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Quantitative Detection of *Helicobacter pylori* Gene Expression In Vivo and Relationship to Gastric Pathology

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The *iceA* locus of *Helicobacter pylori* includes one of two mutually exclusive gene families, *iceA1* and *iceA2*. Colonization with *iceA1* strains is associated with enhanced acute mucosal inflammation, and adherence to gastric epithelial cells in vitro induces expression of *iceA1* but not *iceA2* mRNA; however, both transcripts can be detected in vivo. The aim of this study was to determine whether differing levels of *iceA* transcription in vivo may contribute to disease pathogenesis. RNA from 41 *H. pylori*-positive gastric biopsy specimens was reverse transcribed to cDNA. Quantitative PCR was performed using biotinylated iceA1, iceA2, and 16S rRNA primers, and binding of biotinylated products to streptavidin-coated plates was detected by hybridization with a fluorescein-labeled probe. *iceA* genotypes were determined by PCR and sequence analysis. All 41 samples contained detectable *H. pylori* 16S rRNA, with similar levels in *iceA1*- ($n = 10$) and *iceA2* ($n = 31$)-colonized patients ($P = 0.34$). Biopsy specimens from four (40%) and 19 (61%) persons colonized with *iceA1* or *iceA2* strains, respectively, had detectable *iceA* RNA. Acute inflammatory scores were significantly higher in *iceA1* RNA-positive patients than in *iceA1* RNA-negative, *iceA2* RNA-positive, or *iceA2* RNA-negative subjects ($P \leq 0.05$ for each). Within the *iceA2* RNA-positive group, *H. pylori* strains with a single 35-amino-acid cassette were associated with significantly higher mucosal *iceA2* transcript levels ($P = 0.014$ versus strains with two cassettes). These results indicate that the levels of transcription of *H. pylori iceA1* and *iceA2* and of 16S rRNA are independent and that particular *iceA2* gene structures are associated with enhanced transcription. The finding that *iceA1* transcription levels are significantly associated with the intensity of neutrophilic infiltration suggests that heterogeneity in inflammatory scores among persons colonized with *H. pylori iceA1* strains reflects levels of *iceA1* transcription in vivo.

Helicobacter pylori induces gastric inflammation in virtually all colonized individuals, and such gastritis increases the risk for peptic ulcer disease and distal gastric adenocarcinoma (8, 14, 15, 21, 23, 27). However, only a minority of persons carrying *H. pylori* develop clinical sequelae, suggesting that particular bacterial products may contribute to pathogenesis (4). The first strain-specific gene identified in *H. pylori* was *cagA*, a component of the *cag* pathogenicity island (1, 7, 9, 30); persons colonized with *H. pylori cagA*-positive strains are at increased risk for developing peptic ulceration and distal gastric cancer compared to persons harboring *cagA*-negative strains (5, 10–12, 22, 24, 29). The gene *vacA* represents a second *H. pylori* locus of heterogeneity, and strains that possess a *vacA* s1 signal sequence allele are also associated with peptic ulcer disease (3, 32). However, the majority of persons colonized with *cagA*$^+$ *vacA* s1 strains remain asymptomatic, suggesting that additional *H. pylori* genes may also be important in disease pathogenesis. Recently, *iceA* (a designation derived from the phrase "induced by contact with epithelium") was identified in *H. pylori* following transcriptional up-regulation on contact with gastric epithelial cells (26). *iceA* exists as two distinct genotypes, *iceA1* and *iceA2*, and only *iceA1* RNA is induced following adherence in vitro (26). The deduced *H. pylori iceA1* product (Fig. 1A) demonstrates strong homology to a restriction endonuclease, *Nla*III, in *Neisseria lactamica* (19); however, mutations and deletions found in the majority of *iceA1* sequences preclude translation of a full-length homolog. In vivo, carriage of *H. pylori iceA1* strains has been found in some (20, 26, 32) but not all (18, 36) studies to be associated with peptic ulceration and enhanced acute neutrophilic infiltration. However, substantial heterogeneity among gastric inflammatory scores exists within *iceA1*-colonized populations (20, 26).

In contrast to *iceA1*, *iceA2* has no homology to known proteins, and its structure reveals patterns of repeated peptide cassettes (Fig. 1B). In its most common form, *iceA2* can encode a protein of 59 amino acids (aa) with two conserved outer domains of 14 and 10 aa, respectively, that flank three internal peptide domains of 13, 16, and 6 aa, respectively (16). Sequence analysis of several *H. pylori iceA2* strains shows that the internal 35-aa cassette (composed of the 13-, 16-, and 6-aa domains) may be absent or repeated up to three times, resulting in deduced proteins of 24, 59, 94, or 129 aa (Fig. 1B) (16). Although substantial differences exist between the *iceA1* and *iceA2* sequences, both genes are transcribed in vivo (26), leading to the hypothesis that levels of *iceA* transcription within the host environment may contribute to disease development. Therefore, the aims of this study were to quantitate specific *iceA1* and *iceA2* transcription levels in gastric mucosa and to determine whether such levels were related to bacterial numbers in the same biopsy specimen, *iceA* sequence heterogeneity, gastric inflammation, peptic ulcer disease, and the presence of other *H. pylori* genotypes.

