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cDNA Array Analysis of *cag* Pathogenicity Island-Associated *Helicobacter pylori* Epithelial Cell Response Genes

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Helicobacter pylori **strains containing the *cag* pathogenicity island (PAI) induce NF-κB activation and interleukin-8 secretion in gastric epithelial cells. The aim of this study was to investigate changes in epithelial gene expression induced by *cag* PAI-positive and -negative strains of *H. pylori* using high-density cDNA array hybridization technology. Radio-labeled cDNA prepared from *H. pylori*-infected Kato 3 gastric epithelial cells was hybridized to high-density cDNA arrays to identify changes in epithelial gene expression compared to noninfected controls. In vivo expression of selected, differentially expressed genes was examined by reverse transcription-PCR analysis of *H. pylori*-positive and -negative gastric mucosa. Screening of ca. 57,800 cDNAs identified 208 known genes and 48 novel genes and/or expressed sequence tags of unknown function to be differentially expressed in Kato 3 cells following *H. pylori* infection. Marked differences in gene expression profiles were observed following *cag* PAI-positive and *cag* PAI-negative infection with 15 novel cDNAs and 92 known genes being differentially expressed. *H. pylori* was found to change the expression of genes encoding growth factors and cytokine/chemokines and their receptors, apoptosis proteins, transcription factors and metalloprotease-disintegrin proteins (ADAMs), and tissue inhibitors of metalloproteinases. Gastric differential expression of selected known genes (amphiregulin and ADAM 10) and a novel gene (*HPYR1*) was confirmed in vivo in patients with *H. pylori* infection. Confirmation of the in vivo expression of selected genes demonstrates the usefulness of this approach for investigating pathogen-induced changes in host gene expression.**

The gastric pathogen *Helicobacter pylori*, which adheres closely to the luminal surface of the human gastric epithelium in vivo (27), is the causative agent of active chronic gastritis and a predisposing factor for the development of both peptic ulcer disease and gastric cancer (14, 70). Molecular genetic analysis of *H. pylori* has shown that approximately 50 to 60% of strains have a 40-kb DNA segment called the *cag* pathogenicity island (PAI), which encodes a multicomponent type IV secretion system (2, 9). Whilst there is marked global variation in the frequency of *cag*$^+$ *H. pylori* strains (71), in many populations, infection with *cag*$^+$ strains has been associated with increased risk of severe gastritis (15, 52, 62, 76), peptic ulceration (12, 13, 15, 72, 76), atrophic gastritis (33, 71), and distal gastric cancer of the intestinal type (7, 51, 63, 67).

The enhanced inflammatory response induced by *cag*$^+$ *H. pylori* strains is thought to have a key role in disease pathogenesis. In vivo gastric mucosal C-X-C chemokines are increased in those infected with *cag*$^+$ strains (52, 62, 76, 77), and a major source of neutrophil chemoattractant chemokines such as interleukin-8 (IL-8) is the gastric epithelium (16). In vitro studies modelling bacterial-epithelial interactions have shown that *cag*$^+$, but not strains lacking the *cag* PAI, induce IL-8 in gastric epithelial cells (17, 18, 60). Induction of this epithelial response, which involves mitogen-activated protein (MAP) kinase (32) and NF-κB activation (1, 31, 45, 61), is dependent on multiple genes throughout the *cag* PAI (29, 34). The *cag* PAI is also essential for the translocation of the bacterial protein CagA into gastric epithelial cells (4, 66) where it becomes tyrosine phosphorylated and induces cytoskeletal changes in epithelial cells (3, 4, 50, 59, 66).

The integral role of the epithelium in mucosal defense has become increasingly appreciated (30). Whilst several studies have focused on *cag* PAI-dependent differential expression of chemokines in gastric epithelial cells, the effects of *H. pylori* on the expression of other epithelial genes have not been investigated in detail. Secreted products such as chemokines, intracellular proteins, and immunologically relevant membrane proteins may all be differentially expressed in epithelial cells after microbial exposure (30). Recent studies show that even commensal intestinal bacterial flora modulate epithelial gene expression (8).

Several approaches have been adopted to detect differentially expressed genes, including subtractive hybridization (64), differential display (36, 65), serial analysis of gene expression (69), and, more recently, cDNA arrays (10, 20, 22, 26). The advantages of cDNA array technology are that it allows simultaneous expression analysis of thousands of genes to be monitored in parallel and permits identification of quantitative differences in expression of both genes of known function and novel genes and/or expressed sequence tags (ESTs) of unknown function. To date few studies have used this approach to investigate bacterially induced changes in host gene expression (21, 29). In this study the heterogeneity in gene expression profiles of a gastric cancer epithelial cell line infected with a wild-type *cag* PAI-positive *H. pylori* strain and a wild-type strain lacking the *cag* PAI has been examined by differential screening of cDNA arrays. The aims of the study were to

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identify differences in epithelial gene expression of possible relevance to the enhanced virulence of *cag* PAI-positive strains and to identify novel, differentially expressed genes of potential importance in the pathology of *H. pylori*-related disease.

MATERIALS AND METHODS

Bacteria and cell culture. Wild-type *H. pylori* strains (*cag* PAI-positive NCTC 11637 and G50, which lacks the *cag* PAI [12]) were cultured on blood agar base number 2 (Oxoid, Basingstoke, Hampshire, United Kingdom) incorporating 7% fresh horse blood under microaerobic conditions at 37°C. Prior to use the bacteria were harvested on day 3 into RPMI 1640 medium (Gibco Life Technologies, Paisley, United Kingdom) supplemented with 10% heat-inactivated fetal calf serum (Sera Lab, Crawley, Surrey, United Kingdom) and 2 mM glutamine (Life Technologies) and were used immediately.

Kato 3 gastric epithelial cells (European Collection of Animal Cell Cultures) were routinely maintained in RPMI 1640 medium containing 10% fetal calf serum, 2 mM glutamine, and 40 μg of gentamicin/ml in 5% CO_2 at 37°C. For coculture experiments Kato 3 cells were resuspended in antibiotic-free medium and cultured at 2×10^5/ml in 24-well tissue culture plates (Corning Costar, High Wycombe, Bucks, United Kingdom) with or without wild-type *H. pylori* strains NCTC 11637 (*cag* PAI positive) and G50, which lacks the *cag* PAI (12) at a bacterium-to-cell ratio of 50:1 as previously described (17, 18). In contrast to NCTC 11637, G50 does not stimulate IL-8 gene transcription (34) or IL-8 protein secretion (17, 18) in gastric epithelial cells. At 45 min, 3 h, and 24 h postculture, mRNA was immediately extracted from the epithelial cells using the direct mini message maker kit (R & D Systems, Abingdon, United Kingdom) and stored at −70°C. IL-8 concentrations in 24-h culture supernatants of control and *H. pylori*-infected cells were assayed by enzyme-linked immunosorbent assay as previously described (17, 18) to ensure the characteristic secretion of IL-8 in cells challenged with *cag* PAI-positive NCTC 11637. mRNA from seven independent experiments was pooled for probe preparation to minimize possible interexperimental variation.

