THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 275, No. 5, Issue of February 4, pp. 3629–3636, 2000

Printed in U.S.A.

Helicobacter pylori Activates the Histidine Decarboxylase Promoter through a Mitogen-activated Protein Kinase Pathway Independent of Pathogenicity Island-encoded Virulence Factors*

(Received for publication, October 4, 1999, and in revised form, November 18, 1999)

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***Helicobacter pylori* infection of the gastric mucosa is accompanied by an activated histamine metabolism. Histamine plays a central role in the regulation of gastric acid secretion and is involved in the pathogenesis of gastroduodenal ulcerations. Histidine decarboxylase (HDC) is the rate-limiting enzyme for histamine production, and its activity is regulated through transcriptional mechanisms. The present study investigated the effect of *H. pylori* infection on the transcriptional activity of the human HDC (hHDC) promoter in a gastric epithelial cell line (AGS) and analyzed the underlying molecular mechanisms. Our studies demonstrate that *H. pylori* infection potently transactivated the hHDC promoter. The *H. pylori*-responsive element of the hHDC gene was mapped to the sequence +1 to +27 base pairs, which shows no homology to known *cis*-acting elements and also functions as a gastrin-responsive element. *H. pylori* regulates the activity of this element via a Raf-1/MEK/ERK pathway, which was activated in a Ras-independent manner. Furthermore, we found that *H. pylori*-induced transactivation of the hHDC promoter was independent of the *cag* pathogenicity island and the vacuolating cytotoxin A gene and therefore may be exerted through (a) new virulence factor(s). A better understanding of *H. pylori*-directed hHDC transcription can provide novel insights into the molecular mechanisms of *H. pylori*-dependent gene regulation in gastric epithelial cells and may lead to new therapeutic approaches.**

Helicobacter pylori has been identified as a major pathogen associated with the development of chronic gastritis and gastroduodenal ulcer disease as well as gastric adenocarcinoma and mucosa-associated lymphoid tissue lymphoma (1–5). *H. pylori* strains expressing the vacuolating toxin A and genes encoded by the cytotoxin-associated gene A (*cagA*)[1]-associated pathogenicity island (PAI) have been considered associated with enhanced pathogenic potential of the bacterium (1, 6–8). Individuals infected with PAI-positive *H. pylori* strains display more severe courses of gastric inflammation and gastroduodenal ulcer disease and appear to develop more frequently gastric adenocarcinomas and mucosa-associated lymphoid tissue lymphomas (9, 10).

The PAI represents a cluster of 31 genes that encode presumably a specialized secretion system that enables the bacterium to expose or secrete particular proteins that are involved in induction of intracellular signaling cascades of target cells (7). A molecular concept for the enhanced pathogenicity of PAI-positive *H. pylori* strains has been provided by the observation that genes located within the PAI are indispensable for activation of NF-κB and AP-1 as well as stimulation of IL-8 production and secretion in gastric epithelial cells and therefore seem to elicit a more pronounced immune response (7, 11–15). In addition to *cag*-related genes, factors outside the PAI also appear to contribute to the differences in the pathogenic potential of *H. pylori* (16, 17).

The gastric inflammatory response to *H. pylori* is characterized by mucosal infiltration of neutrophils and lymphocytes leading to enhanced release of cytokines and chemokines (3, 18). In addition, increased gastric histamine secretion appears to contribute to the inflammatory changes and tissue damage associated with chronic *H. pylori* infection of the gastric mucosa (19–21). Histamine represents the major stimulus controlling gastric acid secretion and is produced and secreted by enterochromaffin-like (ECL) cells of the corpus mucosa (22–26). Histidine decarboxylase (HDC) is the key enzyme for histamine production in gastric ECL cells, and its enzymatic activity is to a large extent regulated through enhanced transcription of the HDC gene (27–30). In addition to its central role in regulation of gastric acid secretion, increased HDC gene expression has also been found to be associated with gastric inflammation and

* This study was supported in part by Deutsche Forschungsgemeinschaft Grants NA 292/6-1 (to M. N.) and HO 1288/6-1 (to M. H.), by Fonds der Chemischen Industrie grants (to M. N. and T. F. M.), and by Verum Stiftung and Mildred Scheel Stiftung grants (to B. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: *cagA*, cytotoxin-associated gene A; PAI, pathogenicity island; NF-κB, nuclear factor κB; AP-1, activator protein 1; IL, interleukin; ECL, enterochromaffin-like; HDC, histidine decarboxylase; hHDC, human HDC; JNK, c-Jun NH_2-terminal kinase; MAP kinase, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; PMA, phorbol 12-myristate 13-acetate; EGF, epidermal growth factor; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; kb, kilobase(s); TK, thymidine kinase; m.o.i., multiplicity of infection; IκBα, inhibitory protein κBα; luc, luciferase; EMSA, electrophoretic mobility shift assay; GAS-RE, gastrin-responsive element; bp, base pair(s); GAS-RE-BP, GAS-RE-binding protein; HIV, human immunodeficiency virus.

development of gastroduodenal ulceration (31–35). Furthermore, we found that oxidative stress, which is commonly increased in ulcerative and inflammatory diseases affecting the gastric mucosa, is capable of transactivating the hHDC promoter *in vitro* (36). Aside from its ulcerogenic potential, histamine has also been shown to possess immunomodulatory properties in the context of mucosal inflammations, contributes to the healing of ulcerative lesions of the gastric and intestinal mucosa, and has also been shown to stimulate the growth of gastric epithelial cells (37–41). A current *in vivo* study demonstrated that *H. pylori* infection is associated with increased mucosal histamine levels as well as an expansion of the gastric ECL cell lineage (42). The hypothesis that these changes could be at least in part be attributed to a direct effect of *H. pylori* on ECL cells has been substantiated by the finding that *H. pylori* can stimulate histamine secretion from isolated rat ECL cells as well as ECL cell proliferation *in vitro* (43). Although these findings strongly suggested that *H. pylori* can directly influence the histamine metabolism of gastric ECL cells, potential molecular mechanisms that could underlie this effect are unclear.