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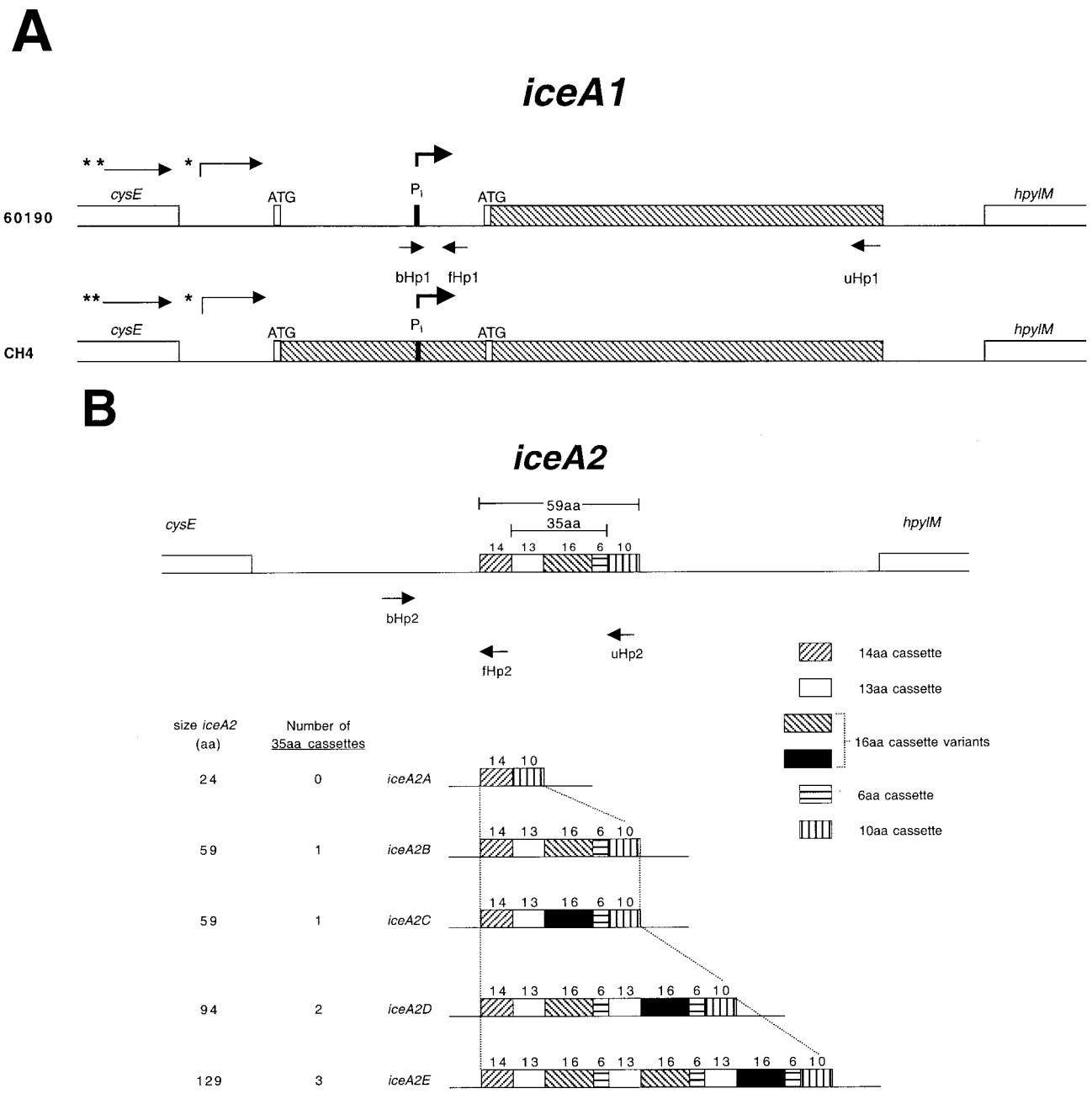

FIG. 1. Schematic representation of the genetic organization of *iceA1* (A) and *iceA2* (B) and the flanking genes, *cysE* and *hpyIM*. (A) The hatched regions represent continuous ORFs homologous to *nlaIIIR*. The top schematic represents *cysE*, prototype *iceA1*, and *hpyIM* from *H. pylori* strain 60190, and the bottom schematic is a variant from strain CH4 containing an ORF homologous to the complete *N. lactamica nlaIIIR* ORF. Two potential ATG start sites are shown, as is the major transcriptional start site P_1 (large arrows) recently identified in strain 60190 (13). The presence of minor transcripts originating in the intergenic region between the *cysE* and *iceA1* ORFs is indicated by the small arrows with single asterisks. Read-through transcription from *cysE* into *iceA1* is indicated by the small arrows with two asterisks. The size of the intergenic region between the end of the *cysE* ORF and the first *iceA1* ATG codon is 84 nucleotides for strain 60190 and 25 nucleotides for strain CH4. The positions of PCR primers are indicated by arrows below the 60190 schematic. (B) The top schematic represents *cysE*, prototype *iceA2*, and *hpyIM* from *H. pylori* strain J178, encoding a protein of 59 aa. The diagrams below represent subsequently identified *iceA2* variants (16). Each of the five *iceA2* peptide motifs, of 14, 13, 16, 6, and 10 aa, respectively, is represented by a box. The existence of two distinct 16-aa domains is indicated by different patterns. The positions of PCR primers are indicated by arrows. The total number of amino acids in each *iceA2* ORF is shown for each variant.