cDNA arrays. cDNA arrays utilized were (i) a rearrayed Integrated Molecular Analysis of Genomes and Their Expression (I.M.A.G.E.) library (46,302 clones) obtained from the I.M.A.G.E. consortium (Washington University–Merck Pharm) in collaboration with the Human Genome Mapping Project, Hinxton, United Kingdom, (ii) an array containing oligodT)-primed standard spleen cDNA library clones (10, 752 clones) (Life Technologies), (iii) a custom array designed to represent 136 inflammatory genes (Glaxo Wellcome, Stevenage, Hertfordshire, United Kingdom), and (iv) a Clontech Atlas Human cDNA Expression Array 1 (588 clones). For preparation of the arrays (i to iii), each cDNA was amplified by PCR using vector-specific primers and arrayed in duplicate onto positively charged nylon membranes (Boehringer Mannheim, Lewes, East Sussex, United Kingdom) by use of a robot (Q bot) (Genetix Ltd., Christchurch, Dorset, United Kingdom). An array of control housekeeping genes, including glyceraldehyde-3-phosphate dehydrogenase (GAPDH), elongation factor 3, ribosomal L3, and β-actin in a range of dilutions, was used as normalizing genes. The intensity of signal from the control gene arrays was used to measure the relative specific activity of probes.

The prepared cDNA arrays were initially screened with an oligonucleotide probe to assess the quantity of PCR product gridded at each position on the array. The M13F oligonucleotide dCGCCAGGGTTTTCCCAAGTCACGAC (Promega, Southampton, United Kingdom) was used for the rearrayed human I.M.A.G.E. library and inflammatory-gene array, and the SP6 oligonucleotide dTATTTAGGTGACACTATAG (Promega) was used to screen the human spleen library. The oligonucleotide annealing site vector-specfic sequence was situated within the PCR-priming sites of cDNA PCR products. The oligonucleotides were end labeled using 5 μl of 10× polynucleotide kinase buffer (700 mM Tris-HCl buffer, pH 7.6, containing 100 mM $MgCl_2$ and 50 mM dithiothreitol) (New England Biolabs, Hitchin, Herts, United Kingdom), 1 μl of oligonucleotide (20 pmol/μl), 5 μl of [α-^{33}P]dATP (3,000 Ci/mmol) (Amersham, Little Chalfont Bucks, United Kingdom), 2 μl of polynucleotide kinase (10 U/μl), and 37 μl of distilled water at 37°C for 1 h. Probes were purified using Sephadex G-25 columns, and the level of incorporation of label was measured as described above.

Prior to hybridization the arrays were preincubated in digoxigenin Easy Hyb (Boehringer Mannheim) at 45°C subsequent to hybridization for 16 h with the oligonucleotide probes (see above) at 45°C. Posthybridization arrays were washed with 6× SSC (0.9 M NaCl and 0.09 M sodium citrate containing 0.5% [wt/vol] sodium dodecyl sulfate) (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) at 45°C. The phosphorimages of the hybridized arrays were captured using a Storm scanner 860 (Molecular Dynamics, Chesham, Bucks, United Kingdom).

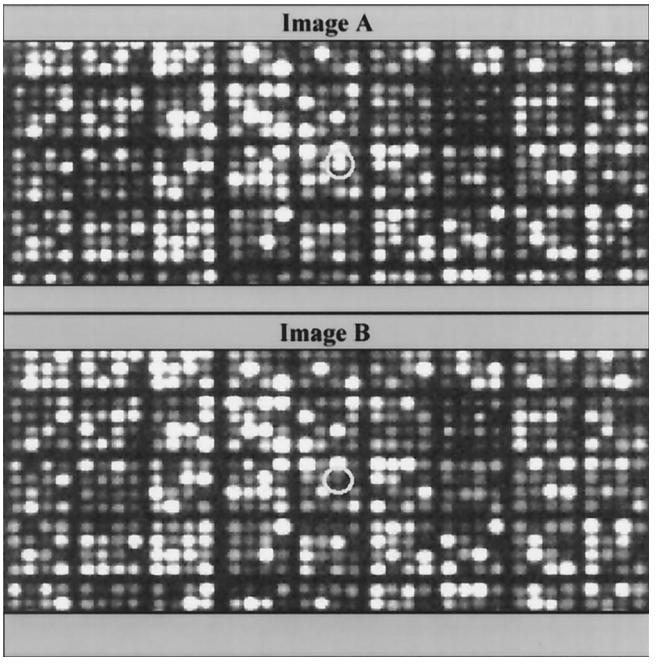

FIG. 1. Example of I.M.A.G.E. cDNA array hybridized with epithelial cell-derived probe from *H. pylori*-infected Kato 3 gastric epithelial cells (A) and a probe derived from uninfected Kato 3 cells (B). The circles highlight a differentially expressed gene.

Epithelial probe preparation and hybridization. Epithelial mRNA was quantified using a UNICAM UV-visible light scanning spectrophotometer, and the integrity was assessed electrophoretically prior to radiolabeling. mRNA (1 to 2 μg) was incubated with 1 μl of $d(T)_{15}V$(A, C, G)N(A, G, C, T) (1.44 μg/μl) at 70°C for 10 min prior to the addition of 4 μl of 5× reaction buffer (Life Technologies) 2 μl of 0.1 M dithiothreitol, 1 μl of deoxynucleoside triphosphate mix (1 mM dATP, dGTP, and dTTP and 0.01 mM dCTP), 1 μl of RNasin (40 U/μl) (Boehringer Mannheim), and 4 μl of [α-^{33}P]dCTP (3,000 Ci/nmol) (Amersham). The mixture was incubated at 42°C for 1 min prior to addition of 2 μl of Superscript II (200 U/μl) (Life Technologies) and further incubation for 90 min. Probes were purified by passage through Sephadex G-50 columns (Pharmacia Biotech, St. Albans, Herts, United Kingdom). The incorporation rate of label was measured using a Bioscan QC-4000 (Bioscan, Washington, D.C.), and the size range of the probe was estimated electrophoretically using Tris-borate-EDTA-urea–6% polyacrylamide gel electrophoresis with radioactive size standards.

The arrays were prehybridized as described above for the oligonucleotide probe hybridizations. ^{33}P-radiolabeled, single-stranded cDNA probes (50 μl) were equalized on counts (counts per minute) and were quenched with 5 μl of $poly(A)_{80}$ (1 μg/μl), 10 μl of human Cot-1 DNA (1 μg/μl), and 435 μl of digoxigenin Easy Hyb (10 min, 100°C; and 90 min, 45°C) before hybridization to the cDNA arrays and control grids at 45°C for 3 days. Following stringent posthybridization washing in 0.1× SSC containing 0.1% Sodium dodecyl sulfate at 68°C, the phosphorimages of the hybridized arrays were scanned as done before.