It has been demonstrated that in gastric epithelial cells *H. pylori* infection elevates the abundance of "classical" second messenger molecules such as Ca^{2+}, cyclic adenosine monophosphate (cAMP), and inositol trisphosphate (44). Although *H. pylori* has been shown to stimulate phosphorylation of several cellular proteins in gastric cancer cells *in vitro* (44, 45), intracellular signaling cascades activated by the bacterium are largely unknown. In a current study, we investigated the effect of *H. pylori* on the AP-1 transcription factor complex in gastric cancer cells *in vitro* and found that AP-1 activity is regulated by *H. pylori* through activation of c-Jun NH_2-terminal kinase (JNK) (13), which belongs to the superfamily of "mitogen-activated protein kinases" (MAP kinases) (46, 47). In addition to the JNK cascade, the MAP kinase superfamily comprises the extracellular-regulated kinase (ERK) pathway (46). Although some overlap between both pathways has been described (46), the JNK cascade activates primarily transcription factors involved in the "stress response" of eukaryotic cells, whereas the ERK pathway has been linked to genes involved in cellular proliferation and differentiation (46–48). Recently we demonstrated that ERK-related signaling cascades also play a central role in the transmission of the effects of gastrin and oxidative stress on the hHDC promoter in gastric cancer cells, whereas the JNK pathway is not involved in hHDC gene regulation (36, 49). Therefore, it is highly likely that the ERK cascade represents a potential target signaling route through which activators of hHDC gene transcription exert their transactivating effect on the hHDC promoter.

To investigate whether *H. pylori* can directly influence the transcriptional activity of the hHDC promoter, we performed *in vitro* studies employing hHDC-luciferase reporter gene constructs as well as various hHDC promoter mutants. Furthermore, we aimed to analyze the signal transduction pathways and nuclear factors responsible for transmission of this effect on the hHDC promoter. Finally, we analyzed the virulence factors involved in regulation of the hHDC promoter by *H. pylori*.

EXPERIMENTAL PROCEDURES

Cell Culture—The human gastric adenocarcinoma cell line (AGS) was grown in RPMI 1640 (Life Technologies, Inc., Heidelberg, Germany) supplemented with 4 mM glutamine, 100 units ml^{-1} penicillin, 100 $\mu g\ ml^{-1}$ streptomycin, and 10% fetal calf serum (Life Technologies, Inc.) in a humidified 5% CO_2 atmosphere. AGS-B cells express the cholecystokinin-B/gastrin receptor through stable transfection and were described before (30). Where indicated, cells were treated with 50 nM phorbol 12-myristate 13-acetate (PMA, Sigma, St. Louis, MO) for 4 h or treated with 200 ng/ml EGF (Promega, Heidelberg, Germany) for 6 h. AGS-B cells were stimulated with 10^{-7} M gastrin (Calbiochem, San Diego, CA) for 12 h. To block MEK activation, cells were preincubated with 50 μM PD98059 (Calbiochem) for 30 min before infection.

Bacteria and Infection—The following *H. pylori* strains were used for infection experiments: *P12* strains (wild type) and the isogenic *cagA** (*cagA* with a probable polar effect), *vacA* (50), and *PAI* (missing the *cag* pathogenicity island (PAI); and *G27* (wild type) and the isogenic *cagI* strain (8). For the construction of the *PAI* strain two approximately 2-kb DNA fragments upstream of the *cag*-PAI (region 545254–547164) and downstream of the PAI (584570–586563) (51) were amplified by polymerase chain reaction and cloned into pBluescript separated by a kanamycin resistance gene. The plasmid was transformed into *H. pylori* (*P12*), and one transformant was analyzed by polymerase chain reaction for correct allelic exchange of the PAI with the resistance gene. *H. pylori* strains were grown on agar plates containing 10% horse serum in a microaerophilic atmosphere (generated by Campy-Gen, Oxoid, Basingstoke, U. K.) at 37 °C for 48–72 h. 24 h after infection *H. pylori* was harvested in phosphate-buffered saline (pH 7.4), diluted corresponding to the multiplicity of infection (m.o.i.), and incubated with the epithelial monolayer. Infection with *H. pylori* was monitored routinely by light microscopy.

Transient Transfections and Luciferase Reporter Assays—24 h prior to transfection, cells were seeded in tissue culture plates and grown to 60–70% confluence. Transient transfections of 1–2 μg of reporter constructs were carried out using cationic liposomes (Dac-30, Eurogentec, Sart Tilman, Belgium) according to a protocol reported previously (13, 52). Transactivation of hHDC-luciferase reporter gene constructs was measured after transfection of 5′-deletion constructs (hHDC1000, hHDC480, hHDC400, hHDC125, +1 to +27 TK luc) as described previously (49, 53, 54). Transactivation activity of NF-κB and AP-1 was measured as described previously (13, 52). Cotransfection of dominant negative kinase cDNAs (DNERK1(K71R), DNERK2(K52R), DNRaf-1, DNRas15(G15A), DNRas17(S17N), and DNMEKK1(K432M)) with appropriate hHDC constructs has been described previously (49, 52). After transfection the cells were deprived of serum and maintained in RPMI 1640 supplemented with 4 mM glutamine and 0.1% fetal calf serum for 20–24 h. The expression of the transfected dominant negative kinase constructs was controlled by immunoblotting. For measurement of transactivation activity transfected cells were harvested, and luciferase activity was assayed as recommended by the manufacturer's instructions (Promega). The results were recorded on a Wallac 409 β-counter (Berthold-Wallac, Bad Wildbach, Germany). The data represent the mean ± S.D. calculated from three independent experiments as fold activation compared with the control. Activities varied <15% among transfection experiments.

Immunoblotting—To detect activated MEK1/2 and ERK1/2 total AGS cell extracts were prepared in 20 mM Tris (pH 7.5), 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 10 mM K_2HPO_4, 1 mM Na_3VO_4, 10 mM NaF, 1.25% Nonidet P-40, and 10% glycerol. Equal amounts of protein extracts were separated in SDS-polyacrylamide gel electrophoresis and blotted on membranes. Western blot analysis was performed using phospho-specific antibodies (New England Biolabs, Beverly, MA) to detect pMEK1/2 and pERK1/2. Each sample was probed with anti-MEK1 and anti-ERK2 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) to indicate equivalent protein amounts in all lanes. To detect IκBα, samples were probed with an anti-IκBα antibody (Santa Cruz Biotechnology, Santa Cruz, CA).

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts were prepared by using a non-ionic detergent method as described previously (55). For the detection of gastrin-responsive element (GAS-RE) DNA binding activity, equal amounts of nuclear protein extracts were incubated with labeled oligonucleotides containing the +1 +27 GAS-RE binding site sequence of the human HDC promoter:

5′-ACCCTTTAAATAAAGGGCCCACACTGG-3′

5′-CCAGTGTGGGCCCTTTATTTAAAGGGT-3′.