MATERIALS AND METHODS

Clinical specimens. Patients at the Nashville Department of Veterans Affairs Medical Center Gastroenterology Clinic were prospectively enrolled in this study after giving written informed consent. The study was approved by the Vanderbilt University and Nashville Department of Veterans Affairs Institutional Review Boards. Exclusion criteria included the following: use of nonsteroidal medications; history of gastric surgery; malignancy; infection with human immunodeficiency virus; positivity for hepatitis B surface antigen; active gastrointestinal bleeding; use of steroids or other immunosuppressive drugs, antibiotics, or bismuth compounds before endoscopy; and detection of *H. pylori* by histology but not culture. Patients with peptic ulcer disease were included in the study, and ulceration was defined as a mucosal break at least 1.0 cm in diameter, with depth and covered by exudate, noted at the study endoscopy. Five gastric biopsy specimens were taken from the greater curvature of the antrum, between 2 and

TABLE 1. Oligonucleotide primers used in this study

Gene	Primer	Modification	Sequence (5′ to 3′)	Strand
iceA1	bHp1	Biotinylated	GTGTTTTTAACCAAAGTATC	+
iceA1	uHp1		CTATAGCCATTATCTTTGCA	−
iceA1	fHp1	Fluorescein	TGCTTATAGTGGTTTTTAATGTC	−
iceA2	bHp2	Biotinylated	GTTGGGTATATCACAATTTAT	+
iceA2	uHp2		TTACCCTATTTTCTAGTAGGT	−
iceA2	fHp2	Fluorescein	GTATTTTGCCATTAACGACTTT	−
16S rRNA	bHp16s	Biotinylated	GCTAAGAGATCAGCCTATGTCC	+
16S rRNA	uHp16s		TGGCAATCAGCGTCAGGTAATG	−
16S rRNA	fHp16s	Fluorescein	CCTACCTCTCCCACACTCTA	−

5 cm from the pylorus, and distributed as follows: culture ($n = 2$), histological examination ($n = 2$), and reverse transcriptase (RT) PCR ($n = 1$). Biopsy specimens for RNA isolation were immediately frozen at −70°C. To isolate *H. pylori*, gastric biopsy specimens were immediately placed in normal saline at 4°C and homogenized in 250 μl of normal saline with a tissue grinder (Micro Kontes, Vineland, N.J.). Fifty microliters was plated onto Trypticase soy agar with 5% sheep blood (BBL) and incubated for 96 h under microaerobic conditions, as described previously (25). The *iceA*, *cagA*, and *vacA* genotypes were determined by PCR of *H. pylori* genomic DNA, as previously reported (26).

Quantitation of *H. pylori* gene expression in vivo. Individual gastric biopsy specimens were placed in sterile vials (Nunc, Roskilde, Denmark) and stored at −70°C until they were used for RNA preparation for RT-PCR. Total biopsy specimen RNA was isolated (25) and treated with DNase (1 U/μg), and 1 μg of the RNA was reverse transcribed to cDNA using random hexamers as primers (Pharmacia LKB Biotech, Piscataway, N.J.) as previously described (25). *H. pylori iceA1* (bHP1 and uHP1 [Table 1]), *iceA2* (bHP2 and uHP2 [Table 1]), and 16S rRNA (bHP16S and uHP16S [Table 1]) primers were designed based on known *H. pylori* genomic sequences (26). *iceA1* genes demonstrate considerable strain variation, with insertions, deletions, and potential differences in transcription start sites (16). These sequence variants could affect the ability to detect *iceA1* transcripts in gastric mucosa if they compromise hybridization of the oligonucleotides used either for amplification or detection of the amplified products. To assure that transcripts from all potential sequence variants would be detected, the *iceA1* amplification primers (bHP1 and uHP1 [Table 1]) and the fluorescein-conjugated detection oligonucleotide (fHP1 [Table 1]) were designed to hybridize to conserved regions downstream of the most 3′ transcription start site in the *iceA1* gene (P_1 [Fig. 1A]) (16). *iceA2* genes also show extensive strain variation in sequence. In contrast to *iceA1*, *iceA2* sequences contain variable numbers (0, 1, 2, or 3) of a 35-aa cassette that possesses one of two distinct 16-aa domains (Fig. 1B). Since all *iceA2* sequences determined to date contain a conserved 14-aa domain (Fig. 1B), the *iceA2* fluorescein-conjugated detection oligonucleotide (fHP2 [Fig. 1B and Table 1]) was designed to hybridize within this motif region (Fig. 1B and Table 1).

The upstream (forward) oligonucleotide of each primer pair was biotinylated (Genosys Biotechnologies, Inc., The Woodlands, Tex.). PCR mixtures included 40 ng of cDNA in 50 μl with 2.5 mM $MgCl_2$ and 0.4 μM concentrations of the primers (forward, 0.2 μM biotinylated and 0.2 μM unlabeled; reverse, 0.4 μM unlabeled) with amplification for 40 cycles (94°C, 1 min; 52°C [55°C for 16S rRNA], 1 min; 72°C, 2 min) and 7 min of extension at 72°C. Biotinylated amplimers were then added to streptavidin-coated 96-well microtiter plates in triplicate and denatured, and the resultant single-stranded streptavidin-bound PCR products were hybridized for 15 min at 50°C with an internal fluorescein-conjugated oligonucleotide probe specific to the gene of interest (Table 1) (2, 6). The fluorescein-labeled probes were then detected with an anti-fluorescein alkaline phosphatase-conjugated antibody followed by the addition of chemiluminescent substrate (Tropix, Bedford, Mass.), and light emission was quantitated using a luminometer. As a positive control for both PCR primer and internal oligonucleotide hybridizations, genomic DNA isolated from each *H. pylori* strain grown in vitro was used for PCR amplification. Genomic DNA served as the positive control, and RNA that had not been reverse transcribed was the negative control for each PCR. Prototype *iceA1* and *iceA2* sequences from *H. pylori* strains 60190 and J178, respectively, and 16S rRNA from strain 60190 each were subcloned into pT7Blue (Novagen, Madison, Wis.), as previously described (26). Known amounts of plasmid template DNAs were then used to construct standard curves for quantitative PCR.