Image analysis. Phosphorimages were edited using the ImageQuant package (Molecular Dynamics) and were processed using DGEnt PC software (Glaxo-Wellcome) whereby a specific intensity value was determined and assigned for each spot on an array and corresponding control genes. Each array was processed twice to provide data for PCR product loading (oligonucleotide hybridizations) and the epithelially derived sample probe hybridization for each spot. Comparisons of gene expression profiles were undertaken using the DGEnt PC software (Fig. 1). This package incorporates data from the edited ImageQuant images and allows comparison of two arrays probed with different epithelially derived samples. The comparison data were adjusted to compensate for differing probe-specific activity using control genes which included β-actin, elongation factor 3, ribosomal L3, and GAPDH. The Clontech Atlas arrays included their own set of control genes, which were used to standardize the arrays relative to probe strength.

TABLE 1. Primer sequences for RT-PCRs

Primer	Primer sequence	Product size (bp)
Amphiregulin-F	dTTCTGCATTCACGGAGAATGC	390
Amphiregulin-R	dTGATCCACTGGAAAGAGGACC	
HPYR1-F	dGTAGGATCTTGTTTTGCTGC	319
HPYR1-R	dCTACAGCTCTGTTGGTTACC	
ADAM 10-F	dCTTCCTAGTGCCTACAATGG	300
ADAM 10-R	dCCTGCAAGTGAAGAAAATGC	
GAPDH-F	dGAGTCAACGGATTTGGTCGT	158
GAPDH-R	dGGTGCCATGGAATTTGCCAT	
ureA	dGCCAATGGTAAATTGTT	411
	dCTCCTTAATTGTTTTTAC	
cagA	dGATAACGCTGTCGCTTCATACG	409
	dCTGCAAAAGATTGTTTGGCAGA	

Secondary hybridizations of cDNA putatively differentially expressed from initial screen. Clones from the I.M.A.G.E. and splenic libraries considered to be carrying potential differentially expressed cDNAs from the first hybridizations were repicked. Their inserts were amplified by PCR and arrayed as described above. The oligonucleotide and epithelial probe hybridizations were performed as before with the same batch of mRNA, and each cDNA was analyzed to verify the initial changes in gene expression. Repicked cDNA clones were sequenced using a BigDye Terminator sequencing kit (Applied Biosystems Inc., Warrington, Cheshire, United Kingdom) and analyzed on ABI 377 sequencers (Applied Biosystems Inc.).

Confirmation of differential expression of genes in vitro and in vivo. Further analysis of selected differentially expressed genes was undertaken in Kato 3 and AGS gastric epithelial cells (European Collection of Animal Cell Cultures) following stimulation with *cag* PAI$^+$ G27 *H. pylori* and H12-5A, a *cagM* isogenic mutant strain (kindly provided by A. Covacci, Chiron Vaccines, Siena, Italy) which lacks the ability to induce IL-8 in Kato 3 cells (9). Total RNA was extracted at various times poststimulation using Catrimox-14 (VH Bio Ltd., Newcastle upon Tyne, United Kingdom). Following reverse transcription (RT), expression of genes of interest was analyzed by PCR as previously described (65).

The expression of genes of interest was examined in gastric biopsies of patients undergoing routine upper gastrointestinal endoscopy. All subjects provided informed consent, and the study was approved by the local research ethics committee. Total RNA was extracted from endoscopic gastric biopsies using Catrimox-14 (VH Bio Ltd.) and was treated with DNase I (Gibco Life Technologies). Purified RNA was reverse transcribed in 20 μl of solution using 0.5 μg of Random Primers (Promega) as previously described (28, 62). Expression of genes of interest was examined by semiquantitative RT-PCR using primer sequences described in Table 1. The quantity of PCR product for each gene examined was compared to the control gene GAPDH by densitometry using a UVP gel documentation system and GelBase software (GDS 5000; Ultra Violet Products, San Gabriel, Calif.) as previously described (25). PCR was also performed with a sample of the original RNA to confirm the absence of genomic DNA. The *H. pylori* status and *cagA* and *ureA* status of gastric biopsies were determined by biopsy urease test, histology, and RT-PCR as previously described (62, 69).

Statistical analysis. Statistical analysis was undertaken using the two-tailed Fisher exact test and Mann-Whitney U test. A P of less than 0.05 was considered statistically significant.

RESULTS

Quality control evaluation of high-density cDNA arrays. The cDNA arrays were screened initially with M13 and SP6 oligonucleotides in order to estimate the levels of individual PCR products loaded in each spot of the I.M.A.G.E. and spleen and inflammatory-cDNA arrays. A measure of the DNA loading of each spot on the array is essential prior to comparison of signals produced following hybridization with the epithelial cell-derived cDNA probes (see below).

Screening of cDNA arrays. cDNA probes prepared from mRNA extracted from Kato 3 epithelial cells following exposure to NCTC 11637 and G50 for 45 min, 3 h, and 24 h, and uninfected control Kato 3 cells (45 min, 3 h, and 24 h) were hybridized to three I.M.A.G.E. cDNA arrays, one splenic cDNA array, and one inflammatory-cDNA array, comprising 46,302, 10,752, and 136 cDNAs, respectively. A Clontech Atlas array (588 cDNAs) was also included for analysis using the 3-h and 24-h radiolabeled probes. In total, 57,778 cDNAs were screened. Following normalization against the housekeeping genes, the cDNA spot intensity values for each array were compared for the different time points. Each potential differentially expressed cDNA identified with a 1.1-fold alteration in intensity or higher in the initial screen was selected for rescreening using these criteria. Spleen clones (n = 466) and I.M.A.G.E. clones (n = 652) which potentially represented differentially expressed cDNAs were rearrayed and hybridized with cDNA probes prepared as for the first screen using the same mRNA batch. The rescreening was performed to minimize potential errors resulting from labeling, PCR, arraying, and data interpretation. Differential hybridization of 624 cDNAs was confirmed.

Characterization of differentially expressed genes found using cDNA arrays. The level of IL-8 expression in the inflammatory-cDNA arrays was examined initially, as IL-8 is known from previous studies (17, 18, 60) to be upregulated during *H. pylori* infection. Characteristically, IL-8 expression at 3 h was found to be 4.2-fold higher for Kato 3 cells infected with *cag* PAI-positive *H. pylori* than for cells infected with *cag* PAI-negative *H. pylori*. This confirmed that the array system and the manner in which the data had been normalized had produced the expected results for IL-8. We proceeded therefore to perform a large-scale analysis of the remainder of the differentially expressed genes.