The oligonucleotide containing the GAS-RE recognition site was labeled using T4 kinase (Roche Molecular Biochemicals GmbH, Mannheim, Germany) in the presence of [γ-^{32}P]ATP. The DNA binding reactions were performed using a binding buffer containing 10 mM Tris (pH 7.5), 2 μg of poly(dI-dC), 1 μg of bovine serum albumin, 10 mM $MgCl_2$, 100 mM KCl, 1 mM EDTA, 1 mM dithiothreitol, and 4% Ficoll for 20 min at room temperature. For competitions, increasing amounts of an unlabeled oligonucleotide were included in the bandshift reaction. The DNA binding activity of NF-κB was performed with an Igκ oligonucleotide as described previously (55). The DNA binding reactions were performed using a binding buffer containing 20 mM HEPES (pH 8.4), 60 mM KCl, 5 mM dithiothreitol, 1 μg of bovine serum albumin, 2 μg of

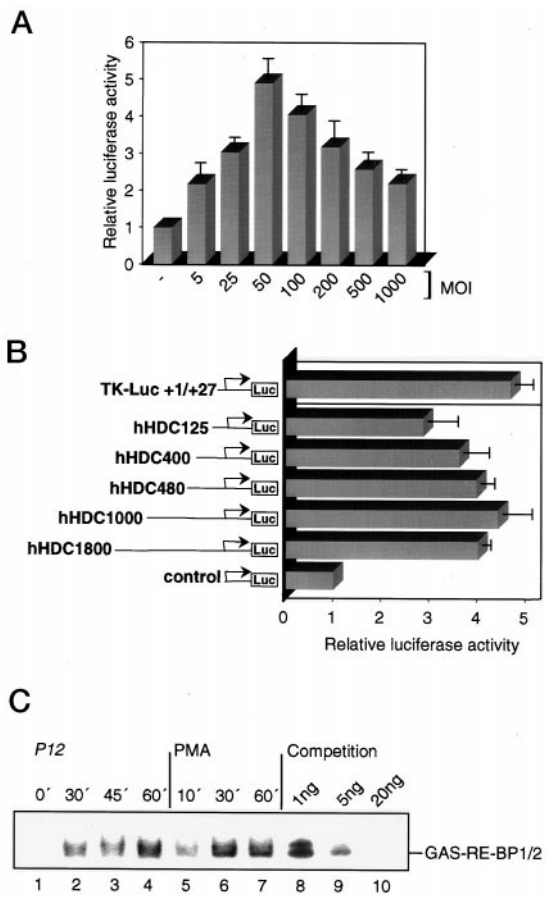

FIG. 1. ***H. pylori* stimulates the transcriptional activity of the hHDC promoter.** *A*, AGS cells were transiently transfected with 1 μg of the 1.8-kb hHDC-luciferase construct and colonized with *H. pylori* (*P12*) at different m.o.i. values for 4 h or left untreated (−). *B*, identification of the *H. pylori*-responsive element of the hHDC promoter by 5′-deletion analysis. AGS cells were transiently transfected with a series of hHDC 5′-deletion constructs or with the empty vector (control) and infected with *H. pylori* (*P12*) at a m.o.i. of 50 for 4 h. In addition, a construct in which luciferase expression is under the control of a single copy of the hHDC +1 to +27 bp element was used. Shown is the fold luciferase activity of three independent experiments against the noninfected cells. *C*, *H. pylori* stimulates the binding of nuclear proteins to the hHDC +1 to +27 bp element. To determine the influence of *H. pylori* infection of AGS cells on the binding of nuclear proteins to the hHDC +1 to +27 bp element, the hHDC +1 to +27 bp sequence was used in EMSAs as ^{32}P-labeled probe. Nuclear extracts were prepared from AGS cells after infection with *H. pylori* (*P12*) at a m.o.i. of 50 (*lanes 1–4*) or treatment with 50 nM PMA (*lanes 5–7*) for different periods of time. For competitions, the indicated amounts of an unlabeled +1 to +27 oligonucleotide were used (*lanes 8–10*). Only the sections of protein-DNA complexes of the autoradiograms are shown. The position of the protein-DNA complex is indicated.

poly(dI-dC), and 10% glycerol for 20 min at 30 °C. The reaction products were analyzed by electrophoresis in a 6% polyacrylamide gel using 12.5 mM Tris, 12.5 mM boric acid, and 0.25 mM EDTA (pH 8.3). The gels were dried and exposed to Amersham TM films (Amersham Pharmacia Biotech) at −70 °C using an intensifying screen.

RESULTS

H. pylori Infection Stimulates HDC Promoter Activity through a Minimal 27-Base Pair Promoter Element—AGS cells were transiently transfected with the hHDC1.8kb-luc construct and colonized with *H. pylori* (*P12*) at different m.o.i. values for 4 h. *H. pylori* stimulated hHDC promoter activity in a m.o.i.-dependent manner already at a m.o.i. of 5 compared with noninfected cells (Fig. 1*A*). Further increase of the infection-dose up to a m.o.i. of 50 raised the hHDC promoter activity to maximal stimulation.

To identify the *H. pylori*-responsive element of the hHDC promoter sufficient for the transcriptional response, we analyzed a series of hHDC 5′-deletion constructs (Fig. 1*B*). Removal of nucleotides from the 5′-end down to 125 bp upstream of the Cap site (+1) had no significant influence on the *H. pylori*-induced luciferase activity. Similar results were obtained in cells treated with PMA (data not shown). Because this region comprises the +1 to +27 bp GAS-RE of the hHDC proximal promoter, we used a luciferase construct in which the GAS-RE sequence hHDC +1 to +27 was ligated upstream of the enhancerless herpes simplex virus 1 thymidine kinase (TK) promoter. We found that this element was capable of conferring *H. pylori* responsiveness to the same extend as the longest hHDC 5′-flanking fragments.

To underline that the GAS-RE is activated by *H. pylori* through enhanced binding of nuclear factors, we analyzed the DNA binding activity of nuclear proteins to the +1 to +27 sequence in EMSAs. Enhanced binding of AGS nuclear proteins was observed within 30 min postinfection (Fig. 1*C*, *lanes 1–4*). Treatment of AGS cells with PMA, a strong inducer of hHDC promoter activity, resulted in a strong increase of DNA binding activity of transcription factors within 10 min (Fig. 1*C*, *lanes 5–7*). Because *H. pylori*- and PMA-induced complexes produced identical bandshifts it can be concluded that the *H. pylori*-stimulated complex consists of GAS-RE-binding proteins (GAS-RE-BPs). This was further confirmed by the finding that the complex stimulated by *H. pylori* could be competed away by an excess of unlabeled hHDC +1/+27 bp oligonucleotide (Fig. 1*C*, *lanes 8–10*). Based on these data, *H. pylori* was identified to induce very specific (m.o.i. of 5–50) enhanced hHDC promoter activity through activation of the GAS-RE-BP1/2 transcription factors that bind to the +1 to +27 bp minimal element.

