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Chromatin and DNA methylation dynamics of *Helicobacter pylori*-induced COX-2 activation

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ABSTRACT

COX-2 expression is altered in gastrointestinal diseases. *Helicobacter pylori* (*Hp*) infection may have a critical role in COX-2 disregulation. We undertook this study to investigate possible chromatin and DNA methylation changes occurring early during COX-2 gene activation as a direct consequence of *Hp*–gastric cells interaction. We show that *Hp* infection is followed by different expression, chromatin and DNA methylation changes including: (i) biphasic activation of COX-2 gene; (ii) rapid remodulation of HDACs expression and activity, increased acetylation and release of HDAC from COX-2 promoter; (iii) transient gradual increase of H3 acetylation and H3K4 dimethylation and decrease of H3K9 dimethylation; (iv) late and long-lasting increase of H3K27 trimethylation; (v) rapid cyclical DNA methylation/demethylation events at 8 specific CpG sites (−176, −136, +25, +36, +57, +82, +198, +231) surrounding the COX-2 gene transcriptional start site. Our data indicate that specific chromatin and DNA methylation changes occur at COX-2 gene in the first phases of *Hp* exposure in cultured gastric cells as a primary response to host–parasite interaction.

Introduction

Helicobacter pylori (*Hp*) is a Gram-negative, spiral-shaped microaerophilic bacterium that is involved in several gastric diseases. Individuals infected by *Hp* develop gastritis, and up to 10% of infected individuals develop duodenal ulcer disease. Persistent infection with *Hp* may cause chronic atrophic gastritis, with development of intestinal metaplasia, dysplasia, and gastric carcinoma (Parsonnet et al., 1991; Erkisi et al., 1997; Komoto et al., 1998; Wang et al., 1998; You et al., 2000; Peek and Blaser, 2002). This carcinogenesis pathway is reinforced by experiments in animal models (Peek and Blaser, 2002; Watanabe et al., 1998; Zheng et al., 2004). In 1994, the International Agency for Research on Cancer classified *Hp* as a carcinogenic agent class I.

The mechanism of *Hp* pathogenicity is not well understood, although both bacterial virulence and host susceptibility factors have been associated with the development of chronic gastric inflammation and gastric carcinogenesis (Peek and Blaser, 2002; Watanabe et al., 1998; Zheng et al., 2004; Gerhard et al., 1999; van Doorn et al., 1998; Franco et al., 2008).

Hp, through TLRs receptors, induces expression of host inflammatory genes such as TNF-α, which in turn activates NF-kB, a transcription factor whose target genes include COX-2 (Chang et al., 2004; Suganuma et al., 2006; Romano et al., 1998; Takahashi et al., 2000). It is well established that COX-2 expression is upregulated in *Hp* gastritis and some gastrointestinal cancers (Fu et al., 1999; Lim et al., 2000; Eberhart et al., 1994). In gastric cancer, COX-2 expression is involved in several tumor progression-related mechanisms, such as angiogenesis, inhibition of apoptosis and invasiveness (Saukkonen et al., 2003). Therefore, COX-2-oriented therapy was proposed and showed promising anticancer efficacy (Buecher et al., 2005).

It has been suggested that COX-2 expression may be regulated by epigenetic mechanisms. DNA methylation status of COX-2 has been associated with the clinical outcome of gastric cancer (de Maat et al., 2007). In particular COX-2 gene hypomethylation correlates with COX-2 hyperexpression and poor prognosis. Moreover, transcriptional activity of the COX-2 gene is associated with increased histone H3 and H4 acetylation (Park et al., 2004; Nie et al., 2003). Therefore, epigenetic mechanisms may play a critical role in the progression of gastric cancer strongly influencing the clinical outcome.

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A critical and still unanswered question is whether epigenetic changes observed in gastric diseases may be a direct consequence of *Hp* infection. To date, a few studies have addressed the possibility that *Hp*-induced host target genes activation may be controlled by epigenetic mechanisms. One recent study indicated that a change in H3S10 phosphorylation status at IL-6 gene promoter is related to *Hp* infection through the NF-kB/ERK/p38 pathway (Pathak et al., 2006).