The intrinsic redundancy of the splenic cDNA array also provided further confidence in the identification of differentially expressed genes, as several instances of detection of the same differentially hybridizing gene from independent cDNA spots were encountered (e.g., eukaryotic translation elongation factor 1 alpha 1 gene [EEF1A1] and tumor-associated protein [p23]). Other genes, such as ferritin light chain and α-tubulin, were identified as being differentially hybridized from both the I.M.A.G.E. and spleen libraries.

Analysis of all arrays demonstrated that the interaction of either *H. pylori* strain with Kato 3 epithelial cells in comparison to noninfected control cells resulted in the upregulation of 100 known genes and 34 genes of unknown function (including ESTs) and downregulation of 108 known and 12 novel genes in the epithelial cells. Furthermore, comparative analysis of gene expression between *cag* PAI-positive and *cag* PAI-negative infected Kato 3 epithelial cells indicated that 91 known genes and 15 novel cDNAs were differentially expressed by 1.3-fold or greater. Novel genes demonstrated differential expression levels ranging from 1.3 to 29. Following *cag* PAI-negative infection, a higher number of both known and novel cDNAs were found to be upregulated than following *cag* PAI-positive infection. Screening the I.M.A.G.E. (Table 2), splenic (Table 3), inflammatory- (Table 4), and Clontech Atlas (Table 5)

TABLE 2. I.M.A.G.E. array[a]

Time (category)[b]	Encoded protein	Gene accession no.	Fold difference
0.75 h (I)	AAC-11 (apoptosis inhibitor)	U83857	12.8
	Protein disulfide isomerase related-protein P5	D49489	7.2
0.75 h (D)	α-NAC	X80909	3.7
	HnRNP A1 isoform	M84334	2.3
	p23 tumor-associated protein	Z46805	2.2
	Poly(A) binding protein	Y00345	2.2
	TRA-1 (tumor rejection antigen)	X15187	2.2
	Elongin B	L42856	2.0
	L apoferritin	X03742	1.8
	Sm protein G	X85373	1.9
	α-Tubulin	K00558	1.6
	Ribosomal protein L28	U14969	1.7
	Guanine nucleotide binding protein G, α subunit	M14631	1.7
3 h (I)	Poly(A) binding protein	Y00345	2.1
3 h (D)	AAC-11 (apoptosis inhibitor)	U83857	5.1
24 h (D)	Transcription factor BTF3	P20290	8.8
	HSP90	D87666	6.2
	Thymosin β4	M17733	5.5
	VDUP-1	S73591	3.5
	NADH-ubiquinone	U94586	3.0
	Iduroate sulfate	L35485	2.9
	L apoferritin	X03742	2.8
	Integral membrane protein E16	M80244	2.7
	β-Tubulin	V00599	2.3
	Sm protein G	X85373	2.3
	A52 extension protein	S79522	2.1
	Guanine nucleotide binding protein G, α subunit	M14631	2.0
	AAC-11 (apoptosis inhibitor)	U83857	1.7

[a] Gene transcripts in Kato 3 gastric epithelial cells that demonstrate differential expression at 45 min and 3 and 24 h after culture with *H. pylori cag* PAI$^+$ NCTC 11637 and G50, which lacks the *cag* PAI.
[b] I, NCTC 11637 > G50; D, NCTC 11637 < G50.

cDNA arrays identified differences in expression of known genes following culture with *cag* PAI-positive and *cag* PAI-negative *H. pylori* ranging from 61.5 to 1.3.

Clontech Atlas and inflammatory-cDNA arrays. The Clontech Atlas arrays were included in the hybridizations with probes made from mRNA extracted from cells infected with *cag* PAI-positive and *cag* PAI-negative *H. pylori* at 3 and 24 h. The 3-h results revealed increased expression in 13 genes and decreased expression of 47 genes with *cag* PAI-positive-infected cells compared to results for *cag* PAI-negative-infected cells. At 24 h, 15 genes were found to be upregulated by the *cag* PAI-positive *H. pylori* strains compared to unstimulated control cells and 50 genes were found to be downregulated (data not shown).

The inflammatory-cDNA arrays which were included in every hybridization contained many cytokines, cytokine receptors, and inflammatory mediators. Examples of genes expressed in uninfected Kato 3 gastric epithelial cells and their relative intensity values are described in Table 6. Levels of expression ranged from 29 (oncostatin M) to 641 (tissue inhibitor of metalloproteinase 1 [TIMP-1]) relative grey level units. Probes made from *H. pylori*-infected Kato 3 cells hybridized to several other genes not expressed in unstimulated Kato 3 cells, including CD55, CD68, CD138, JAG1, IL-17, IL-3 α and β receptors, IL-4 receptor, IL-9 receptor, IL-13 receptor, BAX, BCL2, VEGF, and several members of the ADAMs (a disintegrin and metalloprotease) family. Compared to uninfected controls, 32 genes were upregulated and 25 genes were downregulated by *cag* PAI-positive *H. pylori* infection over the time course. Similar analysis following *cag* PAI-negative *H. pylori* infection demonstrated that 24 genes were upregulated and that 17 genes were downregulated throughout the time course. Thirty-one genes were more highly expressed and 22 genes were less expressed by *cag* PAI-positive infection than by *cag* PAI-negative infection (Table 4).

In vivo verification of differentially expressed genes. The expression of three genes found to be upregulated in gastric epithelial cells following infection with *H. pylori* was further examined in gastric biopsies of patients with and without *H. pylori* infection by semiquantitative RT-PCR. In the antral mucosa, increased mRNA transcripts for ADAM 10 (Fig. 2A), amphiregulin (Fig. 2B), and a novel gene in the I.M.A.G.E. library upregulated at 0.75 h following *cag* PAI-positive infection (*H. pylori* responsive 1 gene *HPYR1*, accession no. AF200341) (Fig. 2C) were present in *H. pylori*-infected patients but were infrequently observed in *H. pylori*-negative patients. *HPYR1* transcripts were observed in the antral mucosa of 42% of patients with *cagA*-positive infection ($n = 24$) but in only 15% of patients infected with *cagA*-negative strains ($n = 13$) and 16% of uninfected subjects ($n = 19$). In contrast, ADAM 10 mRNA expression was observed significantly ($P < 0.05$) more frequently in those with *cagA*-negative *H. pylori* infection ($n = 14$) than in uninfected patients ($n = 19$) or those with *cagA*-positive infection ($n = 28$) (Table 7). Amphiregulin mRNA expression in antral ($n = 59$) and corpus ($n = 39$) biopsies was significantly increased ($P < 0.05$) in the antrum but not in the corpus in *H. pylori* infection (Fig. 3). Levels of amphiregulin mRNA were similarly increased in both *cagA*-positive and *cagA*-negative infection.