Activation of the hHDC Promoter Is Independent of cag-PAI-encoded Gene Expression—To investigate the role of *cag* genes for activation of the hHDC promoter by *H. pylori*, AGS cells were colonized with different isogenic mutants lacking certain *cag* genes. AGS cells transfected with the 1.8-kb hHDC-luc construct exerted after infection with *H. pylori* wild type strains (*P12* and *G27*) a strong increase in luciferase activity (Fig. 2*A*, *left panel*). Similar activation was obtained in AGS cells, infected with isogenic mutants, lacking the *vacA, cagA**, and *cagI* genes. Additionally, the isogenic *H. pylori* strain *PAI*, which lacks the entire PAI, potently induced the hHDC promoter activity, supporting the notion of a *cag*-independent activation of the hHDC promoter by *H. pylori*. In contrast, in AGS cells transfected with a reporter gene construct in which luciferase expression was under control of the NF-κB consensus element of the HIV promoter, *H. pylori* mutants lacking *cagA*-cagI* genes or the entire PAI did not stimulate reporter gene expression (Fig. 2*A*, *right panel*). The *cag*-independent activation of the HDC promoter activity was also investigated by EMSA. Enhanced DNA binding of the GAS-RE-BP1/2 to the HDC enhancer element was observed in AGS cells infected with any *H. pylori* (Fig. 2*B*, *lane 1–8*, *left panel*). Using the Igκ-NF-κB oligonucleotide to detect NF-κB DNA binding activity, colonization with the *H. pylori* strains *P12*, *vacA*, and *G27* induced strong activation of NF-κB (Fig. 2*B*, *lanes 1–3* and *6*, *right panel*), whereas the knockout mutants *cagA**, *cagI*, and *PAI* showed a strongly reduced NF-κB DNA binding activity (Fig. 2*B*, *lanes 4*, *7*, and *8*, *right panel*).

H. pylori-stimulated hHDC Promoter Activity Involves Activation of the ERK/MEK Kinases—To study the signaling that is induced by *H. pylori* in a *cag*-independent manner, we investigated the capability of *H. pylori* to induce activation of certain MAP kinase pathways. Subconfluent monolayers of

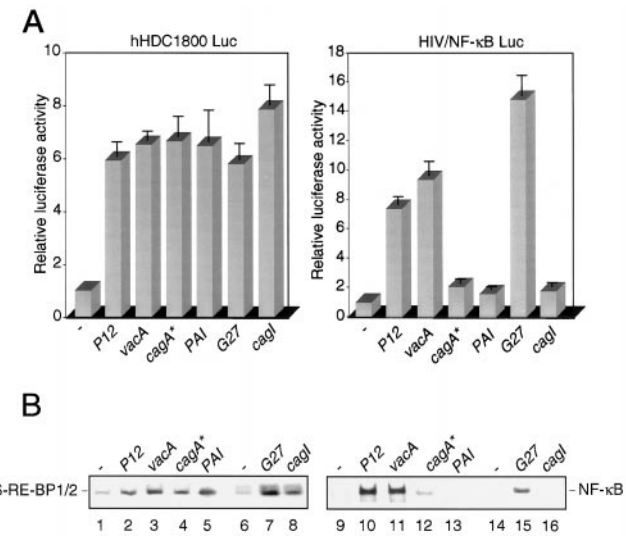

FIG. 2. ***H. pylori*-induced activation of the hHDC promoter is independent of the expression of *vacA* and *cag* genes.** *A*, AGS cells were transfected with 1 μg of the 1.8-kb hHDC promoter luciferase construct and infected with *H. pylori* strains *P12* and *G27*, their isogenic mutants *vacA*, *cagA**, *PAI*, and *cagI* at a m.o.i. of 50 for 4 h, or left untreated (−) (*left panel*). As a control, the effect of *H. pylori* on NF-κB-dependent transactivation was analyzed in AGS cells transfected with 0.5 μg of a HIV-NF-κB luciferase reporter construct (*right panel*). Results of three independent experiments are expressed as fold induction compared with the control. *B*, the influence of various isogenic *H. pylori* mutants on the binding of nuclear factors to the hHDC +1 to +27 bp element was analyzed after infection of AGS cells with *H. pylori* strains at a m.o.i. of 50. For EMSAs, nuclear extracts were prepared after a 60-min infection with *H. pylori* strains and analyzed for DNA binding activity to the hHDC +1 to +27 bp sequence using this sequence as a probe (*left panel*). As a control, nuclear extracts were analyzed for NF-κB DNA binding activity using a ^{32}P-labeled probe representing a consensus Igκ NF-κB binding site. For these experiments, nuclear extracts were prepared after a 90-min infection with *H. pylori* strains (*right panel*). Only the sections of protein-DNA complexes of the autoradiograms are shown. The positions of protein-DNA complexes are indicated with *arrows*.