In this work, we hypothesized that *Hp* infection may have a direct impact on the epigenetic status of COX-2 gene in gastric cells and that the *Hp*-induced COX-2 activation may be mediated by rapid epigenetic changes at COX-2 gene-regulatory regions. Here, we show that a complex series of epigenetic events that include both chromatin and DNA methylation modifications, accompany *Hp*-induced COX-2 activation in gastric epithelial cells.

Materials and methods

Human gastric epithelial cell culture

Since a normal human gastric epithelial cell line is not available, we used MKN28 cell line derived from a human gastric tubular adenocarcinoma and showing moderate gastric-type differentiation. This cell line has proven to be a suitable in vitro model for the study of interactions between *Hp* and the gastric epithelium (Motoyama et al., 1986; Romano et al., 1988). MKN28 cells were grown as monolayers in DMEM Ham's nutrient mixture F-12 (1:1; Sigma, St. Louis, MO) supplemented with 10% FCS (Life Technologies, Inc.) at 37 °C in a humidified atmosphere of 5% CO_2.

Bacterial strains and coculture conditions

Hp strains used in this study were *Hp* wild-type strains 60190 (ATCC 49503, VacA$^+$/*cag* PAI$^+$) and CCUG 17874 (National Collection of Type Cultures, London, England, 11637, VacA$^+$/*cag* PAI$^+$) containing the *cag* pathogenicity island and secreting an active form of VacA (Le'Negrate et al., 2001). In contrast, the Hp mutant strain Tx30a (ATCC 51932, VacA$^-$/*cag* PAI$^-$) (Atherton et al., 1995) lacks the *cag* pathogenicity island and is unable to translocate CagA product into the host gastric epithelial cells and produces a VacA protein that fails to induce vacuolation in vitro (Aras et al., 2003; Cover et al., 1990). In addition, we also used *E. coli* strain DH5α (Stratagene).

Bacteria were grown in brucella broth (DIFCO Laboratories, Detroit, MI) supplemented with 1% Vitox (Oxoid, Basingstoke, United Kingdom) and 5% FCS (Life Technologies, Inc., Paisley, United Kingdom) for 24–36 h at 37 °C in a thermostatic shaker under microaerobic conditions. Bacteria were harvested by centrifugation and added to gastric cells at concentration of 5×10^7 CFU/ml in DMEM supplemented with 10% FCS at a multiplicity of infection (MOI) of 100. Cells were incubated in the absence (controls) or in the presence of bacteria for the indicated times. As controls, we also used the CCUG 1784 (VacA$^+$/*cag* PAI$^+$) *Hp* strain, the VacA$^-$/*cag* PAI$^-$ *Hp* strain Tx30a and the *E. coli* strain DH5alpha (Stratagene). The cells were suspended in phosphate-buffered saline (PBS), and the density was estimated by spectrophotometry (*A*405) and by microscopic observation. To avoid the influence of serum, gastric cells were serum-starved for 16 h before and throughout the period of treatment in all experiments.

Preparation of cell extracts and Western blot analysis

Antibody for COX-2 or IkB-a (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and horseradish peroxidase-conjugated secondary antibody against goat or rabbit IgG (Amersham Pharmacia Biotech, Buckinghamshire, UK) were used. Western blot analyses of each sample were performed at least 3 times. Protein levels were quantified using the software Quantity One (Bio-Rad). Detailed methods are reported in Supporting Information.

RNA analysis by quantitative real-time PCR

The comparative method of relative quantification ($2^{-\Delta\Delta Ct}$) (Livak and Schmittgen, 2001) was used to calculate expression levels of each target gene, normalized to the housekeeping gene G6PD. Detailed methods and primer sequences are reported in Supporting Information.

HDAC activity assay

Assays were performed using the colorimetric HDAC activity assay kit (Upstate) according to the manufacturer's instructions. The detailed method is reported in Supporting Information.

Chromatin immunoprecipitation (ChIP) assays

Detailed methods and primer sequences are reported in Supporting Information.

Sodium bisulfite treatment

The detailed method is reported in Supporting Information.

DNA methylation analysis by MALDI-TOF

DNA methylation analyses were performed using SEQUENOM MassARRAY platform. This system utilizes MALDI-TOF mass spectrometry in combination with RNA base-specific cleavage (MassCLEAVE) (Ehrich et al., 2005). A detectable pattern is then analyzed for methylation status.

Detailed methods and primer sequences are reported in Supporting Information.