Histology. Sections of biopsy specimens were examined without knowledge of the experimental PCR results by one experienced histopathologist (K.T.T.). Histologic parameters were scored from 0 to 3 as outlined in the Sydney System and included assessment of the degree of activity (neutrophil infiltration), lymphocyte and plasma cell infiltration, epithelial degeneration, mucus depletion, epithelial erosion, glandular atrophy, intestinal metaplasia, and colonization density, as previously described (26). Modified Giemsa stain was used for identification of *H. pylori* (17).

Sequence analysis. Sequence analysis was performed on genomic DNA from 38 of the 41 *H. pylori* strains isolated (7 *iceA1* and 31 *iceA2*) using an ALF-Express automated sequencer and DNAMAN PC Gene Software (Lynn on Biosoft).

Statistics. Mean 16S rRNA transcript levels, and inflammation and injury scores were compared between groups using the Mann-Whitney U rank sum test. Differences in *H. pylori* strain-specific genotypes (*cagA* and *vacA*) and the prevalence of peptic ulcer disease were compared among groups using the Mantel-Haenszel test. A P value of <0.05 was defined as significant.

RESULTS

Quantitation of *iceA1* and *iceA2* transcripts in vitro. Substantial heterogeneity exists among inflammatory scores within *iceA1*-colonized populations (26), raising the possibility that levels of *iceA* transcription within the host environment may contribute to such differences or to disease development. Therefore, we developed a quantitative RT-PCR assay to measure RNA transcripts for *iceA1*, *iceA2*, and *H. pylori* 16S rRNA in gastric biopsy specimens. We first determined the sensitivity of the quantitative PCR assay for detection of *H. pylori iceA* sequences by amplifying serial dilutions of plasmid DNA containing the *iceA1* or *iceA2* inserts from *H. pylori* strains 60190 (*cag*$^+$ *vacA* s1a/m1 [where s1a is the signal sequence allele and m1 is the midregion allele] *iceA1*) and J178 (*cag*$^+$ *vacA* s1a/m1 *iceA2*) (26), respectively, with a lower limit of detection by PCR for both *iceA1* and *iceA2* of 10^{-22} mol of plasmid DNA (Fig. 2A). Preliminary studies of eight gastric biopsy specimens demonstrated that all reverse-transcribed samples with detectable *iceA* cDNA gave amplification within this linear range. Samples that had not been reverse transcribed showed no amplification above negative controls, indicating the absence of detection of contaminating DNA. There is extensive sequence variation in *iceA1* and *iceA2* genes. Therefore, we next established that the primers used to amplify and detect the prototype sequences (Fig. 2A) would detect all sequence variants in the 41 isolated strains and that these primers were specific. Accordingly, quantitative PCR was performed using 10 ng of genomic DNA from each of the 41 isolates (Fig. 2B). The mean relative light units for each *iceA1* or *iceA2* DNA sample tested were >1,000-fold higher than baseline in the respective *iceA1* or *iceA2* PCRs (Fig. 2B). These results demonstrated that the reagents could detect transcripts of all *iceA1* and *iceA2* sequence variants if they were expressed in these biopsy specimens. In addition, the primers used demonstrated absolute specificity for the appropriate genotypes, as no amplification above background was seen with the reciprocal gene.

Detection of *iceA* transcripts in gastric biopsy specimens. Of the 41 patients studied, all were positive for *H. pylori* by both serology and culture. Of these, 10 were colonized with *iceA1* strains and 31 harbored *iceA2* strains (Table 2). Fifteen (37%) of the 41 patients had duodenal ulcer disease; no gastric ulcers were identified (Table 2). Quantitative RT-PCR to measure *iceA* RNA expression in gastric tissue revealed that 4 (40%) of

