 0 to 3 h. An equal amount of protein extract was fractionated by sodium dodecyl sulfate-PAGE and electrophoretically transferred to a polyvinylidene difluoride membrane, and an enhanced chemiluminescence detection assay (Amersham Pharmacia Biotech, Piscataway, NJ) was performed.

Quantifying RANTES mRNA levels in gastric antral mucosal tissues. To investigate whether the in vitro data reflected the in vivo phenomenon, antral biopsy specimens were examined to determine their RANTES mRNA levels. Samples were selected from patients with gastritis for whom the cag PAI and OipA status had been previously characterized (38). Total RNAs were extracted from the gastric antral mucosal biopsy specimens using TRIZOL reagent (Invitrogen). The RANTES mRNA levels and β-actin mRNA levels were determined using Quantikine mRNA colorimetric quantification kits (R&D Systems Inc.). The RANTES mRNA levels were expressed as ratios of RANTES mRNA to β-actin mRNA (1,000 × RANTES mRNA/β-actin mRNA). The biopsy specimens had been obtained under protocols approved by local ethics committees, and informed consent had been obtained from all patients.

Statistical analysis. Each experiment was performed at least three times. Analysis of variance (ANOVA) was used when the data were normally distributed (in vitro data). Differences identified by ANOVA and the Kruskal-Wallis test were pinpointed by the Student-Newman-Keuls test and the Tukey-Kramer test, respectively (26). Results are presented as medians along with 25th and 75th percentiles when the data were not distributed normally and as means and standard errors (SE) when they were. P values of <0.05 were accepted as statistically significant.

RESULTS

H. pylori induces RANTES in gastric epithelial cells. RAN- TES mRNA was detectable 1 h after coculture with wild-type H. pylori strain JK51 and reached maximal levels at 4 h (Fig. 1A). RANTES mRNA levels at 4 h in MKN45 cells cocultured with wild-type H. pylori strain JK51 were significantly increased compared with those in uninfected control cells (% of β-actin mRNA, 11.9 ± 0.3 versus 1.3 ± 0.4) (P < 0.05) (Fig. 1B). H. pylori infection-related RANTES mRNA levels were significantly lower following infection with the oipA mutants than after infection with the wild-type strain JK51 (7.4 ± 0.4 or its cag PAI mutant (4.5 ± 0.3) (P < 0.05). Infection with cag PAI oipA double mutants led to a further reduction in RANTES mRNA levels (2.2 ± 0.2) compared with a single knockout of either oipA or cag PAI (P < 0.05).

RANTES protein was also induced by wild-type H. pylori infection and reached maximal levels at 24 h (Fig. 1C). Similar to mRNA levels, H. pylori-induced RANTES protein levels
were both cag PAI and OipA dependent (Fig. 1D). We used eight clones of each mutant. The RANTES mRNA and protein levels were similar among the clones (data not shown). We therefore used one or two of these clones of each mutant in subsequent experiments.