Expression kinetics and *cag* PAI specificity of *HPYR1*. As *HPYR1* was confirmed to be upregulated in vivo in the antral

TABLE 3. Spleen array[a]

Time (category)[b]	Encoded protein	Gene accession no.	Fold difference
0.75 h (I)	MAD-3	M69043	2.0
3 h (I)	Thymosin β4	M11948	3.9
	MAD-3	M69043	2.5
	HUMNK4	AJ003147	2.0
	TSC-22	U35048	1.9
3 h (D)	Similar to Sm22	D21261	4.8
	L apoferritin	X03742	2.6
24 h (I)	TFIIIA homologue	U20272	2.0
	HLA-B	Y13567	1.5
24 h (D)	Neutrophil adherence factor, αM subunit	J04145	2.9
	β2 microglobulin	M36501	2.5

[a] Gene transcripts in Kato 3 gastric epithelial cells that demonstrate differential expression at 45 min and 3 and 24 h after culture with *H. pylori cag* PAI$^+$ NCTC 11637 and G50, which lacks the *cag* PAI.
[b] I, NCTC 11637 > G50; D, NCTC 11637 < G50.

TABLE 4. Inflammatory-cDNA array[a]

Time (category)[b]	Encoded protein	Fold difference	Time (category)[b]	Encoded protein	Fold difference
0.75 h (I)	Oncostatin M	2.3	0.75 h (D)	TNFRII	12.5
	p53	2.0		c-*fos*	3.1
	ADAM 11	1.8		TIMP-1	2.6
	IL-8	1.6		TNFRI	2.9
				IFN-γβR	1.8
3 h (I)	IL-17	61.3		ADAM 15	1.4
	Oncostatin M	25.8			
	IL-8	4.0	3 h (D)	Bclx	13.1
	NF-κB	2.5		ADAM 15	2.9
	CD55	1.7		ADAM 11	2.8
	JAGGED	1.7		ADAM 21	2.8
	IFN-γβ/R	1.5		ADAM 20	2.6
	JAGGED	1.5		CD68	2.4
				CD11a	2.0
24 h (I)	IL-7	6.9		CD11c	1.7
	G-CSFR	3.6		BAX α	1.5
	OSMRβ1	2.6			
	ADAM 21	2.5	24 h (D)	IL-8	3.1
	CD138	2.4			
	TNFRI	2.2			
	TIMP-1	2.1			
	TIMP-2	1.9			
	JAGGED	1.8			
	IFN-α/βR	1.7			
	p53	1.6			
	BAX α	1.6			
	ADAM 10	1.6			
	ADAM 11	1.5			
	INOS	1.5			

[a] Gene transcripts in Kato 3 gastric epithelial cells that demonstrate differential expression at 45 min and 3 and 24 h after culture with *H. pylori cag* PAI$^+$ NCTC 11637 and G50, which lacks the *cag* PAI.
[b] I, NCTC 11637 > G50; D, NCTC 11637 < G50.

mucosa of patients infected with *cagA*$^+$ strains, further in vitro studies were undertaken to investigate the kinetics of expression of this gene in *H. pylori*-stimulated Kato 3 and AGS gastric epithelial cells. As wild-type strains with and without the *cag* PAI were used for the cDNA array hybridization experiments, the *cag* PAI specificity of the *HPYR1* response was examined using a *cagM* isogenic mutant strain, H12-5A, and the *cag* PAI-positive parental strain G27. Increased *HPYR1* mRNA expression was observed at 45 min postinfection with the *cag* PAI-positive G27 strain in both Kato 3 cells and AGS cells. At 3 and 6 h post-G27 infection, *HPYR1* mRNA was evident in Kato 3 cells but was not detected at 24 h poststimulation (Fig. 4). No increase in *HPYR1* mRNA expression relative to unstimulated control cells was observed in cells cultured with the *cagM* isogenic mutant strain H12-5A (Fig. 4), suggesting that upregulation of *HPYR1* in gastric epithelial cells was *cag* PAI dependent. No amplification products were evident in PCR analysis of non-reverse transcribed controls, confirming the absence of DNA contamination.

DISCUSSION

High-density cDNA array technology permits the simultaneous, multiple screening of tens of thousands of genes to identify host gene expression patterns and identify new, previously uncharacterized, disease-associated genes. We have used this approach to examine in vitro the transcriptional response of gastric epithelial cells to *H. pylori*. The splenic and custom inflammatory-cDNA arrays used in this study have identified many previously unknown *H. pylori*-induced immune-response genes in epithelial cells. The I.M.A.G.E. library similarly has allowed identification of multiple known genes and novel ESTs. The Clontech Atlas array, which includes genes considered to be important in human disease, provided a further screen for candidate epithelial response genes.

One advantage of the I.M.A.G.E. and splenic arrays was that a large number of cDNAs (circa 58,000) could be screened simultaneously. On the primary screen, approximately 1.9% of genes were identified as differentially expressed. These results strongly support the earlier observations of Eckmann et al. (21) that the intestinal epithelial response to bacterial pathogens is very specific and narrow. Interestingly, in the latter study mRNA expression analysis of ca. 4,300 genes in intestinal epithelial cells following infection with *Salmonella* demonstrated that immune regulatory genes such as IL-17 and oncostatin M were upregulated in a manner similar to that observed in the present study. However, the study of Eckmann et al. (21) reported only on genes whose expression was upregulated. It is clear from the present study that infection of gastric epithelial cells with *H. pylori* results also in the downregulation of the expression of multiple host epithelial genes. In addition, many genes showed marked temporal changes in expression, being both upregulated and downregulated relative to uninfected control epithelial cells at various time points postinfection.

Several hundred differentially expressed genes were identified that exhibited changes in expression levels following infection with *H. pylori* over the three time points studied. In-

TABLE 5. Clontech Atlas array[a]

Time (category)[b]	Encoded protein	Fold difference	Accession no.
3 h (I)	IL-8	2.1	Y00787
	60S ribosomal protein L6	1.3	X69391
	Amphiregulin	1.3	M30704
3 h (D)	MAP kinase kinase 3	5.1	L36719
	Transducin β-2	4.5	M36429
	Bclx	4.2	Z23115
	Hepatoma-derived growth factor	3.9	D16431
	Tyrosine protein kinase csk	2.7	X59932
	SHB adapter protein	2.6	X75342
	Tyrosine protein kinase cak	2.1	X74979
	Repair protein RAD-23	2.5	D21235
	NADPH-cytochrome P450 reductase	2.4	S90469
	Cyclin D1	2.1	X59798
	Fibroblast growth factor receptor	1.9	U11814
	VEGF growth factor	1.7	M32977
	Heat shock 60-kDa (P1)	1.7	M34664
	Nucleobindin	1.6	M96824
	Integrin β4	1.6	X53587
	GADD45	1.4	M60974
	EZRIN	1.4	X51521

[a] Gene transcripts in Kato 3 gastric epithelial cells that show differential expression 3 h after culture with *H. pylori cag* PAI$^+$ NCTC 11637 and G50, which lacks the *cag* PAI.