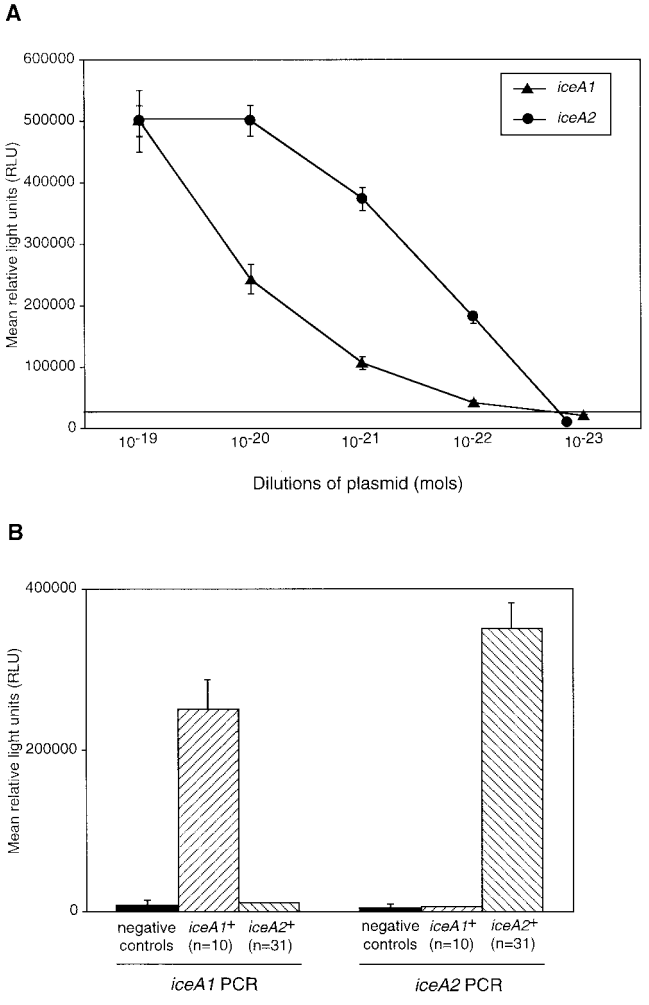

FIG. 2. Sensitivity (A) and specificity (B) of quantitative *iceA1* and *iceA2* PCR on DNA. (A) Dilutions of plasmid containing either 60190 *iceA1* or J178 *iceA2* DNA were used as templates for PCR with *iceA1*- or *iceA2*-specific oligonucleotide primers (Table 1). The results shown (mean ± SD) are for dilutions corresponding to plasmid DNA from 10^{-19} to 10^{-23} mol from a representative standard curve. A line representing mean relative light unit values for negative controls with no template DNA which were run concomitantly is shown for reference. The limit of detection for *iceA1* and *iceA2* by this assay was 10^{-22} mol of plasmid DNA. (B) Genomic DNA (10 ng) from each of the 41 *H. pylori* strains was used as a template for quantitative PCR with biotinylated *iceA1*- and *iceA2*-specific primers (Table 1). Streptavidin-bound PCR products were hybridized with an allele-specific internal fluorescein-conjugated probe (Table 1), and chemiluminescence was quantitated using a luminometer. Mean relative light units + SD for *iceA* PCRs are shown for *iceA1* and *iceA2* strains and for negative controls (solid columns) run concomitantly and consisting of reactions with no template DNA.

10 *iceA1*- and 19 (61%) of 31 *iceA2*-colonized persons had detectable *iceA* RNA. The patients were stratified into the following four groups: *iceA1* RNA positive, *iceA2* RNA positive, *iceA1* RNA negative, and *iceA2* RNA negative. To confirm that detection of *iceA1* and *iceA2* expression in vivo reflected the genotype of the colonizing strain, the results of PCR using bacterial genomic DNA as a template were compared to RT-PCR results on biopsy specimen cDNA. In all 23 *iceA* RNA-positive tissue samples, the biopsy specimen *iceA* status corresponded to the *iceA* genotype of the strain isolated ($P < 0.0001$), demonstrating the high specificity of these assays.

TABLE 2. Patient and strain characteristics

Characteristic	No. of patients	
	iceA1	*iceA2*
Total no. of patients ($n = 41$)	10	31
Clinical characteristics		
Duodenal ulcer disease[a] ($n = 15$)	6	9
Nonulcer disease ($n = 26$)	4	22
Esophagitis ($n = 9$)	2	7
Barrett's esophagus ($n = 3$)	0	3
Gastric adenocarcinoma ($n = 1$)	0	1
Gastric erythema alone ($n = 13$)	2	11
Strain characteristic ($n = 41$)		
cagA positive ($n = 24$)	8	16
cagA negative ($n = 17$)	2	15
vacA s1a ($n = 5$)	3	2
vacA s1b ($n = 18$)	6	12
vacA s2 ($n = 18$)	1	17
Ulcer-derived strains ($n = 15$)	6	9
cagA positive ($n = 12$)	6	6
cagA negative ($n = 3$)	0	3
vacA s1a ($n = 4$)	2	2
vacA s1b ($n = 7$)	4	3
vacA s2 ($n = 4$)	0	4
Non-ulcer-derived strains ($n = 26$)	4	22
cagA positive ($n = 12$)	2	10
cagA negative ($n = 14$)	2	12
vacA s1a ($n = 1$)	1	0
vacA s1b ($n = 11$)	2	9
vacA s2 ($n = 14$)	1	13

[a] Duodenal ulcer disease was defined as a circumscribed break in the mucosa with a diameter of at least 1.0 cm with apparent depth and covered by an exudate noted at endoscopic examination.

Quantitation of constitutively expressed *H. pylori* 16S rRNA transcripts in gastric tissue. *H. pylori iceA* expression in vivo may be dependent upon local host conditions, such as pH, osmolarity, or nutrient concentrations, or, as found in vitro, bacterial growth phase (13). Alternatively, the amount of *iceA* mRNA may simply reflect bacterial colonization density. Therefore, to determine whether bacterial density correlated with levels of mucosal *iceA* RNA in the same tissue specimen, we also quantitated levels of the constitutively expressed 16S rRNA. All 41 gastric biopsy samples contained detectable *H. pylori* 16S rRNA, although considerable variation was observed (Fig. 3). *H. pylori* 16S rRNA transcript levels were similar among persons colonized by *iceA1* strains and persons colonized with *iceA2* strains ($P = 0.34$). When patients were stratified on the basis of *iceA* expression in vivo, mucosal levels of 16S rRNA were not significantly different of *iceA1* RNA-positive and *iceA1* RNA-negative patients ($P = 0.1$ [Fig. 3]). *H. pylori* 16S rRNA transcript levels among *iceA2* RNA-positive persons were higher ($P = 0.008$) than those of *iceA2* RNA-negative persons (Fig. 3). However, among the *iceA2* RNA-positive group, levels of *iceA2* RNA did not correlate with the 16S rRNA levels from the same patients ($P = 0.6$). In addition, there were no significant associations found between the number of organisms quantified histologically in adjacent antral biopsies and *iceA1* or *iceA2* expression levels ($P = 0.25$ and 0.41, respectively) (data not shown). Since the differences in 16S rRNA expression are quite small, these results suggest that the presence of *iceA* transcripts does not simply reflect bacterial load.