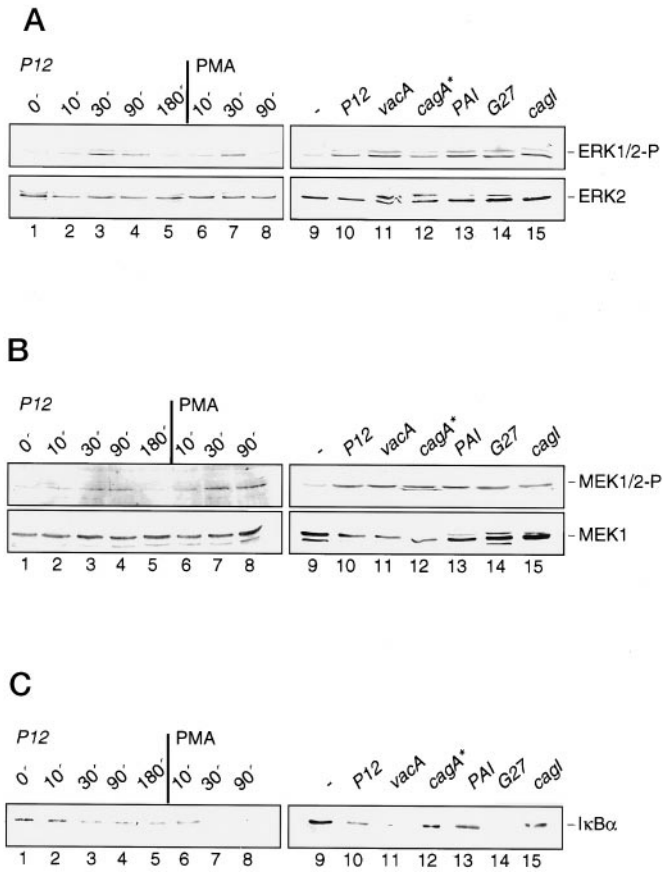

FIG. 3. **Infection with *H. pylori* stimulates phosphorylation of ERK1/2 and MEK1/2 kinases.** AGS cells were infected with *H. pylori* (*P12*) at a m.o.i. of 100 or stimulated with 50 nM PMA for the indicated time points (*left panels*). Studying the *H. pylori* mutants, AGS cells were infected for 30 min (*right panels*). Cells were lysed, and 30 μg of lysates were separated by SDS-polyacrylamide gel electrophoresis and blotted onto membranes. Phosphorylation of ERK1/2 (*A*) and MEK1/2 (*B*) was detected using phospho-specific antibodies in Western blot analysis (*upper panels*). As a loading control, the same amounts of the cell lysates were blotted and probed with non-phospho-specific anti-ERK2 and anti-MEK1 antibodies (*lower panels*). Additional bands, beside ERK2 and MEK1, observed in some lanes represent a cross-reactivity with other antigens recognized by the antibodies. *C*, as an additional control we determined the IκBα abundance in response to *H. pylori* colonization using an IκBα antibody for immunodetection. The positions of the recognized proteins are indicated.

AGS cells were infected with *H. pylori* (*P12*), and cell lysates were prepared after different time points postinfection and analyzed for activated and phosphorylated ERK1/2 and MEK1/2 by Western blot analysis using phospho-specific antibodies. *H. pylori* activated both ERK1 and ERK2 in AGS cells within 30 min after infection (Fig. 3*A*, *lanes 1–5*), comparable to the activation induced by stimulation with PMA (Fig. 3*A*, *lanes 6–9*). Similar results were obtained using phospho-specific antibodies to detect activated MEK1/2. MEK1 and MEK2 were also phosphorylated within 30 min after *H. pylori* infection or PMA treatment (Fig. 3*B*, *lanes 1–9*). To investigate whether the *H. pylori*-induced activation of the ERK/MEK kinases is *cag*-independent, we studied the effects of the *H. pylori* mutants on the activation of ERK1/2 and MEK1/2. In contrast to the AP-1-activating kinase pathway (13) the infection of AGS cells with *H. pylori* knockout mutants (*vacA*, *cagA, PAI*, and *cagI*) in all cases stimulated activation of ERK1/2 and MEK1/2 (Fig. 3*A* and *B*, *right panel*). As a control, we used the same extracts to analyze the *H. pylori*-infected cells for degradation of the NF-κB inhibitor IκBα. Colonization of AGS cells with *H. pylori* (*P12*) for different periods of time resulted in decreasing amounts of IκBα (Fig. 3*C*, *lanes 1–5*) as well as treatment of the cells with PMA (Fig. 3*C*, *lanes 6–8*). Corresponding to the NF-κB activation (Fig. 2*B*, *lower panel*), IκBα is degraded in response to infection with *H. pylori* strains *P12*, *vacA*, and *G27* (Fig. 3*C*, *lanes 10*, *11*, and *14*) but remains unaffected after infection with *H. pylori* strains *cagA**, *PAI*, and *cagI* (Fig. 3*C*, *lanes 12*, *13*, and *15*). These data indicate that *H. pylori* infection has the capacity to induce ERK/MEK activation independent of *H. pylori cag* gene expression.

To examine whether these *H. pylori*-activated MAP kinases are involved in the upstream signaling regulating the hHDC promoter activity, we cotransfected AGS cells with the 1.8-kb hHDC-luciferase construct and dominant negative ERK1 (DNERK1) and ERK2 (DNERK2) constructs. Infection of AGS cells with *H. pylori* or PMA treatment resulted in a 3-fold induction of the HDC promoter activity compared with non-treated cells (Fig. 4*A*, *left panel*). After cotransfection of single inhibitory kinase constructs (DNERK1 or DNERK2), the *H. pylori*- and PMA-stimulated transactivation activity of the HDC promoter was completely inhibited, just as after expression of both DNERK1 and DNERK2 together. As an additional control we used the AGS-B cell line, which has been stably transfected with the human cholecystokinin-B/gastrin receptor (30). Cells exposed to gastrin reacted with a strong increase of the hHDC promoter transactivation activity after 12 h, which was diminished by expression of DNERK1 and DNERK2 mutants (Fig. 4*A*, *left panel*). The selective effect of the DNERK1 and DNERK2 kinase constructs was demonstrated using a

