# Helicobacter pylori Induces Gene Expression in Human Gastric Cells Identified by mRNA Differential Display

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Changes in gene expression patterns in gastric cells infected with *Helicobacter pylori* were characterized by means of mRNA differential display. Total RNA preparations were extracted from the *H. pylori* infected gastric cells and paired non-infected cells, and were probed with candidate clones identified after screening up to 6,000 mRNA species. Among them, four clones, 04G-1, 04G-2, 01G-1, and Cppa-2 show significant expression in the infected cells by Northern blot analysis, and they are 199 bp, 196 bp, 228 bp, and 276 bp in length, respectively. Database search revealed that nucleotide sequences of these clones share very low identity with any known sequence. These results indicate that *H. pylori* can significantly affect gene expression in gastric cells. Furthermore, mRNA differential display can be used in pathogenesis studies to identify new genes in gastric cells in response to insults such as *H. pylori.*   © 1996 Academic Press, Inc.

*Helicobacter pylori* infection is strongly associated with chronic gastritis (1,2). However, peptic ulcer disease and gastric carcinoma occur in only a subset of individuals infected chronically with *H. pylori* (3,4). Therefore, both bacterial and host factors are presumed to contribute to this differential response. In regard to bacterial factors, the success of *H. pylori* as a gastric pathogen is dependent on virulence factors such as those that allow *H. pylori* to survive in the hostile environment of the gastric lumen, including spiral shape and motility (5), adaptive enzymes and proteins (6-9), ability to adhere to gastric mucosal cells and mucus (10). Additional factors such as those that lead to disruption of the gastric mucosal barrier, including toxins (11,12), mediators of inflammation (13), and contribute of gastric acid (14), are considered as pathogenic mechanisms of *H. pylori.*

In contrast, little is known regarding host factors in the *H. pylori* infection, particularly the changes in gene expression in the infected gastric cells. Comparisons of gene expression in pathological changes that arise in diseases such as peptic ulcers and even cancer provide the underlying information we need to analyze the biological processes induced by *H. pylori* infection (15). The aim of this study is to elucidate the molecular basis for gastric cells pathogenesis, and first it is necessary to isolated genes which are differentially expressed. Technique of differential display has recently been described to investigate differentially expressed mRNAs (16,17). In the present report, *H. pylori* lysate was used as the stimulus, and we described a group of novel genes whose expression was significantly increased in the *H. pylori* stimulated gastric adenocarcinoma cells by the method of differential display. We also determined the nucleotide sequences of these cDNA fragments and compared homologies with those deposited in the DNA data base.

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## MATERIALS AND METHODS

*Bacterial strains and cultivation.* The vacuolating toxin positive strain of *Helicobacter pylori* from ulcer patients were kindly given by Dr. N. Lee of Chang Gung memorial hospital, Taiwan. The bacteria were cultured on chocolate agar plates at 37° C in a microaerophilic atmosphere of 10 % $CO_2$ in air and 99 % humidity for three to four days. *H. pylori* appeared as curved-shaped rod under microscopy, and was examined by urease tests.

*Preparation of H. pylori lysate.* The *H. pylori* cells grown on agar plates were washed out with phosphate buffer saline (PBS, pH 7.2). The suspension of bacterial cells were disrupted by sonication (Heat-System Model XL 200, NY) in ice bath at 35 % pulse with 30 sec intervals for 5 min. The solution was centrifuged at $15,000 \times g$ for 10 min at 4° C. The supernatant was used as lysate for assay. Protein concentration was determined by Bradford method with a Bio-Rad protein assay kit according the manual instruction.

*Cell culture.* Human gastric adenocarcinoma cell line (AGS) was a gift from National Defense Medical Center, Taiwan. AGS cells were maintained and cultured as a monolayer in 50-$cm^2$ tissue culture flasks with RPMI1640 medium containing 10 % fetal bovine serum, 100 $\mu g/ml$ penicillin, and 100 $\mu g/ml$ streptomycin. Cell culture was grown in an atmosphere of 5 % $CO_2$ at 37 °C. Numbers of cells were counted after trypsinization by a Neubauer hemocytometer (VWR, Scientific Corp. Philadelphia, PA). Before isolation of RNA, the cells were divided into two groups, one was stimulated with 1.5 mg/ml *H. pylori* lysate for 4 h, and the other one without adding *H. pylori* lysate was used as the normal cells.