Results

Hp infection induces activation of COX-2 gene in MKN28 gastric cells.

Hp infection of gastric mucosa leads to activation of the COX-2 gene (Takahashi et al., 2000). In order to investigate the early transcriptional events occurring at the human COX-2 promoter gene upon induction by *Hp*, we utilized the human gastric epithelial cell line MKN28 as a model. MKN28 cells were grown to confluence, incubated with bacterial suspension of the *Hp* 60190 (wild-type) strain or the mutant *Hp* Tx30a or the *E. coli* (DH5alpha), and then the levels of COX-2 mRNA were measured. A time-dependent increase of COX-2 mRNA in response to *Hp* infection was found (Fig. 1A). COX-2 mRNA expression showed a first peak after 60–120 min of infection, a second more pronounced peak at 12 h, and declined after 24 h (Fig. 1A). The same experiments using a different *Hp* strain (CCUG 17874) gave comparable results (data not shown) indicating that the observed pattern of COX-2 induction was not related to a specific strain. Moreover, when MKN28 cells were incubated with a VacA$^-$/cagPAI$^-$ *Hp* strain (Tx30a), a comparable increase in COX-2 expression was observed (Fig. 1A) showing that the COX-2 activation pattern was not dependent on VacA/CagPAI status. Finally, to determine whether similar effects were induced by different Gram-negative bacteria, we studied the effects of *E. coli*. The infection of cells with *E. coli*, DH5a strain, did not have any effect on COX-2 expression (Fig. 1A) suggesting that the observed effects were