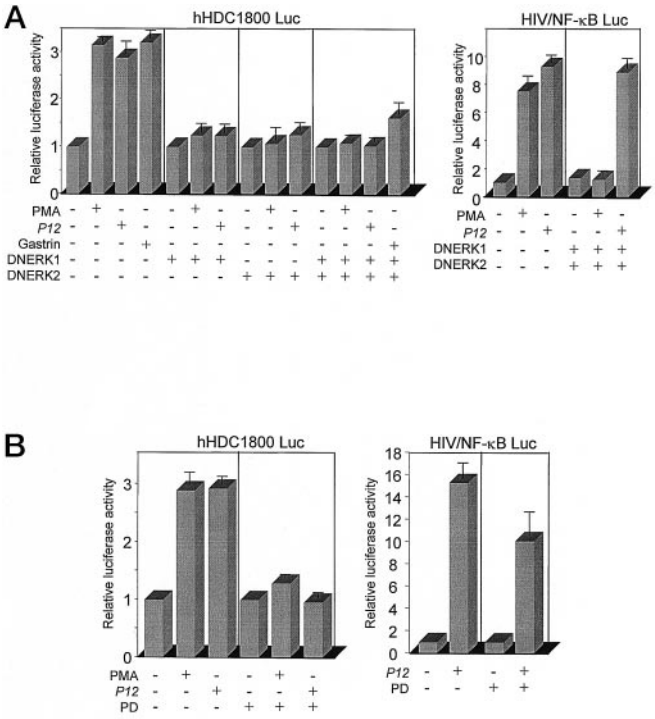

FIG. 4. ***H. pylori*-induced hHDC promoter activity involves ERK and MEK kinases.** *A*, AGS cells were transfected with 1 μg of the 1.8-kb hHDC promoter luciferase construct and either 0.5 μg of a dominant-negative ERK1 mutant (DNERK1), 0.5 μg of an ERK2 mutant (DNERK2) (or in combination 0.25 μg for each construct), or empty vector. The total amount of plasmid DNA was kept constant. Transfected cells were either infected with *H. pylori* (*P12*) at a m.o.i. of 50, treated with 50 nM PMA for 4 h, treated with 10^{-7} M gastrin (AGS-B cells) for 12 h, or left untreated (*left panel*). Further, as a control AGS cells were transfected with a NF-κB-dependent luciferase reporter construct (HIV/NF-κB Luc) and DNERK constructs (*right panel*). *B*, to determine the role of the upstream ERK-activating kinase MEK, AGS cells were transfected with 1 μg of the 1.8-kb hHDC reporter construct and treated with 50 μM PD98059 (*PD*) for 30 min followed by infection with *H. pylori* (*P12*) at a m.o.i. of 50 or treatment with 50 nM PMA for 4 h (*left panel*). Further, as a control, AGS cells were transfected with a NF-κB-dependent luciferase reporter construct and treated as described above (*right panel*). The data represent the means ± S.D. calculated from three independent experiments as fold induction compared with the control.

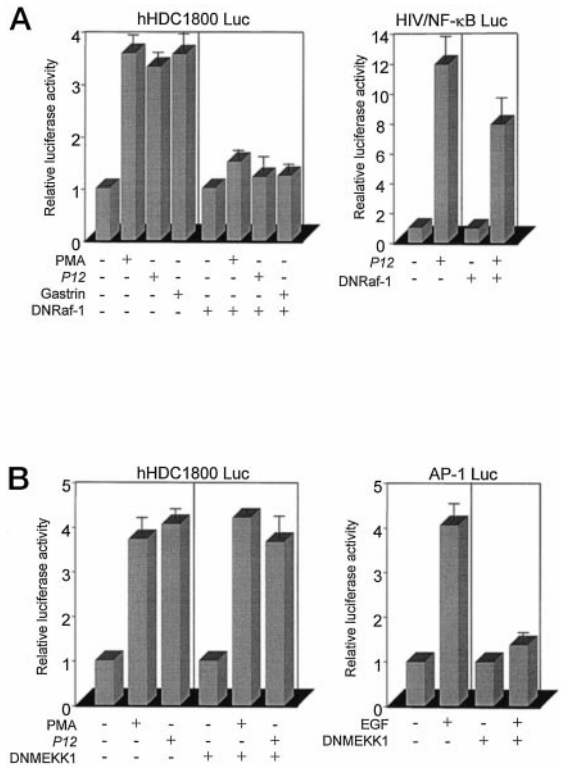

FIG. 5. ***H. pylori*-stimulated activation of the hHDC promoter involves Raf-1 but not MEKK1.** *A*, AGS cells were transfected with 1 μg of 1.8-kb HDC reporter construct and either 0.5 μg of dominant negative Raf-1 (DNRaf-1) or empty vector. Transfected cells were either infected with *H. pylori* (*P12*) at a m.o.i. of 50, treated with 50 nM PMA for 4 h, treated with 10^{-7} M gastrin (AGS-B cells) for 12 h, or left untreated (*left panel*). As a control, AGS cells were transfected with 0.5 μg of the HIV-NF-κB reporter reporter construct and 0.5 μg of DNRaf-1 (*right panel*). *B*, AGS cells were transfected with 1 μg of the 1.8-kb HDC reporter construct and either 0.5 μg dominant negative MEKK1 (DNMEKK1) or empty vector. Transfected cells were colonized with *H. pylori* (*P12*) at a m.o.i. of 50 or treated with 50 nM PMA for 4 h, or left untreated (*left panel*). As a control, AGS cells were transfected with 1 μg of an AP-1 luciferase reporter construct and 1 μg of DNMEKK1 and stimulated with 200 ng/ml EGF for 6 h (*right panel*). Results of three independent experiments are expressed as fold induction of untreated cells.

luciferase construct driven by a HIV-NF-κB enhancer element. In contrast to the PMA-induced NF-κB transactivation, overexpression of dominant negative mutants of ERK1 and ERK2 did not block the *H. pylori*-induced NF-κB transactivation (Fig. 4*A*, *right panel*). These data indicate a strong and selective involvement of ERK kinases in the HDC promoter-activating pathway.

To block MEK activation in response to *H. pylori* colonization, we preincubated the AGS cells with the MEK-selective inhibitor PD98059. Pretreatment of AGS cells for 30 min with 50 μM PD98059 resulted in a total inhibition of the hHDC promoter transactivation activity induced by *H. pylori*, indicating the involvement of MEK in the upstream signaling of the hHDC promoter activity (Fig. 4*B*, *left panel*). The selectivity of the targeted kinases was also investigated using a HIV-NF-κB luciferase construct. The *H. pylori*-induced NF-κB transactivation was not affected by PD98059 pretreatment (Fig. 4*B*, *right panel*).

Raf-1-dependent Activation of the hHDC Promoter in Response to H. pylori Colonization—Possible upstream activators of MEK1/2 are represented by molecules like Raf-1 or MEKK1 (MEK kinase 1) (46). Cells expressing a DNRaf-1 construct and infected with *H. pylori* or treated with PMA or gastrin were analyzed for *trans*activation activity of the hHDC promoter. Compared with mock-transfected cells, the hHDC promoter transactivation activity was strongly reduced (Fig. 5*A*, *left panel*), whereas expression of DNRaf-1 had no influence on the activation of NF-κB induced by *H. pylori* (Fig. 5*A*, *right panel*). These data lead to the suggestion that the Raf-1 kinase lies upstream in the specific signal pathway leading to MEK/ERK-directed activation of the hHDC promoter. The possible role of MEKK1 in the *H. pylori*-induced activation of the hHDC promoter was investigated using constructs expressing DNMEKK1. Colonization of transiently transfected AGS cells with *H. pylori* or treatment with PMA induced hHDC promoter activity that was not affected by overexpression of dominant negative MEKK1 (Fig. 5*B*, *left panel*). To show the functional dominant negative effect of the MEKK1 construct, we cotransfected the DNMEKK1 cDNA with the AP-1 luciferase construct. Expression of DNMEKK1 inhibited the EGF-induced AP-1 activation (Fig. 5*B*, *right panel*).