We next examined the roles of MAPK and NF-κB signal transduction pathways in RANTES gene expression by using specific inhibitors, including U0126, a specific inhibitor of MEK1/2 (MAPK/extracellular signal-regulated kinase 1/2 [ERK1/2]), which is upstream of ERK1/2; SB203580, a specific inhibitor of p38; SP600125, a specific inhibitor of Jun N-terminal kinase (JNK); and the proteasome inhibitor MG-132, which inhibits NF-κB activation. MKN45 cells were pretreated with each inhibitor for 30 min prior to H. pylori infection. Even at concentrations as high as 30 μM, none of these inhibitors affected RANTES mRNA levels in uninfected MKN45 cells (data not shown). Each inhibitor significantly suppressed the induction of wild-type H. pylori-induced RANTES mRNA (Fig. 2A). We also used the siRNA technique to knockdown NF-κB expression to confirm the effect of NF-κB on RANTES mRNA expression. RANTES mRNA levels were suppressed approximately 50% by the NF-κB p65 siRNA expression plasmid compared with empty plasmid (P < 0.05) (Fig. 2B). Induction patterns of protein levels paralleled those of mRNA levels, confirming that H. pylori-induced RANTES production was via MAPK pathways and NF-κB pathways (Fig. 2C and D). H. pylori infection induces RANTES promoter activity. To determine whether H. pylori infection of gastric epithelial cells is associated with the induction of RANTES gene transcription, we transiently transfected MKN45 cells with a construct containing the first 974 nucleotides (nt) of the human RANTES promoter linked to the luciferase reporter gene (pGL2-974).
Previous studies have shown that this fragment of the promoter is sufficient to drive regulated luciferase expression in a variety of cell types (21). The luciferase activity reached maximal levels at 6 h postinfection for the wild-type H. pylori strain, and therefore luciferase activity was measured after 6 h of treatment in all subsequent experiments. The peak luciferase activity was significantly higher for the wild-type H. pylori strain JK51 (normalized to the activity by the Renilla luciferase vector [19.4 ± 2.3] and increased 3.5- ± 0.4-fold compared to the uninfected control) than for its oipA mutants (1.9- ± 0.1-fold) or cag PAI mutants (1.5- ± 0.1-fold) (P < 0.05) (Fig. 3). The activity was further decreased for the cag PAI oipA double mutants (1.1- ± 0.02-fold increase) (data not shown). In these experiments, we used two clones of each mutant. The results were similar between the clones (data not shown). These results are consistent with the data shown above suggesting that the cag PAI and OipA regulate RANTES gene transcription through different pathways.

Effects of 5′ deletions of the RANTES promoter sequence on H. pylori-inducible activity. To define the regions of the RANTES promoter involved in regulating RANTES gene expression after H. pylori infection, MKN45 cells were transiently transfected with plasmids containing serial 5′-to-3′ deletions of the RANTES promoter linked to the luciferase reporter gene. Cells were infected with H. pylori and harvested at 6 h postinfection, which corresponds to peak reporter gene induction, to

![Graph A](image1.png)

**FIG. 2.** Effect of MAPK pathways and NF-κB pathways on RANTES mRNA expression and protein production in MKN45 cells stimulated by H. pylori. Each inhibitor (5, 10, and 30 μM) was added to MKN45 cells 30 min prior to wild-type (WT) H. pylori infection, and then the cells were incubated for 4 h with the WT strain for measurements of mRNA levels (A) and for 24 h for measurements of protein levels of RANTES (B). SB, SB203580; SP, SP600125. An NF-κB p65 siRNA expression plasmid or empty plasmid was transfected into MKN45 cells 72 h prior to H. pylori infection, and then the cells were incubated for 4 h with the WT strain for measurements of mRNA levels (C) and for 24 h for measurements of protein levels of RANTES (D). Three independent coculture experiments, each measured in duplicate, were performed. Since the data were parametric, we analyzed them using ANOVA pinpointed with the Student-Newman-Keuls test (means ± SE). *, P < 0.05 compared with the WT strain without inhibitors and with empty vector.
measure luciferase activity. The pGL2-220 plasmid contains five binding sites, for the transcription factors CRE, ISRE, NF-IL-6, NF-κB1, and NF-κB2 (Fig. 3A). The proximal NF-κB site was defined as NF-κB2, and the distal NF-κB site was defined as NF-κB1.

Deletions from nt −974 to −220 did not affect H. pylori-induced luciferase activity or basal activity (data not shown). As shown in Fig. 3B, deletion to nt −195 reduced wild-type H. pylori-induced luciferase activity (2.5-±0.1-fold) (P < 0.05 compared to a deletion from nt −220), indicating that the