*Differential display of mRNA from H. pylori-infected and normal cells.* Differential display of mRNA was performed as previously described with minor modification (16,17). DNA-free RNA from the gastric cells was prepared by treatment with RNase-free DNaseI (Gibco BRL). Total RNA was isolated by TRIzol reagent-chloroform (Gibco BRL) extraction (18). After isopropanol precipitation, DNA-free total RNA (0.1 $\mu g$) was reverse transcribed in 1X MMLV buffer which contained 2 $\mu M$ of either $T_{11}A$, $T_{11}G$ or $T_{11}C$ as the 3′-primer oligonucleotide, 10 mM DTT, and 250 $\mu M$ each of dGTP, dATP, dTTp, and dCTP. After the solution was heated at 70° C for 10 min and cooled to 42° C, 200 units MMLV Superscript-II (Gibco BRL) was added for 50 min and then at 70° C for 15 min. Polymerase chain reaction (PCR) was performed by adding 0.1 volume of reverse transcription reaction mixture in 1X PCR buffer which contained 2.5 $\mu M$ each of dNTP, 5 $\mu C_i$ $^{35}$S-dATP, 0.2 $\mu M$ of the respective $T_{11}N$ oligonucleotide, 0.2 $\mu M$ specific multiple arbitrary 10-mer oligonucleotide sets (Genosys Biotechnologies Co.), and 0.8 units Taq DNA polymerase (Promega Inc.). PCR reaction was performed in the Rapidcycler (Idaho Technology Inc.) at 94° C for 30 sec and 40 cycles at 94° C for 2 sec, 40° C for 40 sec, 72° C for 30 sec and followed by 72° C for 5 min. Loading buffer was added to each PCR product solution and the samples electrophoresed on 6 % acrylamide DNA sequencing gels under denaturing conditions. The gel was transferred onto 3 MM paper and dried at 80° C for 45 min, and exposed to Kodak XAR-5 film (Eastman Kodak) for 16 h.

*Reamplification of PCR products and Northern blot analysis.* PCR fragments that were differentially expressed between the *H. pylori* infected and non-infected cells were cut out of the 3 MM paper, phenol-chloroform extracted, and ethanol precipitated. PCR was repeated by using the same set of primers and conditions used in the first amplification reaction for differential display. The reamplified PCR products were separated on a 3 % agarose gel, visualized by ethidium bromide staining, and eluted for using as a probe for Northern blot analysis or subcloning. For Northern blot, total cytoplasmic RNA (10 $\mu g$) obtained from normal and *H. pylori* infected cells were separated on formaldehyde-denaturing 1 % agarose gels and transferred to nylon membranes (Schleicher and Schuell). After UV crosslinking, blots were prehybridized at 40° C in 1× SSC, 1 % SDS, 1× Denhardt's solution, 200 $\mu g/ml$ salmon sperm DNA, 20 mM Tris for 4 h. Hybridization was continued at 40° C overnight in the presence of $1 \times 10^6$ cpm/ml of random prime $^{32}$P-labelled probe. Following a wash at room temperature 2× SSC/0.1 % SDS, blots were washed at 65° C for 20 min with 1× SSC/0.1 % SDS. The washed membrane was autoradiographed for about 6 h. The blots were stripped and reprobed with a $^{32}$P-labeled human glyceraldehyde-3-phosphate (GAPDH) gene probe.

*Cloning and sequencing.* Those samples showing significant changes by Northern blot analysis were subcloned into the pGEM-T vector with the TA cloning system from Promega. After the subcloned inserts were isolated and confirmed by Northern blot analysis, DNA sequencing was performed directly from the TA cloning vector using Sequenase version 2.0 kit (US Biochemical). T7 and SP6 promoter primers were used to locate the cloned insert. DNA sequences were analyzed by the FASTA search program of GCG package and were screened for homology against the GenBank/EMBL database sequences.