Activation of the H. pylori-induced hHDC Promoter Activity Based on a Ras-independent Signaling—The small G-protein Ras is one known upstream regulator of Raf-1. In the following we studied whether Ras activation contributes to the stimulation of the hHDC promoter activity. To explore the capacity of Ras to induce *H. pylori*-mediated hHDC promoter activity, we

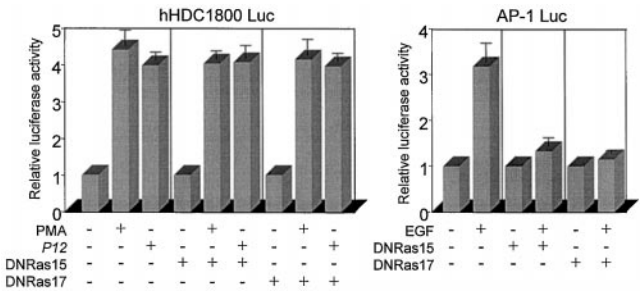

FIG. 6. ***H. pylori* transactivates the hHDC promoter through a Ras-independent mechanism.** AGS cells were transfected with 1 μg of the 1.8-kb hHDC-luc construct and with 0.5 μg of the dominant negative Ras mutants DNRas15 or DNRas17 or empty vector. Transfected cells were infected with *H. pylori* (*P12*) at a m.o.i. of 50 or treated with 50 nM PMA, or left untreated for 4 h (*left panel*). As a control, AGS cells were cotransfected with 0.5 μg of the AP-1 luciferase reporter construct and 0.5 μg for the dominant negative Ras mutants DNRas15 or DNRas17, respectively, and treated with 200 ng/ml EGF for 6 h (*right panel*). Results of three independent experiments are expressed as fold induction compared with the activity in the absence of *H. pylori*, PMA, or EGF.

tested if dominant negative Ras (DNRas15 and DNRas17) blocks activation of the hHDC promoter. In contrast to the inhibition by DNERKs and DNRaf-1, neither DNRas15 nor DNRas17 blocks the *H. pylori*-induced hHDC promoter activity (Fig. 6, *left panel*), whereas the EGF-induced AP-1 transactivation activity was inhibited significantly by expression of DNRas15 and DNRas17 (Fig. 6, *right panel*).

DISCUSSION

In the present study we demonstrated that colonization of a permanent gastric epithelial cell line with *H. pylori* stimulates the transcriptional activity of the hHDC promoter *in vitro*. Furthermore, we found that the transactivating effect of *H. pylori* is independent of the *vacA* gene and genes encoded by the *cag*-associated pathogenicity island, which have been implemented in more severe clinical outcomes of chronic gastric *H. pylori* infections (1, 7, 8). The analysis of *cis*- and *trans*-activating factors involved in *H. pylori*-dependent hHDC regulation revealed that a proximal element at + 1 to +27 bp, which has been identified previously to be responsible for gastrin-dependent regulation of the hHDC gene, is also mediating the effect of *H. pylori* on the hHDC promoter (53, 54).

H. pylori has been shown to stimulate the expression of proinflammatory cytokines such as IL-1β, IL-6, tumor necrosis factor-α, and IL-8 (18, 19). Studies about *H. pylori*-induced IL-8 secretion (11, 14, 15) demonstrated that activation of NF-κB is a central mechanism through which *H. pylori* transactivates the IL-8 gene promoter, whereas the *H. pylori*-dependent transactivation of the hHDC promoter does not involve NF-κB. The proximal hHDC +1 to +27 element does not display any homology to known transcription factor binding sites, and competition studies with oligonucleotides representing consensus binding elements of various known transcription factors demonstrated that this element is not bound by NF-κB or other well characterized nuclear factors such as AP-1, Sp1, or CREB (53). The view that *H. pylori*-dependent transactivation of the hHDC promoter does not require NF-κB is substantiated further by the finding that isogenic *H. pylori* mutants (*cagA*, *PAI*, *cagI*), which were not able to transactivate a NF-κB-luciferase reporter gene construct, stimulated the binding of nuclear proteins to the hHDC +1 to +27 element and transactivated a hHDC 1.8-kb luciferase reporter construct in AGS cells. Therefore, it can be concluded that the hHDC +1 to +27 bp element represents a new nuclear target sequence of *H. pylori* through which the bacterium can influence gene expression independent of *vacA*- or PAI-encoded genes in gastric epithelial cells (Fig. 7).

Previous analysis of the nuclear proteins regulating the hHDC +1 to +27 bp element demonstrated that it is bound by two so far unknown transcription factors (54). These proteins with a molecular size of 35 and 52 kDa, respectively, were termed GAS-RE-BPs because their binding to the +1 to +27 element is indispensable for full gastrin responsiveness of the hHDC promoter (54). Similar to gastrin and PMA, *H. pylori* infection of AGS cells stimulated binding of nuclear proteins to this element. EMSA analysis of *H. pylori*- and PMA-stimulated AGS cells demonstrated that the nuclear proteins stimulated by both factors displayed identical bandshifts. Additionally, the *H. pylori*-induced complex binding to the hHDC +1 to +27 sequence could be competed away with an excess of cold +1 to +27 bp oligonucleotide. Therefore, it appears very likely that the transcription factors binding to the +1 to +27 bp element in response to *H. pylori* represent the previously described GAS-RE-BPs. Because the GAS-RE-BPs represent two novel transcription factors, further analysis of these factors may lead to the identification of genes that have so far not been linked to the epithelial response to *H. pylori*. Further, our findings indicate that in addition to the well characterized *vacA* and *cag* genes, *H. pylori* expresses (a) virulence factor(s), which enable(s) the bacterium to activate alternative target genes. The fact that this/these factor(s) appear to be independent of genetic regions that have been associated with enhanced pathogenic potential of *H. pylori* strains indicates that additional gene loci outside the so far characterized virulence factors may contribute to the overall pathogenesis of *H. pylori* on the gastric mucosa.