FIG. 3. Schematic representation of RANTES promoter deletion constructs. (A) Locations of the putative binding sites for CRE, ISRE, NF–IL-6, and NF-κB are illustrated. Numbering is relative to the transcription initiation site. (B) Effect of 5′ deletions in the RANTES promoter sequence on H. pylori-inducible activity. (C) Effect of site mutations in the RANTES promoter sequence on H. pylori-inducible activity. MKN45 cells were transiently transfected with pGL2-974 and infected with the wild-type (WT) strain and its oipA mutants or cag PAI mutants for 6 h at an MOI of 100. Untreated plates served as controls. For each plate, the luciferase activity was normalized to Renilla luciferase vector DNA. Three independent coculture experiments, each measured in duplicate, were performed. When we used isogenic mutants, we picked two clones of each mutant. The data for the two clones were combined and analyzed. Since the data were parametric, we analyzed them using ANOVA pinpointed with the Student-Newman-Keuls test. Data are expressed as means ± SE of normalized luciferase activity. Numbers on error bars refer to fold increases in luciferase activity in H. pylori-infected cells relative to untreated controls. *, P < 0.05 compared with pGL2-220; #, P < 0.05 compared with pGL2-195; and †, P < 0.05 compared with pGL2-120.
CRE site was activated during wild-type *H. pylori* infection-related RANTES gene transcription. An additional 5' deletion to −150 nt did not affect the wild-type *H. pylori*-induced luciferase activity, whereas deletion to −120 nt further reduced the inducibility of the luciferase activity (2.0 ± 0.1-fold), indicating that the ISRE site is also activated during wild-type *H. pylori* infection-induced RANTES gene transcription. Deletion to −90 nt further reduced the wild-type *H. pylori*-induced luciferase activity (1.2 ± 0.04-fold), suggesting that the NF–IL-6 site was also activated by *H. pylori* infection. Since pGL2-90 contains two NF-κB sites, these results indicate that the NF-κB sites alone are insufficient to induce RANTES transcription following *H. pylori* infection.

For the *oipA* mutants, the luciferase activities were similar between pGL2-220 and pGL2-120 and with that of wild-type *H. pylori* with pGL-120, suggesting that OipA is involved in activation of the CRE and ISRE sites (Fig. 3B). The luciferase activity remained below a 1.5-fold increase, irrespective of the plasmid used, when infection was done with the *cag* PAI mutants (Fig. 3B) or the *oipA* *cag* PAI mutants (data not shown). For these experiments, we used two clones of each mutant. The data were similar between the clones (data not shown).

**Effects of site-directed mutations of the RANTES promoter sequence on *H. pylori*-inducible luciferase activity.** To determine the contributions of individual cis elements of the RANTES promoter in conferring responsiveness to *H. pylori* infection, we introduced site-directed mutations of the CRE, ISRE, NF–IL-6, and NF-κB sites and tested the mutant plasmids for *H. pylori* inducibility. Mutation of the NF-κB1 and NF-κB2 sites affected the basal activity (basal activity for uninfected control, 0.8 ± 0.1 and 1.7 ± 0.1, respectively) and reduced wild-type *H. pylori*-induced promoter activation (Fig. 3C), consistent with the NF-κB sites being activated during *H. pylori*-induced RANTES gene transcription.

In contrast, the 5' deletion assays showed that the NF-κB sites alone were insufficient to induce RANTES gene transcription. These results are in agreement with studies of the induction of RANTES gene transcription by respiratory syncytial virus infection in human type II alveolar epithelial cells (4) and are consistent with the notion that the NF-κB sites are necessary but not sufficient to induce RANTES gene transcription. Mutation of the CRE, ISRE, and NF–IL-6 sites also decreased wild-type *H. pylori*-induced luciferase activity, in agreement with the results of the 5' deletion assays. Overall, these data indicate that the full induction of RANTES gene transcription by wild-type *H. pylori* infection requires the CRE, ISRE, NF–IL-6, and NF-κB sites but that each transcription factor alone is not sufficient to induce RANTES gene transcription. With the *oipA* mutants and the *cag* PAI mutants, the luciferase activity was slightly suppressed by site-directed mutations of each site; however, the reduction was not statistically significant (Fig. 3C). The luciferase activity remained below a 1.2-fold induction, irrespective of the plasmid used, for stimulation with the *oipA* *cag* PAI mutants (data not shown). For these experiments, we used two clones of each mutant. The data were similar between the clones (data not shown).