[b] I, NCTC 11637 > G50; D, NCTC 11637 < G50.

fection with both strains induced marked temporal changes in gene expression, emphasizing the importance of longitudinal studies in gene expression profiling using in vitro model systems. This approach has the additional advantage of allowing cluster and principal component analysis (22) of differentially expressed epithelial genes to identify coregulated genes.

As *cag* PAI-positive strains activate NF-κB (31, 45, 61) and MAP kinase pathways (32, 48), it is not surprising that the transcriptional response of gastric epithelial cells induced by *cag* PAI-positive *H. pylori* markedly differed from that induced by the *cag* PAI-negative strain. Understanding the differences in the transcriptional response of gastric epithelial cells to *H. pylori* strains with different virulence characteristics should shed light on bacterial pathogenicity. This study focused on differences in gene expression induced by the wild-type *cag* PAI-positive and -negative strains, as such differences may be potentially relevant to the more severe clinical outcome associated with infection with *cag* PAI-positive strains. Furthermore, a focused approach is essential, given the large number of data generated from screening ca. 58,000 arrayed cDNA sequences. Known genes demonstrating early (0.75 to 3 h) differential expression included several genes involved in cell signaling pathways, which were decreased in *cag* PAI-positive-infected epithelial cells such as MAP kinase kinase 3, tyrosine protein kinase cak, guanine nucleotide binding protein G α subunit, and related transducin β-2. In contrast, *cag* PAI-positive strains induced an early increase in expression of several genes involved in transcriptional regulation, such as MAD-3, the I-κβ inhibitor of NF-κB and TCS-22. At 24 h increased expression of TFIIIA was observed with *cag* PAI-positive-*H. pylori*-infected cells. This protein is involved in initiation of transcription of 5S ribosomal RNA genes. In contrast, a substantial decrease in expression of the transcription factor BTF3, which complexes with RNA polymerase II (81), was observed.

The activation of NF-κB has been linked with suppression of apoptosis via NF-κB-controlled antiapoptotic genes (5, 74). In *H. pylori* infection both gastric epithelial expression and activity of NF-κB (68) and apoptosis (44) are increased. Interestingly, infection with *cag* PAI-positive strains has been associated with a reduced apoptotic index and higher gastric epithelial cell proliferation than that found for *cag* PAI-negative infections (53). Expression of the cell regulatory protein cyclin D1 was decreased following infection with the *cag* PAI-positive strain, and differential expression of genes involved in apoptosis such as AAC-11 and Bclx and also of redox-related genes such as NADPH-cytochrome P450 reductase was observed. p53-induced apoptosis has recently been linked to early transcriptional induction of redox-related genes involved in oxidative damage to mitochondria (54). Infection of gastric epithelial cells with the *cag* PAI-positive strain induced a 12-fold increase in the apoptosis inhibitor AAC-11 at 45 min but decreased expression at 3 and 24 h. AAC-11 inhibits apoptosis in cervical cancer cells, and transfection studies demonstrate that AAC-11 expression is associated with loss of TIMP-2 expression (35). Interestingly, in the present study, changes in expression of TIMP-2 and TIMP-1 (Table 4) were inversely related to that of AAC-11 (Table 2). TIMP-1 and TIMP-2 are expressed in approximately 50% of gastric cancers (47). Further studies to analyze expression of AAC-11, TIMP-1, and TIMP-2 in vivo in relation to *H. pylori* infection and gastric cancer will be of interest.

Infection with *cag* PAI-positive strains was associated with early decreased expression of genes coding for cellular regu-

TABLE 6. Expressed inflammatory mediator genes in control uninfected Kato 3 cells after culture for 24 h[a]

Encoded protein	Abbreviation	Relative level of expression
TIMP-1	TIMP-1	641
TIMP-2	TIMP-2	73
Platelet-derived growth factor receptor alpha	PDGFRA	55
Colony-stimulating factor 1 receptor	CSF1R	54
Leukemia inhibitory factor	LIF	145
Tumor protein p53	TP53	136
CD11A antigen	CD11A	136
Integrin α M	ITGAM	47
Integrin α X	ITGAX	203
CD5 antigen	CD5	96
TNF-α	TNF	58
TNFRI	TNFRI	33
TNFRII	TNFRII	228
IFN-α/β receptor	IFNAR1	277
IFN-γ receptor α	IFNGRA	301
IFN-γ receptor β	IFNGRB	223
IL-2	IL-2	221
IL-5	IL-5	38
IL-7	IL-7	120
IL-8	IL-8	525
IL-3 receptor α	IL3RA	52
IL-6 receptor β	ILRB	72
Oncostatin M β receptor	OSMRB	274
Oncostatin M	OSM	29

[a] Genes expressed in uninfected Kato 3 cells. Their relative intensity values are calculated by dividing the normalized Kato 3-derived probe intensity by the normalized PCR loading intensity multiplied by 1,000.