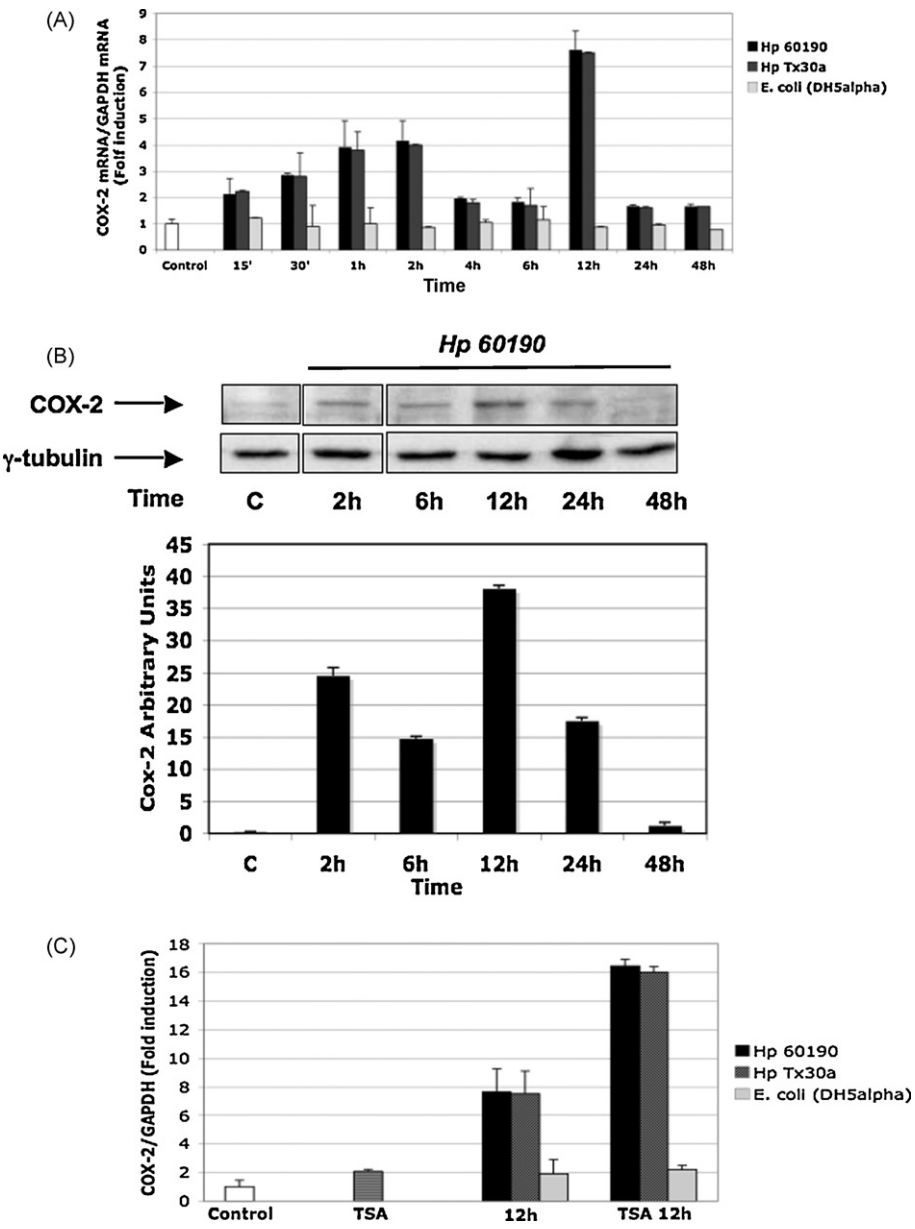

Fig. 1. *Hp* induces expression of the COX-2 gene. MKN28 cells were grown to confluence and incubated with DMEM (control) or with *Hp* 60190, *Hp* Tx30a, and *E. coli* (DH5alpha). (A) Total RNA was isolated at indicated time points after infection and used in real-time PCR reactions. The COX-2 levels were evaluated relative to time point 0 (control) and normalized to GAPDH levels. Data points represent the average of triplicate determinations ± SD. Similar results were obtained in 3 independent experiments. (B) Lysates were collected at the indicated time points after infection with wild-type *Hp* in RIPA buffer, and 50 μg of protein samples were loaded for electrophoresis. Top: The expression levels of COX-2 were detected using antibody against COX-2. The levels of γ-tubulin were used to demonstrate equal loading. Bottom: Protein levels were quantified using the software Quantity One (Bio-Rad). The COX-2 protein levels were normalized to γ-tubulin levels and expressed as relative to untreated control cells (C, control). Data points represent the average of 3 independent experiments ± SD. A representative blot is shown. (C) MKN28 cells were stimulated with *Hp* 60190, *Hp* Tx30a, or *E. coli* (DH5alpha) in the presence or absence of 10 mM tricostatin A (TSA) for 12 h. Total RNA was isolated and used in real-time PCR reactions. The COX-2 levels were evaluated relative to control (untreated and not infected MKN28 cells) and normalized to GAPDH levels. Data points represent the average of triplicate experiments ± SD.

specific for *Hp*. Western blot analyses showed that the levels of COX-2 protein increased following a pattern consistent with that predicted by the mRNA levels (Fig. 1B).

Role of histone acetylation on Hp-induced COX-2 expression

To test the involvement of histone acetylation on the *Hp*-induced COX-2 expression, we treated MKN28 cells with an inhibitor of histone deacetylases (tricostatin, TSA) and measured COX-2 mRNA levels by real-time PCR (Fig. 1C). Both basal and wild-type *Hp*-induced COX-2 mRNA levels at 12 h showed a 2-fold increase upon TSA treatment indicating a role of histone acetylation state in COX-2 gene regulation. Because the infection with a VacA$^-$/cagPAI$^-$*Hp* mutant strain (Tx30a) showed similar effects (Fig. 1C) while the infection with *E. coli* did not show any changes in COX-2 expression, we conclude that the observed effects were specific for *Hp* and not dependent on the VacA/cagPAI status. We then measured the levels of HDAC-1, -2, and -3 mRNAs after *Hp* infection. Constitutive expression of HDAC-1, -2, and -3 mRNAs was detectable in untreated MKN28 cells (Fig. 2A–C). Interestingly, *Hp* infection induced a time-dependent reduction in the levels of all 3 histone deacetylases, which were partially restored after 24 h. Then, we analyzed the global HDAC activity in nuclear extracts of MKN28 cells exposed or not exposed to *Hp*. We found a time-dependent decrease of HDAC activity in exposed cells compared to non-exposed MKN28 (Fig. 2D). Taken together, these data