**Role of CRE binding proteins in RANTES promoter inducibility following *H. pylori* infection.** Because luciferase reporter gene analysis of the promoter showed that the CRE, ISRE, NF–IL-6, and NF-κB sites were all important regulatory elements in *H. pylori* infection-induced RANTES gene transcription, we performed EMSA to determine whether *H. pylori* infection produced changes in the abundance of DNA binding proteins recognizing these regions of the RANTES promoter. The CRE sites can bind homo- and heterodimeric complexes formed by members of the CREB, ATF, Jun, and Fos transcription factor families. Two DNA binding complexes (C1 and C2) were markedly induced by infection with the wild-type strain (data for 2 h postinfection are shown in Fig. 4A). Induction was evident at 1 h postinfection, peaked at 2 to 4 h, and decreased in intensity by 8 h postinfection. In contrast, infection with both the *cag* PAI mutants and the *oipA* mutants resulted in reduced binding to this element, suggesting that both OipA and the *cag* PAI are involved in inducing binding to the RANTES CRE site. Binding to this element was not further reduced by infection with the *oipA* *cag* PAI double mutants and did not reach the levels in uninfected controls (data not shown), indicating that factors other than OipA and the *cag* PAI are involved in inducing binding to the RANTES CRE site.

The inducible complexes were sequence specific, as demon-
components of the RANTES CRE complex induced by *H. pylori* infection. Anti-c-Fos and anti-ATF-2 antibodies resulted in the appearance of a weak supershifted band, although the reduction of C1 was not clear, suggesting that these members of the CREB/ATF family are components of the *H. pylori*-inducible RANTES CRE complex.

**Role of ISRE binding proteins in RANTES promoter inducibility following *H. pylori* infection.** We also determined whether *H. pylori* infection produced changes in the abundance of DNA binding proteins recognizing the RANTES ISRE site. As shown in Fig. 4B, two nucleoprotein complexes were formed in control cells with the ISRE probe (C1 and C2). C2 was strongly induced following infection with the *H. pylori* wild-type strain. *H. pylori* infection caused increased C2 as early as 1 h postinfection, with a peak in binding intensity at 2 to 4 h postinfection (data not shown). In contrast, the *cag* PAI mutants or the *oipA* mutants suppressed C2 (Fig. 4B), suggesting that both OipA and the *cag* PAI are involved in inducing binding to the RANTES ISRE site. Binding to this element was further reduced, to the levels in uninfected control cells, by infection with the *oipA cag* PAI double mutants (data not shown).

The sequence specificity of the ISRE complexes was examined by competition with unlabeled oligonucleotides using EMSA (Fig. 5B). C2 was competed by the wild-type oligonucleotide but not by the mutated one, indicating binding specificity. Supershift assays showed that anti-interferon regulatory factor 1 (IRF-1) antibody resulted in the disappearance of C2, indicating that IRF-1 is a major component of the *H. pylori*-inducible complex.

**Role of NF–IL-6 binding proteins in RANTES promoter inducibility following *H. pylori* infection.** For the RANTES NF–IL-6 site, two nucleoprotein complexes (C1 and C2) were formed in control cells, while one complex (C1) was strongly induced following infection with the wild-type strain (Fig. 4C). *H. pylori* infection increased C1 as early as 1 h postinfection, with a peak in binding intensity at 2 to 4 h postinfection (data not shown). In contrast, the *cag* PAI mutants failed to induce binding, suggesting that the *cag* PAI is involved in inducing binding to the RANTES NF–IL-6 site. The *oipA* mutants appeared to slightly reduce binding; however, because it was unclear upon simple inspection whether a reduction occurred, we compared the amounts of radioactive probe in the protein-DNA complexes after standardization with a free probe. An approximately 25% reduction for the *oipA* mutants was consistently shown in four different experiments (data not shown), consistent with OipA having a partial role in the activation of the RANTES NF–IL-6 site.