To understand the molecular pathways by which the effect of *H. pylori* colonization of AGS cells is transmitted into the nucleus, we analyzed signal transduction cascades involved in *H. pylori*-dependent hHDC transactivation. We found that the transactivating effect of *H. pylori* is transmitted via a signaling cascade comprising ERKs and their upstream activating kinases MEK and Raf-1, respectively. ERKs and MEKs are activated by *H. pylori* colonization with a similar time course showing peak phosphorylation after 30 min of infection. In contrast to the effect of *H. pylori* on the hHDC promoter, activation of NF-κB and AP-1 by the bacterium strictly required *cag* gene expression (13). Therefore, the virulence factor(s) underlying *H. pylori*-dependent activation of Raf-1/MEK/ERK-dependent signaling cascades resulting in enhanced HDC transcription appear(s) to act independently of the bacterial factors controlling the acute cytokine response featuring activation of stress response signaling pathways and NF-κB activation (Fig. 7). Our results indicate that different virulence factors of *H. pylori* are capable of activating distinct branches of the MAP kinases signaling system, which appear to result in transactivation of different epithelial target genes. This observation further suggests that dependent on the virulence factors expressed, *H. pylori* strains may be able to elicit a differential epithelial signaling response, which may lead to transactivation of a specific set of genes.

A recent study described that incubation of KATO III gastric carcinoma cells with *H. pylori* supernatants resulted in inhibition of ERK-signaling stimulated by EGF and that this effect was vacuolating toxin A-dependent (56). In contrast to this study, we found that exposure of AGS cells to two different strains of intact *H. pylori* (*P12* and *G27*) resulted in enhanced MEK/ERK phosphorylation. Furthermore, in contrast to the findings by Pai *et al.* (56), the effect of *H. pylori* on the MEK/ERK cascade was vacuolating toxin A-independent. Although we did not investigate the effect of *H. pylori* on EGF-stimulated ERK signaling in the AGS model, the robust ERK/MEK phos-

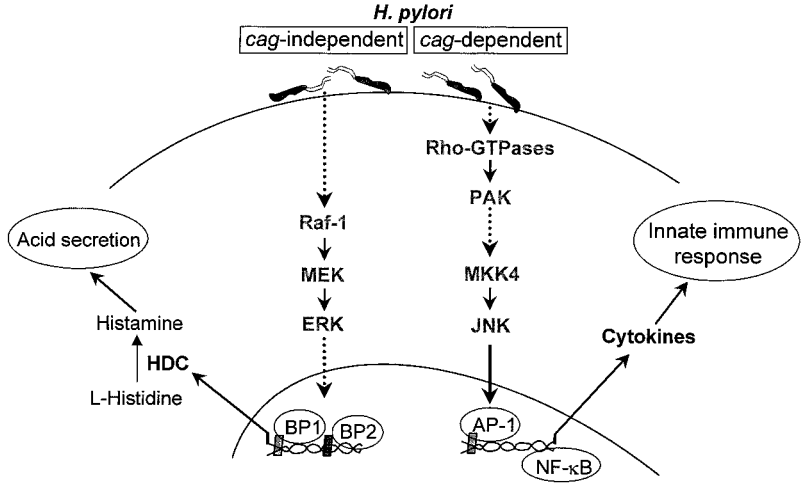

FIG. 7. **Model of the signal transduction pathways induced by *H. pylori* in gastric epithelial cells leading to acid secretion and an innate immune response.** The *H. pylori*-directed signaling comprises the activation of the HDC promoter and the promoters of proinflammatory cytokines and other immunomodulatory genes. Infection with *H. pylori* induces in a *cag*-independent manner the activation of a MAP kinase cascade (Raf-1/ERK/MEK) resulting in an activation of two novel transcription factors (BP1/2). In a *cag*-dependent manner, *H. pylori* induces stress response kinase signaling, which leads to the activation of the immediate early response transcription factors AP-1 and NF-κB, low molecular mass Rho-GTPases, and sequential activation of the protein kinases PAK, MKK4, and JNK, which directs c-Jun phosphorylation controlling AP-1 activation. The *solid arrows* indicate direct activation of downstream targets, and the *dotted arrows* indicate indirect activation through an unknown component.

phorylation in response to intact *H. pylori* demonstrates that the bacterium is capable of stimulating this mitogenic pathway. Activation of the MEK-1/ERK cascade by classical mitogens such as EGF is typically induced through activation of Ras and Raf-1 (46). Because application of two different dominant negative Ras mutants did not influence the transactivating effect of *H. pylori* on the HDC promoter, the functional involvement of Ras in this context is unlikely. Previous studies from our laboratory demonstrated that alternatively to the Ras/Raf-1 sequence, the MEK/ERK cascade in AGS cells can also be activated through a Ras-independent, protein kinase C/Raf-1-dependent pathway (49). Whether protein kinase C-dependent signaling events are involved in the coupling of the Raf/MEK/ERK cascade to upstream signaling events triggered by *H. pylori* is currently under investigation in our laboratory.

Based on the clinical data currently available, the exact impact of *H. pylori*-stimulated HDC transcription on the overall pathophysiology of *H. pylori* in the stomach is currently unclear and has to be further clarified in *in vivo* studies. It can be speculated that dependent on the onset, duration, and/or intensity of activation, enhanced *H. pylori*-stimulated hHDC transcription could contribute to either tissue damage or processes of mucosal restitution and repair in the stomach (32, 33, 37, 38). Furthermore, a direct effect of *H. pylori* on HDC gene expression resulting in enhanced histamine production and secretion could help to maintain an acidic gastric environment, favoring the survival of *H. pylori* over non-acid-resistant bacteria. Overall, *H. pylori*-dependent transactivation of the hHDC gene represents a new molecular model for the interaction of the bacterium with gastric epithelial cells. Our data for the first time demonstrate that *H. pylori* is capable of activating a gastric target gene through an ERK-dependent signaling pathway, independent of vacuolating toxin A- and PAI-related virulence factors. Therefore, a detailed analysis of the molecular mechanisms underlying the effect of *H. pylori* on the hHDC gene could contribute to a better understanding of the molecular pathogenesis of *H. pylori* infections and could probably lead to new approaches for the treatment of *H. pylori*-associated gastric diseases.

Acknowledgments—We appreciate the generous supply of plasmid constructs by John K. Westwick, Melanie H. Cobb, Richard A. Maurer, Kathleen Kelly, Natalie G. Ahn, Michael Karin, John M. Kyriakis, and Geoffrey M. Cooper.

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Helicobacter pylori Activates the Histidine Decarboxylase Promoter through a Mitogen-activated Protein Kinase Pathway Independent of Pathogenicity Island-encoded Virulence Factors

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J. Biol. Chem. 2000, 275:3629-3636.
doi: 10.1074/jbc.275.5.3629