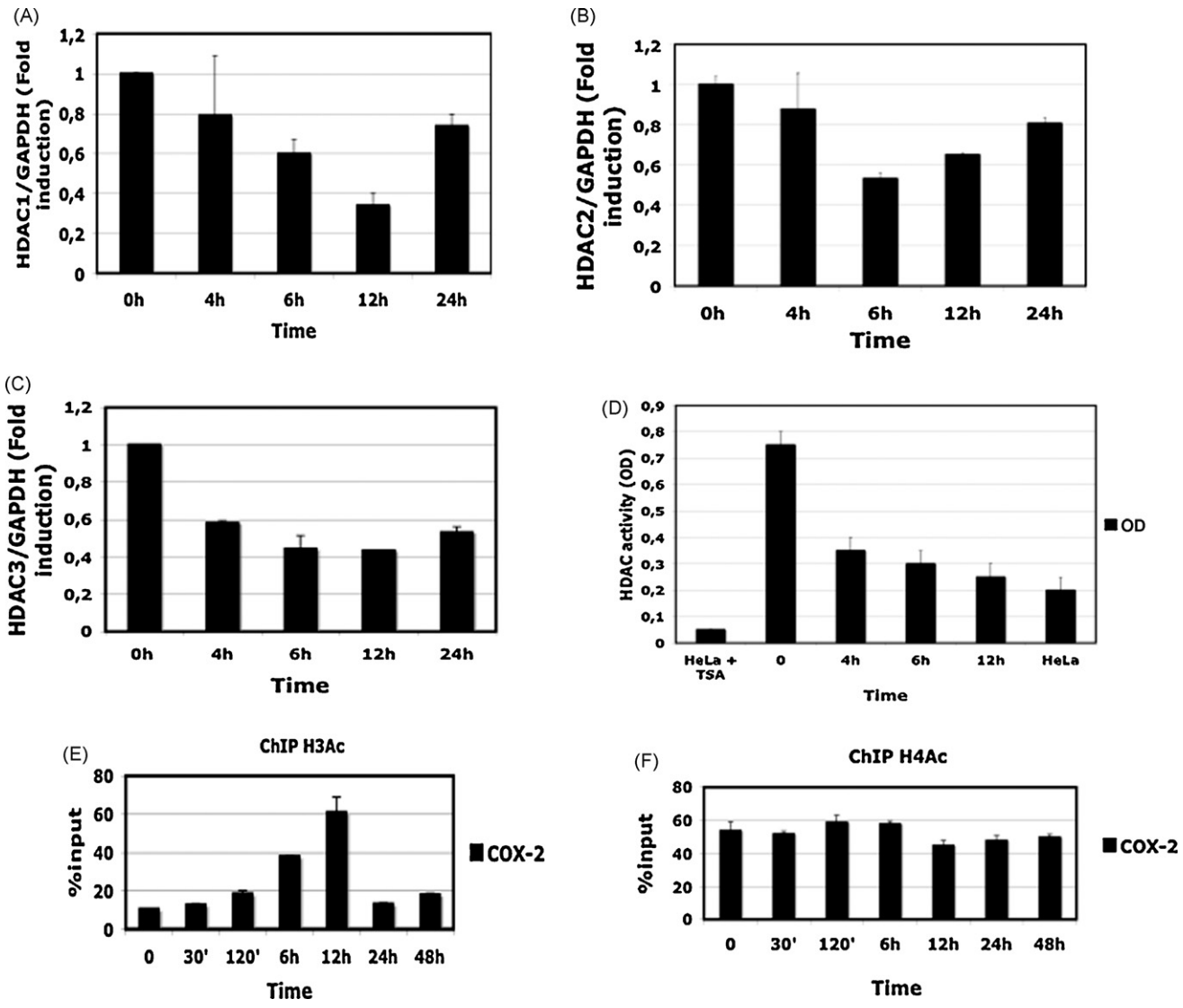

Fig. 2. *Hp* exposure downregulates HDAC-1, -2, and -3 expression and global HDAC activity and induces acetylation of histone H3 at the COX-2 promoter. MKN28 cells were grown to confluence and exposed to *Hp* for the indicated time points. Total RNA was isolated and used in real-time PCR reactions which were evaluated for HDAC-1 (A), HDAC-2 (B), HDAC-3 (C), and GAPDH mRNA levels (A–C). The HDAC-1, -2 and -3 mRNA levels were evaluated relative to time point 0 and normalized to GAPDH levels. Data points represent the average of triplicate experiments ± SD. (D) HDAC activity assays were performed in MKN28 cells after exposure to *Hp* for 0, 4, 6, and 12 h. The HDAC activity was measured in triplicate with a HDAC colorimetric histone deacetylase activity assay kit (Upstate Biotechnology). The nuclear extract of HeLa, treated or not treated with 10 mM Trichostatin A for 12 h, represents negative and positive controls, respectively. Data are expressed as relative OD values per μg of protein sample and are presented as a mean of 3 independent experiments ± SD. (E–F) Chromatin from MKN28 cells was prepared at the indicated time points after infection with *Hp*. Shown are results of ChIP analysis using anti-acetyl-H3 (E) or anti-acetyl-H4 antibodies (F). Recovered DNA fragments were quantified by real-time PCR. Average % input ± SD from 3 independent experiments are plotted.