The inducible complexes were sequence specific, as demonstrated by competition with an unlabeled wild-type, but not mutant, oligonucleotide (Fig. 5A). To determine the composition of the *H. pylori*-inducible complex, we performed supershift assays using a panel of antibodies broadly reacting with the different members of the CREB, ATF, and AP-1 families of transcription factors. Anti-CREB-1 also recognizes ATF-1 and CREB-1; anti-c-Fos recognizes c-Fos, Fos-B, Fra-1, and Fra-2; and anti-c-Jun recognizes c-Jun, Jun-B, and Jun-D. The anti-c-Jun antibody resulted in a reduction of both binding complexes and the appearance of a supershifted band (Fig. 5A), indicating that members of the Jun family are major
control cells with the NF-κB1 probe (Fig. 4D). Infection with wild-type \textit{H. pylori} resulted in a marked increase in binding of a new complex (C2) to NF-κB1. In contrast, both the \textit{cag} PAI mutants and the \textit{oipA} mutants (Fig. 4D) failed to induce C2, suggesting that both the \textit{cag} PAI and \textit{oipA} are involved in inducing binding to the NF-κB1 site. All three binding complexes were competed by the wild-type oligonucleotide but not by the mutated one, indicating binding specificity (Fig. 5D). Supershift assays showed that p50 and p65 are \textit{H. pylori}-inducible NF-κB complexes. The EMSA patterns with the NF-κB2 probe were similar to those with the NF-κB1 probe, although the \textit{oipA} mutants still induced small amounts of C2 (Fig. 4E). Supershift assays for NF-κB2 showed that p65-p65 homodimers formed C1, p50-p65 heterodimers formed C2, and p50-p50 homodimers formed C3.

**Effect of MAPK and NF-κB inhibitors on the activation of transcription factors in the RANTES promoter induced by \textit{H. pylori}**. To investigate which pathway was used by \textit{H. pylori} to activate each transcription factor, we added different inhibitors (SB203580, p38 inhibitor; U0126, MEK1/2 inhibitor; SP600125, JNK inhibitor; and MG-132, NF-κB inhibitor) to MKN45 cells prior to infection with the \textit{H. pylori} wild-type strain and then measured the binding activity of each transcription factor by using EMSA. MKN45 cells preincubated with each inhibitor (10 μM) for 30 min were infected with the wild-type strain for 2 h, and then nuclear proteins were extracted. The binding complex of CRE was slightly inhibited by all three MAPK inhibitors (approximately 35% reduction by U0126 and SP600125 and approximately 20% reduction by SB203580) (Fig. 6A). When we added three MAPK inhibitors together, the binding complex was strongly inhibited, with levels similar to those in unininfected controls (data not shown). The binding complexes of ISRE were strongly inhibited by SB203580 and MG-132, but not by U0126 or SP600125 (Fig. 6B). The binding complexes of NF-κB2 were weakly inhibited by U0126 but were not inhibited by SP600125 (Fig. 6C). As expected, the binding complex of C2 (NF-κB1 and NF-κB2 was inhibited by SP600125 and MG-132, whereas SB203580 and U0126 did not have a significant effect on NF-κB2 binding (Fig. 6D and E).

**Western blot analysis of p38**. Since inhibitor experiments showed that the p38 MAPK pathway plays important roles in RANTES gene transcription, Western blot analysis was performed to assess the phosphorylation of p38. Since we previously reported Western blot analyses of the JNK and ERK MAPK pathways as well as the NF-κB pathway (IκB) in MKN45 cells (38), we focused on p38 in this study. The wild-type strain and the \textit{cag} PAI mutants induced p38 phosphorylation in a time-dependent manner, with similar peak levels of p38 phosphorylation (at 2 h postinfection) (Fig. 7). In contrast, the peak levels were very low with the \textit{oipA} mutants. These findings suggest that the p38 pathway is dependent on OipA but is independent of the \textit{cag} PAI.