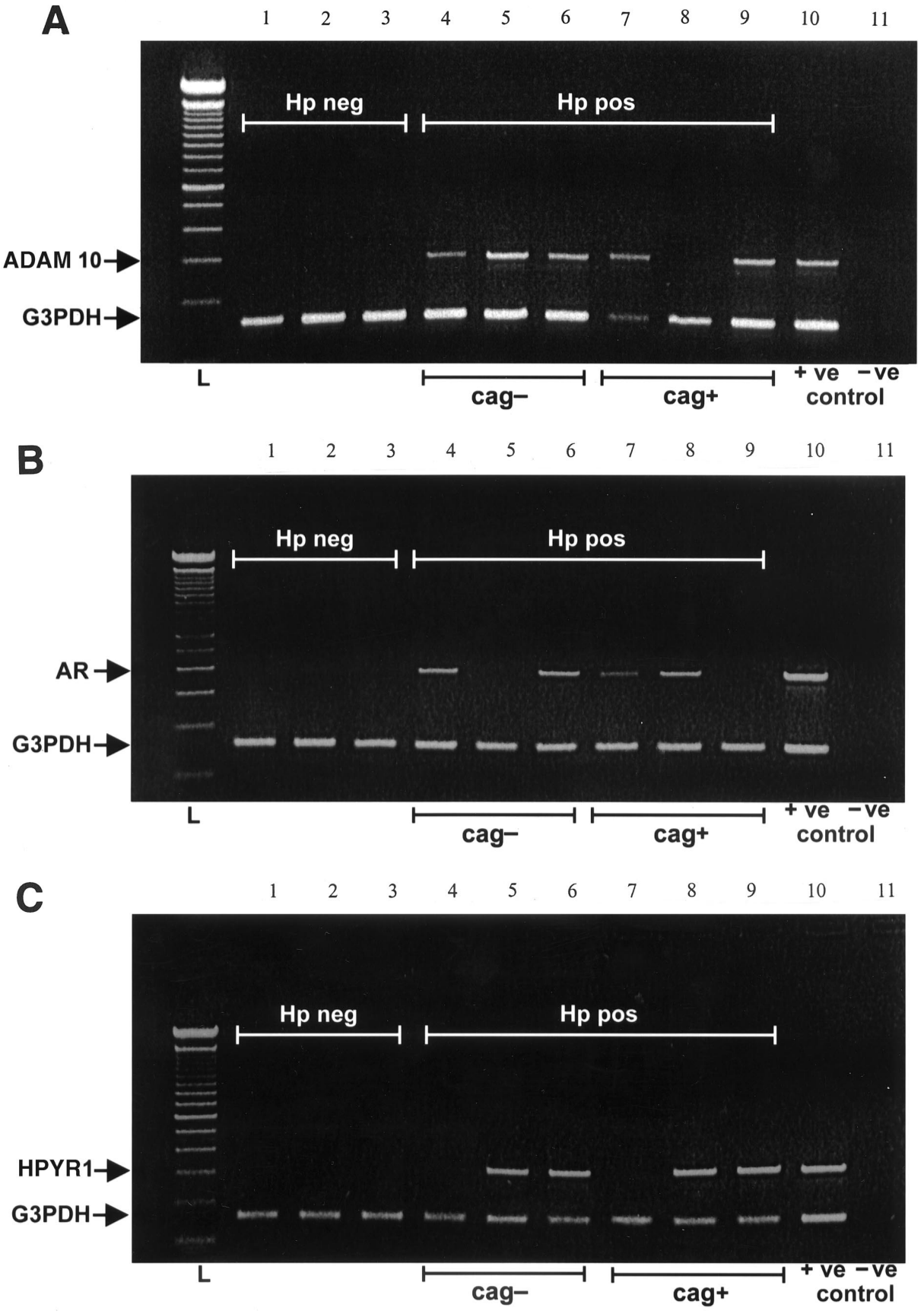

FIG. 2. Representative RT-PCR with primers for ADAM 10 (A), amphiregulin (AR) (B), and *HPYR1* (C) in gastric antral biopsy samples. G3PDH is the control gene GAPDH. Lane L, 100-bp ladder; lanes 1 to 3, *H. pylori* negative normal mucosa; lanes 4 to 6, mucosa from patients with *cagA*-negative *H. pylori*; lanes 7 to 9, mucosa from patients with *cagA*-positive *H. pylori*; lane 10, positive control (*H. pylori*-stimulated Kato 3 gastric epithelial cells); lane 11, negative control. Hp, *H. pylori*.

TABLE 7. Number of patients demonstrating expression of *ADAM 10* and *HPYR1* transcripts in human gastric antral mucosa[a]

Gene	No. of patients demonstrating expression/ no. of patients tested (%)		
	H. pylori negative	*H. pylori* positive	
		cagA negative	*cagA* positive
HPYR1	3/19 (16%)	2/13 (15%)	10/24 (42%)
ADAM 10	3/19 (16%)	8/14 (57%)[b]	6/28 (21%)

[a] Subjects are divided into *H. pylori*-negative patients with histologically normal mucosa and *H. pylori*-positive patients infected with *cagA*-positive and *cagA*-negative *H. pylori* strains.

[b] $P < 0.05$ compared to *cagA*-positive and *H. pylori*-negative patients' results (two-tailed Fisher exact test).

latory proteins, such as elongin B; genes coding for ribosomal proteins Sm of protein G and protein L28; and also genes involved in RNA processing, e.g., α-NAC and poly(A) binding protein. Elongin B, a ubiquitin-like protein, is part of the multifunctional regulatory elongin BC complex. This complex, when bound to the von Hippel-Lindau tumor suppressor gene, is thought to play an important role in negatively regulating hypoxia-inducible proteins by promoting degradation of HIF1α (58) and also the stability of the suppressor of cytokine signaling 1 (SOCS-1) proteins by promoting their degradation (80). As elongin B is a component of the von Hippel-Lindau ubiquitin-protein ligase (E3) complex, there is the potential that it competes for components of other E3s, such as Rbx1/ROC1, that are found in the I-κB SCF^{HOS} complex, thereby reducing the activity of the latter. As recent studies demonstrate that nonvirulent *Salmonella* inhibits I-κB-alpha ubiquitination (49) and thus attenuates acute inflammatory responses, further investigation of the functional importance of regulatory proteins such as elongin B in epithelial responses to enteric bacteria is warranted.

At 24 h postinfection, several interesting genes were identified on the I.M.A.G.E. arrays, which demonstrated decreased expression following infection with the *cag* PAI-positive strain. One of these was thymosin β4, which has been reported to have multiple functions, including inhibition of actin polymerization (19) and promotion of wound healing and angiogenesis (38). The oxidized form of thymosin β4, which is known to be induced in monocytes by glucocorticoids, also acts as an anti-inflammatory agent attentuating neutrophil-associated inflammatory processes (79). The preferential expression of thymosin β4 and also of L apoferritin, a known antioxidant response gene (55), at 24 h following infection with the *cag* PAI-negative strain suggests that these strains may have the potential to attenuate gastric inflammatory responses and protect against mucosal damage. The upregulation of L apoferritin is likely to protect against oxidative damage by enhancing capacity for iron storage. Interestingly, serum ferritin levels are reduced in *H. pylori* infection, but the relation with bacterial *cag* PAI status has not been investigated (42). An additional gene of interest was the membrane protein E16, the expression of which was also increased at 24 h following *cag* PAI-negative infection. The encoded protein of this gene heterodimerizes with CD98 to function as a cationic amino acid transporter (39). Such transporters may regulate the availability of arginine in epithelial cells and thus regulate cellular nitric oxide production (43). Further investigation of the expression of these potentially protective host response genes in vivo in relation to *H. pylori* infection will be important.