indicate that *Hp*-induced transcriptional activation of COX-2 gene expression in gastric epithelial cells is accompanied by reduction of HDAC-1, -2 and -3 expression and global histone deacetylase nuclear activity.

To determine whether the decreased HDAC activity and expression corresponded to increased levels of histones H3 or H4 acetylation in the promoter region of COX-2, we performed a chromatin immunoprecipitation (ChIP) analysis. Cells were incubated with *Hp* for different times, and chromatin was immunoprecipitated with anti-acetyl-H3 and acetyl-H4 antibodies; then, PCR amplifications were performed using promoter-specific primers (see Supplementary Fig. S1 and 'Materials and methods' section). We found that upon infection of MKN28 with *Hp*, the H3 acetylation state was modulated. The histone H3, initially lowly acetylated, was highly acetylated after 12 h while the deacetylated state was restored after 24 h (Fig. 2E). Hyperacetylation of histone H3 is in agreement with the expression pattern of the COX-2 gene. In contrast, the histone H4 acetylation levels remained almost unchanged following *Hp* infection (Fig. 2F).

Effect of Hp on the recruitment of HDAC-1 and HDAC-2 to the COX-2 promoter

Since the expression of COX-2 induced by wild-type *Hp* in MKN28 cells correlates with increased histone H3 acetylation, we hypothesized that *Hp* infection might mediate this effect by reducing the recruitment of HDACs to the COX-2 promoter. To test this hypothesis, we performed ChIP assays, and the immunoprecipitated DNA was quantified by semiquantitative PCR. Both HDAC-1 and -2 were readily detected at the COX-2 promoter (Fig. 3A and B). At 6 h post-infection with *Hp*, we observed a release of HDAC-1, but not HDAC-2, from the COX-2 promoter. Similar results were obtained when MKN28 cells were infected with a VacA$^-$/cagPAI$^-$*Hp* mutant strain (data not shown). These results indicated that the

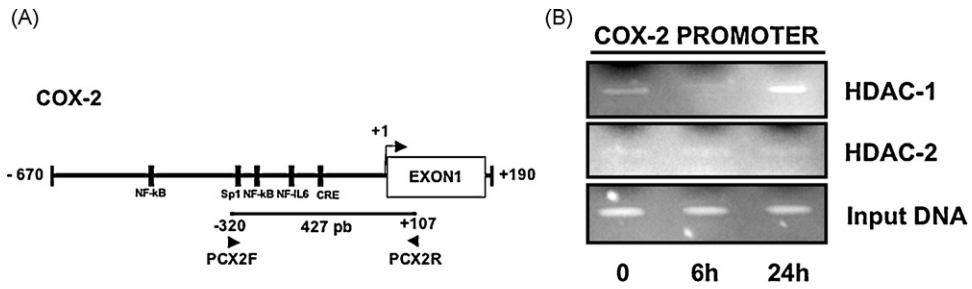

Fig. 3. Time course of *Hp*-induced binding of HDAC-1 and -2 at the COX-2 promoter. (A) Schematic representation of the COX-2 promoter showing the locations of the PCR primer used for ChIP assay. (B) MKN28 cells were grown to confluence and exposed to *Hp* for the indicated time points. Chromatin was harvested and precipitated with anti-HDAC-1 and anti-HDAC-2 antibodies. The relative amount of bound DNA was evaluated by conventional semiquantitative PCR. PCR products were separated by agarose gel electrophoresis and detected by ethidium bromide staining. PCR amplifications of the COX-2 promoter of the total DNA (without prior immune precipitation) were used as a control (Input DNA). Representative gels of 3 separate experiments are shown.

increased H3 acetylation and the induction of COX-2 transcription by *Hp* are accompanied by the release of HDAC-1 from the COX-2 promoter and that these phenomena are not dependent on the VacA/cagPAI status.