**In vitro–in vivo correlation of RANTES mRNA levels in gastric antral mucosa**. To determine whether the in vitro data indicating that RANTES mRNA levels were related to both the \textit{cag} PAI and OipA status reflected in vivo events in the gastric mucosa, we examined antral gastric biopsy specimens from 20 patients infected with \textit{cag} PAI-positive/OipA-positive isolates, 6 patients infected with \textit{cag} PAI-negative/OipA-positive isolates, 5 patients infected with \textit{cag} PAI-positive/OipA-negative isolates, 10 patients infected with \textit{cag} PAI-negative/OipA-negative isolates, and 20 patients without \textit{H. pylori} infection. Antral RANTES mRNA levels were significantly higher in specimens infected with the \textit{cag} PAI-positive/OipA-positive isolates (38.4 [27.8, 44.8]) than in the control [bottom panel] antibodies). \textit{H. pylori} was co-cultured with MKN45 cells for 120 min, and total protein was extracted and used for Western blotting. Three independent coculture experiments were performed, and typical results are shown. When we used isogenic mutants, we picked one clone of each mutant.

![Effect of inhibitors on activation of RANTES promoter](http://iai.asm.org/)

**FIG. 6.** Effect of inhibitors on activation of RANTES promoter region induced by \textit{H. pylori}. Nuclear extracts were prepared from control and wild-type (WT) \textit{H. pylori}-infected MKN45 cells, with or without a treatment with inhibitors (10 μM for each), and used for EMSA. Nuclear extracts were used to bind to a CRE probe (A), ISRE probe (B), NF-κB probe (C), NF-κB1 probe (D), or NF-κB2 probe (E). SB, SB203580; SP, SP600125. Three independent coculture experiments were performed, and typical results are shown.

**FIG. 7.** Western blot analysis of p38 (with phospho-specific [top panel] and control [bottom panel] antibodies). \textit{H. pylori} was co-cultured with MKN45 cells for 120 min, and total protein was extracted and used for Western blotting. Three independent coculture experiments were performed, and typical results are shown. When we used isogenic mutants, we picked one clone of each mutant.
Previously described for virus-mediated RANTES promoter activation in fibroblastic or myeloid cells (10).

Inhibitor studies showed that the JNK inhibitor suppressed the activation of the NF-κB sites, indicating the existence of cross talk between the JNK pathway and the NF-κB pathway. There have been several reports that NF-κB can inhibit JNK signaling induced by tumor necrosis factor (6, 24), and the activation of NF-κB induced by IL-1β has also been reported to be inhibited by a JNK inhibitor in pulmonary epithelial cells (27). We previously reported that the cag PAI, but not OipA, was involved in the activation of the JNK pathway (38). Overall, the data are consistent with the cag PAI/OipA→NF-κB and cag PAI→JNK→NF-κB pathways being involved in the activation of the NF-κB sites in the RANTES promoter (Fig. 9).

We also found that both the cag PAI and OipA were involved in binding to the CRE site in the RANTES promoter. H. pylori-inducible proteins that bound to the CRE site belonged to the c-Fos, ATF, and Jun families of transcription factors. c-Fos, ATF-2, and c-Jun are thought to be involved in signaling of the ERK, p38, and JNK pathways, respectively (9, 20). This is in agreement with our EMSA data for MAPK inhibitors showing that all three MAPK pathways were involved in protein binding to the CRE site. In the present study, we found that OipA, but not the cag PAI, was involved in the activation of the p38 pathway in MKN45 cells. Therefore, we hypothesize that the cag PAI and OipA induce RANTES via different pathways upstream of CRE. It appears likely that the cag PAI→JNK→c-Jun→CRE pathway and the OipA→p38→ATF-2→CRE pathway are involved in activation of the CRE site in the RANTES promoter (Fig. 9).

The relationship between the ERK pathway and the cag PAI in gastric epithelial cells remains unsettled, as several studies have reported that the cag PAI is involved in ERK phosphorylation (12, 13, 19), whereas other studies have not supported this relationship (25, 32, 33, 38). In our previous study, neither the cag PAI nor OipA was involved in the phosphorylation of ERK in MKN45 cells (38). Importantly, we found that CRE binding was not completely abrogated by the cag PAI oipA double mutants, suggesting that factors other than the cag PAI and OipA are at least partially involved in the activation of CRE via the ERK→c-Fos pathway (Fig. 9).