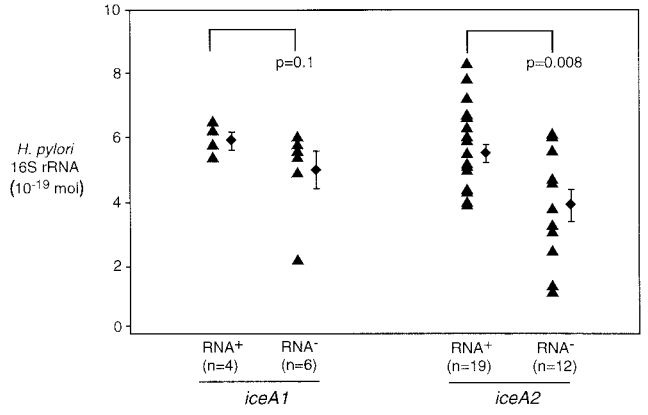

FIG. 3. Relationship of *H. pylori* 16S rRNA levels and detectable expression of *iceA1* or *iceA2* RNA in gastric tissue from 41 *H. pylori*$^+$ persons. Mean values (◆) ± SD are shown adjacent to the data points (▲). Among persons carrying *iceA2* strains, 16S cDNA levels were significantly higher in *iceA2* RNA-positive than in RNA-negative persons (5.4×10^{-19} versus 3.7×10^{-19} mol.; $P = 0.008$); however, 16S cDNA levels were not significantly different among persons carrying *iceA1* strains stratified on the basis of *iceA1* expression in vivo ($P = 0.1$). *P* values were determined by the Mann-Whitney U test.

Relationship of *iceA* expression in vivo to histologic parameters of inflammation and injury. Substantial variability among inflammatory scores exists within *iceA1*- and *iceA2*-colonized patients (26). This heterogeneity prompted us to investigate whether levels of *iceA* mRNA expression in vivo may be associated with the degree of histopathological change. Acute inflammatory scores were significantly higher in *iceA1* RNA-positive patients (2.3 ± 0.3 [mean ± standard deviation {SD}]) than in *iceA1* RNA-negative (0.6 ± 0.3), *iceA2* RNA-positive (0.7 ± 0.1), or *iceA2* RNA-negative (1.2 ± 0.3) subjects (P = 0.01, 0.002, and 0.03, respectively) (Fig. 4). However, the presence of detectable *iceA* transcripts was not related to other parameters of histopathology. Since *iceA1* has been associated with the presence of *cagA* and *vacA* s1 alleles, as well as peptic ulcer disease (26, 32), we also examined the relationship between *iceA* expression and other genetic and clinical markers of *H. pylori* virulence. Mucosal expression of *iceA1* and *iceA2* was not related to *cagA* or *vacA* genotypes or duodenal ulcer disease (data not shown), suggesting that expression of *iceA1* is associated with neutrophilic infiltration independent of the presence of other *H. pylori* virulence-related genes.

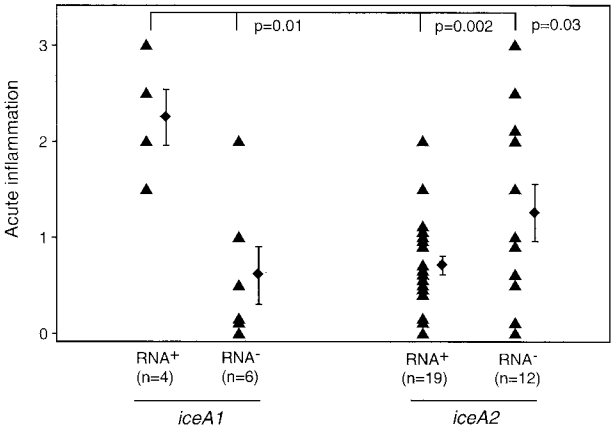

FIG. 4. Relationship between acute-inflammatory scores and expression of *iceA1* or *iceA2* RNA detected in gastric mucosa from 41 *H. pylori*$^+$ persons. Acute inflammation was scored from 0 to 3 by a single pathologist blinded to mucosal levels of *iceA* RNA. Mean values (◆) ± SD are shown adjacent to the data points (▲). Each data point reflects averaged scores from two antral biopsy specimens (one data point per patient). Biopsies from four (40%) of 10 *iceA1*- and 19 (61%) of 31 *iceA2*-colonized persons had detectable mucosal *iceA* RNA. Acute inflammatory scores were significantly higher in *iceA1* RNA-positive patients compared to all other groups. Statistical significance was determined by the Mann-Whitney U test.