Hybridization of the inflammatory-cDNA arrays with epithelially derived probes demonstrated that infection with the *cag* PAI-positive strain resulted in increased expression of several genes involved in immune regulation. Differential expression of transcripts encoding several cytokines (e.g., IL-8, IL-7, IL-17, and oncostatin M), cytokine receptors (tumor necrosis factor receptors I and II, granulocyte colony-stimulating factor receptor, and alpha and beta interferon receptors), and members of the ADAMs family, which have an important function in cytokine, cytokine receptor, and growth factor shedding

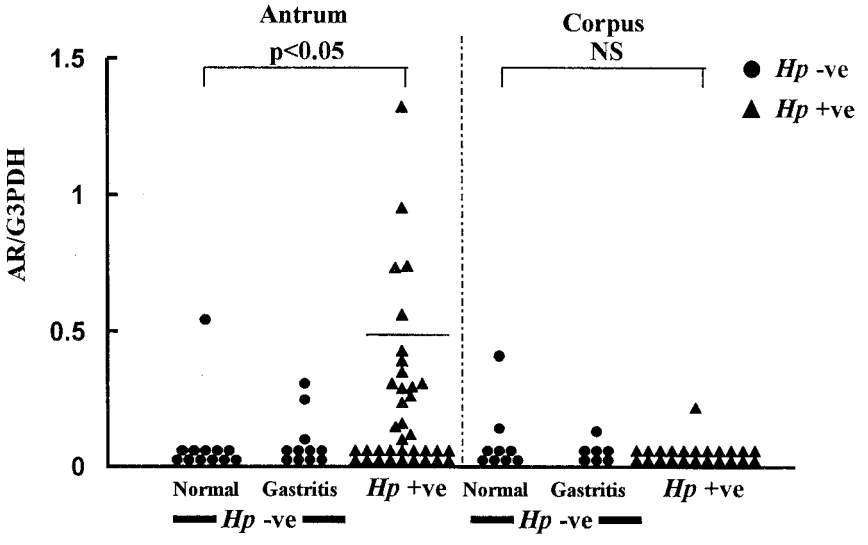

FIG. 3. Semiquantitative analysis of amphiregulin (AR) in antral and corpus mucosa of *H. pylori*-positive (Hp +ve) and -negative (Hp-ve) patients. Levels of amphiregulin relative to GAPDH (G3PDH) are indicated on the *y* axis. *H. pylori*-positive patients had significantly greater ($P < 0.05$) amphiregulin mRNA expression in the antrum than did *H. pylori*-negative patients with normal mucosa. NS, not statistically significant.

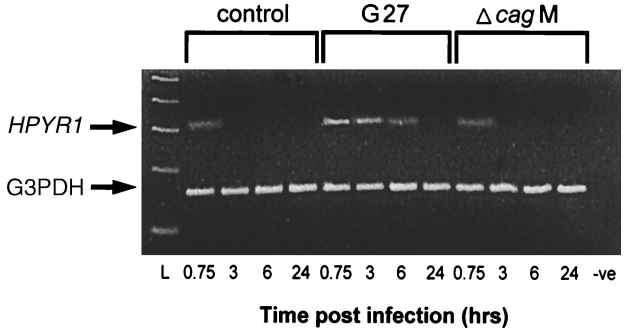

FIG. 4. Representative RT-PCR for *HPYR1* and GAPDH (G3PDH) in Kato 3 gastric epithelial cells following coculture with *cag* PAI-positive strain G27 and an isogenic Δ*cagM* mutant. Cells were harvested for RT-PCR analysis at 45 min and 3, 6, and 24 h postinfection. Lane L, 100-bp ladder; lane -ve, negative.

(75), was observed. Recent studies have confirmed that IL-17 is upregulated in the gastric mucosa in *H. pylori* infection (37). Analysis of expression of the identified genes in patients with *H. pylori* infection of defined *cag* PAI status is required to confirm differential expression in vivo.

In this study two known genes, amphiregulin and ADAM 10, and one gene of unknown function (*HPYR1*) were chosen for further analysis of expression in vivo in the gastric mucosa. Amphiregulin is a member of the epidermal growth factor family, which has a mitogenic effect on epithelial cells. It is present in parietal cells (46) and is overexpressed in gastric carcinomas (11). Previous studies have indicated that soluble products of *H. pylori* induce amphiregulin in MKN28 gastric epithelial cells (56). Our in vivo results confirm that *H. pylori* infection is associated with upregulation of amphiregulin mRNA expression in the antral mucosa. Whilst a low level of differential expression of amphiregulin following infection with *cag* PAI-positive and -negative strains was observed in the cDNA arrays, in vivo similar expression levels were observed in patients infected with *cag* PAI-positive and -negative strains. This confirms earlier in vitro studies that induction of amphiregulin in gastric epithelial cells by *H. pylori* was independent of a functional *cag* PAI (56).

The inflammatory-cDNA arrays showed that *H. pylori* induced temporal changes in gene expression of four members of the ADAMs family of membrane proteins. This recently identified gene family has important functions in the release of cell surface molecules and cell-cell and cell-extracellular matrix interactions (6, 75). ADAM 10, which is expressed in a range of hematological malignancies (73) and in prostate cancer cell lines (40), has both collagenase type IV activity (41) and also cleaves pro-TNF alpha (TNF-α) to the soluble form (57). Our in vivo results confirm that ADAM 10 mRNA expression is increased in *H. pylori* infection in the gastric mucosa; interestingly, expression was more frequent in patients infected with *cag* PAI-negative strains. Recent studies confirm that levels of transcripts coding for ADAM 17, also known as TNF-α converting enzyme, are also increased in gastric *H. pylori* infection (78). Further studies are required to assess the functional importance of ADAM proteins in *H. pylori*-induced gastric pathology.

Another advantage of screening the I.M.A.G.E. and spleen arrays is that, following comparison with nucleotide databases (GenBank and EMBL), new expression data for novel and previously uncharacterized genes, ESTs, have been obtained. Further analysis of these genes is beyond the scope of the present study. However, we investigated in vivo expression of one gene, *HPYR1*, which was upregulated at 0.75 h after exposure to *cag* PAI-positive strains. Our in vivo data confirm an association of *HPYR1* expression in patients with *cag* PAI-positive *H. pylori* infection. Furthermore, our additional in vitro studies with an isogenic *cagM* mutant strain demonstrated that the induction of *HPYR1* is dependent on a functional *cag* PAI and not on the presence or expression of genes at other loci. The sequence used to design the *HPYR1* PCR oligonucleotide-specific primer pairs was from one long contiguous sequence of approximately 1.2 kb found on a BAC clone (543J1) from chromosome 8q24. This sequence may represent the 3′ untranslated region of the transcript, as no open reading frame was identified and a MER68A repetitive sequence element was located 3′ to the amplification region. Alternatively, it may represent a nontranslated RNA transcript (23, 24). It obviously represents a transcript sequence, as RT-negative controls consistently gave negative results, indicating an absence of contaminating genomic DNA.

Further investigation of the previously uncharacterized genes that indicate differential expression in vivo is in progress. As the initial screen used a gastric cancer epithelial cell line, some differentially expressed genes have been found to be tumor specific (R. Stephens et al., unpublished data). Further investigation of these ESTs could lead to the identification of new genes relevant not only to bacterially induced enteric disease but also to gastric neoplasia. The application of cluster analysis and principal component analysis (22) of the temporal data sets obtained in this study may also provide insight into the function of novel genes.

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