We found that both the cag PAI and OipA were involved in binding to the ISRE site in the RANTES promoter. Supershift assays showed that IRF-1 is a component of the H. pylori-inducible complex formed on the RANTES ISRE site. These findings are in agreement with previous studies showing that ISRE complexes containing IRF-1 have been shown to play a role in the induction of RANTES in virus-infected lung epithelial cells (5), IL-1-stimulated astrocytoma cells (21), and IL-1/β interferon-stimulated primary astrocytes (15). Inhibitor experiments showed that the binding complexes of ISRE were strongly inhibited by the NF-κB inhibitor, which is in agreement with the fact that the IRF-1 promoter contains an NF-κB site. The IRF-1 promoter also contains binding sites for signal transducers and activators of transcription 1 (STAT1). Inhibitor experiments showed that the binding complexes of ISRE were strongly inhibited by the p38 inhibitor and OipA but that the cag PAI was not involved in activation of the p38 pathway in MKN45 cells. The p38 pathway is known to be involved in STAT1 phosphorylation (17). Overall, the data...
suggest that the cag PAI/OipA→NF-κB→IRF-1→ISRE pathway and the OipA→p38→IRF-1→ISRE pathway are involved in activation of the ISRE site in the RANTES promoter (Fig. 9). In contrast to the case for CRE, double mutation of the cag PAI and oipA genes completely abolished the activation of the ISRE site, suggesting that the combination of the cag PAI and OipA is sufficient for full activation of the ISRE site.

The cag PAI and OipA were also involved in activation of the NF–IL-6 site in the RANTES promoter. This result contrasts with previous studies using the same cell line that showed that the binding site for NF–IL-6 was not necessary for H. pylori-induced activation of the IL-8 promoter (1, 29, 38). These data are consistent with the notion that H. pylori infections result in the stimulation of transcription factors which differentially affect different promoter regions. We found that the NF–IL-6 site binds H. pylori-inducible proteins belonging to the C/EBP family of transcription factors, which are involved in the regulation of immune functions and tumorigenesis and are regulated through the MAPK and NF–κB pathways (18, 22, 29). In fact, inhibitor experiments showed that the binding complexes of NF–IL-6 were inhibited by the p38 inhibitor and the NF–κB inhibitor. Therefore, we conclude that the OipA→p38→NF–IL-6 pathway and the cag PAI/OipA→NF–κB→NF–IL-6 pathway are involved in activation of the NF–IL-6 site in the RANTES promoter (Fig. 9). The reduction of NF–IL-6 binding by the oipA mutants was less dramatic than that with the cag PAI mutants (Fig. 4). However, the reduction of NF–IL-6 binding was completely abrogated by the p38 inhibitor (Fig. 6), and the p38 pathway was activated by OipA, but not by the cag PAI. One explanation for these findings could be that cag PAI-dependent and OipA-dependent pathways have cross talk in the upstream signaling pathways leading to RANTES gene transcription.

Finally, the results of the in vivo study were mostly consistent with the results in vitro in that the gastric antral mucosal RANTES mRNA levels were highest in antral mucosa of individuals infected with cag PAI-positive/OipA-positive strains, followed by those with cag PAI-negative/OipA-positive strains and then those with cag PAI-positive/OipA-negative strains. RANTES mRNA was not induced in the gastric mucosa of individuals infected with cag PAI-negative/OipA-negative strains, which was different from our in vitro data showing that even cag PAI-negative/OipA-negative strains induced small amounts of RANTES mRNA in gastric epithelial cells. Gastric biopsy specimens contain many nonepithelial cells, including macrophages, and RANTES induction patterns in these cells might be different from those in epithelial cells. In addition, the in vitro-in vivo correlations should be interpreted with some caution, as only one gastric epithelial cancer cell line was used in the in vitro studies. However, it is clear that the cag PAI-dependent and OipA-dependent pathways appear to interact and that the combination of the cag PAI and OipA is involved in RANTES gene expression in the gastric mucosa.

ACKNOWLEDGMENTS

This work was supported in part by National Institutes of Health grants RO1 DK62813 (to Y.Y.) and K08 AI01763 (to A. C.), by Pilot and Feasibility (P/F) awards, by funds from Texas Gulf Coast Digestive