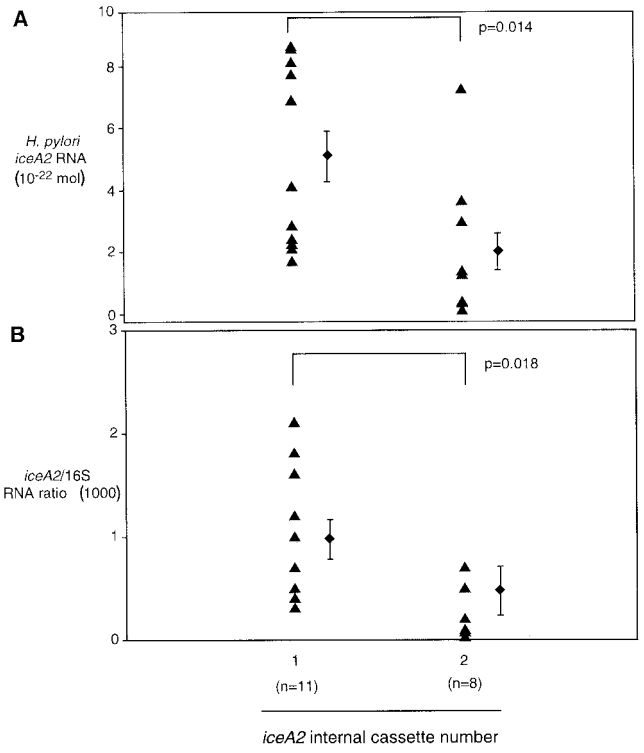

FIG. 5. Relationship of *iceA2* gene structure and levels of *iceA2* expression in vivo. (A) Among patients harboring *iceA2* strains, 19 had detectable levels of *iceA2* transcripts. *iceA2* from corresponding *H. pylori* genomic DNA was sequenced using an ALF-Express automated sequencer. Mean values (◆) ± SD are shown adjacent to the data points (▲). (B) The *iceA2*/16S rRNA ratio was determined by dividing *iceA2* transcript levels by the corresponding 16S rRNA levels. Statistical significance was determined by the Mann-Whitney U test.

***iceA2* gene structures correlate with *iceA2* expression in vivo.** We next sought to determine whether sequence heterogeneity within *iceA1* and *iceA2* might contribute to variation in mucosal *iceA* expression. Among seven *iceA1* sequences, only one contained a continuous open reading frame (ORF) homologous to the *Nla*III full-length ORF and which might be predicted to encode a functional restriction endonuclease (16). Gastric tissue from the patient colonized with this strain did not show *iceA1* RNA expression. Among the four *iceA1* RNA-positive samples, there were no variations in the corresponding *iceA1* DNA sequences that were consistently associated with differences in mucosal *iceA1* RNA (data not shown). Among the 31 *iceA2* strains, 21 possessed a single 35-aa internal cassette while 10 harbored two cassettes. Among the 19 *iceA2* RNA-positive samples, expression levels were higher among persons colonized with strains possessing one versus two internal 35-aa cassettes (Fig. 5A). To evaluate the relationship between cassette structure and *iceA2* and 16S rRNA expression in each biopsy specimen, we also derived an *iceA2*/16S rRNA ratio and compared these ratios with *iceA2* cassette status among the 19 *iceA2* RNA-positive strains. Consistent with the results for *iceA2* RNA alone, *iceA2*/16S rRNA transcription ratios were significantly higher among strains contain-

ing one versus two cassettes ($P = 0.018$) (Fig. 5B). These results suggest that particular *iceA2* gene structures, such as the presence of a single 35-aa internal cassette, are associated with enhanced *iceA2* transcription and that this difference is not dependent upon variations in bacterial density.

DISCUSSION

H. pylori iceA1 strains have been associated with duodenal ulcer disease and enhanced neutrophilic infiltration of the gastric mucosa, yet considerable heterogeneity exists in the levels of inflammation among persons harboring *iceA1* strains (20, 26, 32). Although *iceA1* mRNA expression is induced following adherence to gastric epithelial cells in vitro, this model may differ from events occurring within the gastric mucosa, since it does not include potential contributions by other cells or soluble mediators to bacterial gene expression. *H. pylori iceA2* strains have not been linked with disease outcome, but *iceA2* mRNA has been detected within gastric tissue (26). To explore the hypothesis that the amount of *iceA* expression within the host environment, and not *iceA* genotype per se, may contribute to increased pathology, we quantitated *iceA* expression within gastric tissue and related this to the clinical and histologic outcome, the presence of additional *H. pylori* virulence-associated genes, and *iceA* gene structure. The results showed that, in a limited number of biopsy samples, expression of *iceA1* but not *iceA2* RNA was significantly related to the severity of acute mucosal inflammation. Although *iceA* expression and inflammation were quantified in different tissue samples, these biopsy specimens were harvested from the same antral site during endoscopy to more completely insure that comparisons between these variables were valid. In our population, *iceA1* genotypes are present in approximately 20% of colonized persons, in contrast to recent reports from Europe (32) and East Asia (18, 20), where *iceA1* is the predominant genotype. Since we detected transcripts in 40% of *iceA1*-infected biopsy specimens, only 10% of our total population would be expected to be classified as *iceA1* RNA positive. Of interest, a recent report from Japan (20) has similarly demonstrated that although *H. pylori iceA1* strains are associated with more severe gastritis, heterogeneity exists among inflammatory scores. We therefore look forward to the possibility of having our current results tested in a geographically distinct host population.

We found that in approximately one-half of the patients, *iceA* transcripts could be detected in vivo. One explanation for failure to detect *iceA* transcription may be that sequence variation inherent to *iceA1* or *iceA2* reduced oligonucleotide hybridization. However, amplification of *iceA1* and *iceA2* was successful using DNA from every strain as templates for the PCRs (Fig. 2B). Another possibility is that variation in *H. pylori* colonization densities affected the ability to detect *iceA* mRNA. The fact that there were no substantial differences in the amount of constitutively expressed 16S rRNA between biopsies with and without *iceA* transcripts, though, likely reflects similar *H. pylori* colonization levels. Finally, it is possible that some patients were colonized with multiple *H. pylori* strains, which could have contributed to the present findings. However, as we have previously reported, this appears to be an uncommon event in our patient population (33). Collectively, these data suggest that strain differences in *iceA* expression do not simply reflect bacterial load and that expression of these genes is regulated rather than constitutive. Factors that may contribute to regulation of *iceA* expression include differences in the gastric environment between colonized hosts and intrinsic strain differences, such as the structures of the genes themselves or activation of transcription factors necessary for their expression.