## RESULTS

### Differential Display

The expression of mRNA in *H. pylori* lysate stimulated AGS cells and paired normal cells were analyzed using combinations of forty 10-mers and three $T_{11}N$ primers. Each primer combination displayed 30 - 50 bands, allowing approximately 6,000 different mRNA species
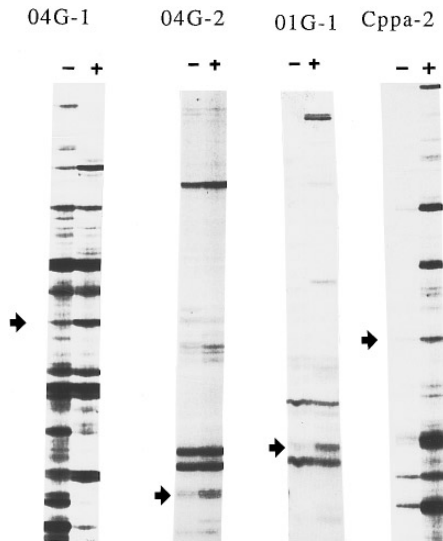
**FIG. 1.** Autoradiogram of differential mRNA display of candidate clones. Candidate cDNA fragments showing altered expresssion by differential mRNA display are marked by arrows and selected for subcloning. Lane + indicates cDNA fragments from normal AGS cells and lane − indicates *H. pylori* lysate stimulated AGS cells.

to be screened in this study. As shown in Fig. 1, arrow heads indicate that clones exhibited differences in their expression between normal and the *H. pylori* stimulated cells. These changes were confirmed by repeating the PCR and differential display procedures. We also use intact *H. pylori* as an infectious agent, the results showed that the differentiation of gene expression in the host cells was not so significant as compared with *H. pylori* lysate as the stimulus.

*Northern Blot Analysis*

When the isolated clones from the display gel were reamplified by PCR, some of the clones exhibited multiple bands different molecular size on agarose gel. Sixteen bands were eluted and subcloned into pGEM-T vector, and the clones were used as probes for Northern blot analysis. By Northern blot analysis using total RNA extracted from normal and *H. pylori* stimulated cells , four clones (04G-1, 04G-2, 01G-1, Cppa-2) were found to be significant changes. As shown in Fig. 2, mRNAs for clone 01G-1 (6.5 kb, 4.4 kb and 2.5 kb), clone 04G-1 (5 kb), clone 04G-2 (1.6 kb), and clone Cppa-2 (0.7 kb) were present in *H. pylori* stimulated cells but not in normal cells.

*Sequence*

The nucleotide sequences of clones 04G-1, 04G-2, 01G-1, and Cppa-2 are showed in Fig. 3. All these clones have variant polyadenylation signals. Searching of GenBank/EMBL databases revealed that these clones do not share any homology to previously known sequences.

## DISCUSSION

Little is known about the choice of which genes are expressed in gastric cells in response to *H. pylori* insult. Studying the altered gene expression in the infected host cells can understand the regulatory mechanism that control pathological changes. *H. pylori* resides at the surface of gastric cells would lyse sometime after death, and the bacterial intracellular materials might
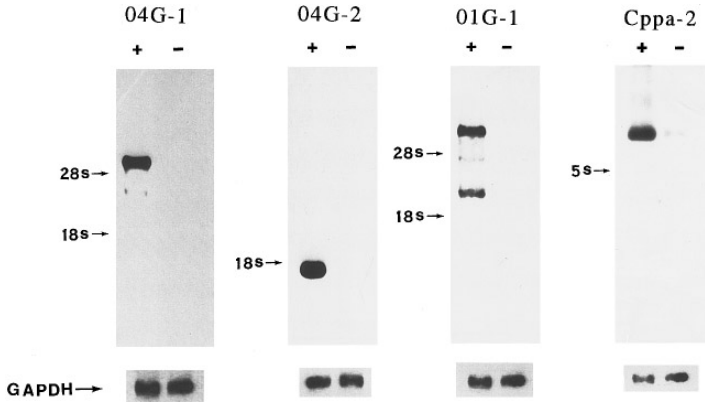
**FIG. 2.** Northern blot analysis using $^{32}$P-dATP labeled cDNA probes derived from reamplification of clones 04G-1, 04G-2, 01G-1, and Cppa-2. The same blots were reprobed with GADPH cDNA as a loading control. Each lane represents 10 $\mu$g of total cytoplasmic RNA isolated from normal or infected cells.

also influence the gastric cells. Therefore, we used the sonicated *H. pylori* lysate as the stimulus to represent the effect of total virulence factors on the host's gene expression. In this study, four genes are identified to be differentially expressed between *H. pylori* stimulated AGS cells and normal cells. We used recently developed differential display method to identify these

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clone 04G-1, length: 196

1     GCAATAAATT AAGCTTCTAA AAATAGTTCA AATTGATTTG GAGAAGAAGT
51    TCATAAATTA AGTGGAAGTT TAAAAGAATT AATTAATTTT GCTTCTTTTA
101   AATATATTTT TGTTGCAAGG GTGAGGATAT TTACAGATTT TCACAATTCT
151   TTCTTTTTCA ACTTTAGTTA ATTGAGTTTT AGTCCAAAAA AAAAAA


clone 04G-2, length: 199

1     TTAGTAAGTC TTTTACTAGG TGGTACTTTT TTACCTGAAA AATAATCTTT
51    TAGAGTTGCA ACACCTGAAT TTATTCACAA TAATTCTAAA TGTTTTTAAG
101   TTTAAAAAAG TCAAGTCATA ATTGTCTAAT TTGTTTTAAG TAATTTTGAG
151   TTCATAATTT CCTTTAAATT TTTAAGTTAA ATTTTAGCAA AAAAAAAAA


clone 01G-1, length: 228

1     TCGAATTAAC TAAAGTTGAA AAAGAAAGAA TTGTGAAAAT CTGTAAATAT
51    CTTCACCCTT GAACAAAAAT GTATTTAAAA GAAGCAAAAT TAATTAATTG
101   TTTTAAACTT CCACTTAATT TATGAACTTC TTCTCCAAAT GAATTTGACA
151   TATTTAAAAA TGATAAAAAA GTTTACTCTC AAATTTCAAA TTTCAAATTA
201   GAAGCTTAAT TTGTTGCAAA AAAAAAAA


clone Cppa-2, length: 276

1     TTTGGCTGCC AAATTTTATA GATGTATCCT GAGTAGGGCA GGGCACGTGA
51    AACCCTGTCT GAATCTGCCG GGACCACCCG TAAGGCTAAA TACTAACAGA
101   CACCGATAGT GAACTAGTAC GTGAGGAAAG GTGAAAAGAA CCCCGAGAGG
151   AGTGAAATAG ATTCTGAAAC CATTTACTTA CAAGTAGTCA GAGCACGTTA
201   AAGTGTGATG GCGTACATCT TGCAGTATGG GCCGGCGAGT TATGTTAATA
251   ATCAAGGTTA AGCAGAAAAA AAAAAA
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**FIG. 3.** Nucleotide sequence of clones 04G-1, 04G-2, 01G-1, and Cppa-2. All contain a poly(A) tail.

genes (16). Those cDNA fragments do not share nucleotide homology with any known gene. The lack of homology for these clones with known sequences does not eliminate the possibility that they may be homologous to known proteins or protein families, since non-coding 3′ region of mRNA species show great species to species variation. Further work to clone the full length cDNAs of these genes are needed to clarify the identify of nucleotide sequence and to study their function as the pathogenic factors in gastric diseases. Although we do not know the role of expression of host cellular genes in the pathogenesis of gastric diseases associated with *H. pylori* infection, we speculate that this event is involved in the inflammatory response, which is an important factor of gastritis, ulcers, and presumably, gastric cancer. Gene expression stimulated by *H. pylori* might also initiate oncogenic transformation as a long-term effect.

Because the different bacterial pathogens share some common virulence factors and also possess unique pathogenic factors, responses of host cells to bacterial insults on the gene expression some are specific and some are non-specific for the invaded bacterium. At this moment, we can not make a conclusion whether or not those novel genes were specifically induced for *H. pylori* lysate, and we would not reject the assumption that those genes may be also highly induced by some other pathogenic bacteria. However, the success of *H. pylori* but not almost other pathogenic bacteria residing in stomach makes gene induction by *H. pylori* lysate have an unique and specific pathogenesis meaning.

Northern blots detected three distinct transcripts for clone 01G-1 in the *H. pylori* lysate stimulated cells but not in normal ones. Several possibilities could contribute explain the occurrence of multiple transcripts, including alternative utilization of polyadenylation sites, alternative splicing of the message, or presence of extended 5′ untranslated region. The availability of full length cDNAs of these clones and the future availability of large amounts of *H. pylori* infected gastric mucosa samples will allow studies to determine if these novel genes or their coding proteins are related to the pathogenesis of peptic ulcers or even the development of the gastric cancer.

## ACKNOWLEDGMENT

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