H. pylori iceA1 has significant homology to *nlaIIIR*, which encodes the restriction endonuclease *Nla*III in *N. lactamica* (19), and is adjacent to *hpyIM*, the *H. pylori* homolog of the cognate *N. lactamica* DNA methyltransferase M.*Nla*III. These data suggest that *iceA1* may be part of a DNA restriction-modification system (26, 34), and data for one strain (CH4) possessing such a full-length ORF demonstrate that it has functional restriction endonuclease activity (16). There are several putative mechanisms by which restriction-modification systems may enhance virulence. For example, DNA ends generated by restriction may stimulate recombination events, and for organisms that colonize potentially hostile niches, such gene exchange may be more important than de novo mutations for maintenance of long-term infection. DNA methylation could also regulate expression of *H. pylori* genes that are required for inducing gastric mucosal inflammation. Recent studies suggest that *iceA1* and *iceA2* strains differ in the level of expression of *hpyIM*, the DNA methyltransferase immediately downstream of *iceA* (Q. Xu, J. P. Donahue, and M. J. Blaser, submitted for publication). Finally, *iceA1* may be directly involved in induction of gastritis, although recent data from our laboratory have demonstrated that inactivation of *iceA1* does not alter interleukin-8 production by gastric epithelial cells in vitro (R. M. Peek, Jr., unpublished data). Although this does not preclude a direct role for *iceA1* in the establishment of gastritis, definitive evidence will require experimental challenge with wild-type and isogenic mutant strains in animal models of *H. pylori*-induced gastric inflammation, and these experiments are under way in our laboratory.

Although *iceA1* may encode a functional restriction endonuclease, the majority of *iceA1* sequences examined to date contain nucleotide insertions and/or deletions that would preclude translation of a full-length protein homologous to *Nla*III (16). The size of the intergenic region between the end of the *cysE* ORF and the first *iceA1* ATG codon (Fig. 1A) also varies, depending on the strain (16); for example, in 60190, the distance is 84 nucleotides, while in CH4, the distance is 25 nucleotides. In addition to sequence variation, different *iceA* transcriptional initiation sites exist among *H. pylori* strains. Northern blot analysis of *iceA1* transcripts in strains 60190 and CH4 indicated that the P_1 promoter shown in Fig. 1A is the major site of transcriptional initiation. However, in strain 60190, primer extension data have also identified the 5′ terminus of an *iceA* transcript in the intergenic region between the *cysE* and *iceA1* ORFs, and RT-PCR results demonstrate readthrough transcription from *cysE* into *iceA1* (13). For strain CH4, which contains a continuous ORF homologous to the *N. lactamica nlaIIIR* ORF, we have demonstrated IceA1 restriction endonuclease activity in cell extracts (16). This indicates that transcripts initiated within the *cysE-iceA1* intergenic region or upstream within *cysE* (possibly at the *cysE* promoter) must also be present in strain CH4. The P_1 promoter resembles an *Escherichia coli* σ^{70} promoter, with nearly consensus -10 and -35 sequences; however, sequences upstream of the putative transcriptional initiation site in the *cysE-iceA1* intergenic region do not resemble consensus sequences shown to be recognized by any of the sigma factor homologs known to be present in *H. pylori* (σ^{80}, σ^{28}, and σ^{54}).

Although our *iceA1* strain population was limited in number ($n = 10$), we did not detect any molecular *iceA1* variant that was consistently associated with expression in vivo, and no expression was observed for a strain with a full-length *Nla*III-like ORF. These results suggest that additional factors, such as adherence, rather than gene structure may more directly reg-

ulate *iceA1* expression. *H. pylori iceA1* was originally identified by its up-regulation following contact with gastric epithelial cells in vitro (26), and thus one physiological event that could potentially regulate *iceA1* expression in vivo is binding to gastric epithelium. Since interaction with host epithelial cells is a stimulus for virulence-associated gene expression for other mucosal pathogens, such as *Yersinia pseudotuberculosis* and uropathogenic *E. coli* (28, 37), contact with gastric epithelial cells also may be a paradigm for induction of *H. pylori* genes linked with pathological outcomes, such as severity of acute inflammation.

In contrast to *iceA1*, our results indicate that strain differences in gene structure may be more important for *iceA2* expression, since higher levels were seen for genes possessing one rather than two 35-aa cassettes. Differences in gene expression related to gene structure have been described for *H. pylori vacA* and *cagA*, which demonstrate either a mosaic cassette structure (3, 31) or variations in 3′ repeat regions (35), respectively. The genetic structure of the *H. pylori iceA2* locus differs considerably from that of *iceA1* in that *iceA2* contains variable numbers of highly conserved peptide repeat regions (Fig. 1B). The five distinct *iceA2* variants that contain zero, one (two forms), two, or three copies of the internal 35-aa cassette have each been isolated from various regions of the world, indicating that the different *iceA2* variants are highly conserved (16). Examination of the overall genetic architecture of the *iceA2* locus has revealed strong (>90%) homology in the nucleotide sequences encoding the 14- and 10-aa external motifs (16). These findings lead to the hypothesis that these external motifs could facilitate recombination between *iceA2* genes containing varying copies of the 35-aa cassette, which could result in differing levels of *iceA2* transcription.

In summary, our experiments demonstrate that RT-PCR is an effective approach for quantitation of both constitutively and conditionally expressed *H. pylori* genes within gastric mucosa. Using this technique, we have demonstrated that *iceA1* expression is significantly related to the host mucosal response while *iceA2* expression may be more highly influenced by gene structure. An additional advantage of this technique is that generation of random-hexamer-primed cDNA provides the ability to quantitate expression of multiple prokaryotic and eukaryotic genes within the same tissue sample and to relate levels of transcription to inherent pathology.

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