Histone methylation at Hp-induced COX-2 gene

To determine whether the induction of the COX-2 gene upon *Hp* infection is accompanied by modifications of the histone methylation state at the COX-2 promoter, antibodies against dimethylated H3K9 (H3K9me2), dimethylated H3K4 (H3K4me2), and trimethylated H3K27 (H3K27me3) were used in ChIP assays. MKN28 cells were infected with *Hp*, and chromatin was prepared at different time points for ChIP analysis. We found that H3K9me2 decreased significantly in gastric epithelial cells by 12 h after *Hp* infection and gradually returned to its basal state at 48 h (Fig. 4A). In contrast, we found that the levels of H3K4me2 were low in non-infected gastric cells, and significantly increased at 12 h after *Hp* infection, returning to a near-basal level by 48 h (Fig. 4B). These results are in agreement with the repressive role of H3K9me2 and with the activating role described for H3K4me2 in gene transcription (Kouzarides, 2007; Jenuwein and Allis, 2001; Turner, 2007; Berger, 2007). By contrast, we found that the H3K27me3 levels, which usually mark a chromatin-repressive state (Kouzarides, 2007; Jenuwein and Allis, 2001; Turner, 2007; Berger, 2007), remained almost unchanged until 6 h post-infection and then significantly and stably increased at the subsequent time points (12, 24, and 48 h, Fig. 4C). This suggests that H3K27me3-repressive mark may serve to limit COX-2 activation and may contribute to the downregulation of the COX-2 gene observed at later times (24 and 48 h) after *Hp* exposure (see Fig. 1).

Because similar results were obtained when MKN28 cells were infected with a VacA$^-$/cagPAI$^-$ *Hp* mutant strain (Tx30a), while the infection with *E. coli* did not show any changes in histone modifications (data not shown), we conclude that also in this case, the observed effects were specific for *Hp* and not dependent on VacA/cagPAI status.

Dynamic changes of CpGs methylation at the COX-2 promoter

It is well established that the COX-2 promoter region is aberrantly methylated in human gastric carcinomas and variation of DNA methylation state may influence the prognosis (de Maat et al., 2007). Thus, we investigated the direct effect of *Hp* exposure on DNA methylation status of the COX-2 promoter. Because it has been recently demonstrated that DNA methylation may transiently and rapidly vary on inducible promoters (Kangaspeska et al., 2008; Métivier et al., 2008) soon after stimulation, we sought to perform an in-depth DNA methylation analysis of the COX-2 promoter at short time intervals after *Hp* exposure. Dynamic variations in the methylation status of 35 CpGs were evaluated, through MALDI-TOF MS technique (Sequenom), on both lower and upper strands, in a large COX-2 regulatory region including the proximal putative NF-kB recognition site (Fig. 5). During the first 2 h of *Hp* exposure, DNA methylation was evaluated at intervals of 7.5 min in order to detect eventual transient changes. Results indicated that a rapid increase in methylation followed by a transient demethylation occurred between 15 and 60 min after *Hp* exposure at 8 CpG sites surrounding the TSS (−176, −136, +25, +36, +57, +82, +198, +231) and exclusively on upper strand. We cannot exclude that further methylation/demethylation cycles may occur after 60 min, but were not easily detectable in unsynchronized cells. No significant changes were detected at the other 27 analyzed CpG sites (data not shown). However, after 2 h, the initial methylation levels were restored even on CpG sites subjected to rapid methylation changes (Fig. 5) and remained almost constant until 48 h (data not shown). These results indicated that the COX-2 promoter region undergoes a rapid cycle of methylation/demethylation at specific CpG sites, but, at least during the first 48 h, no permanent changes in the DNA methylation state occurred. Thus, we believe that the dynamic changes detected during the first 60 min may be part of the mechanism of transcriptional activation, as it has been described for ER-responsive genes (Kangaspeska et al., 2008; Métivier et al., 2008), rather than a stable change in the COX-2 transcriptional program. We hypothesize that the stable aberrant methylation state of the COX-2 promoter observed in various human gastric diseases could be the result of chronic exposure to *Hp* or further different inflammation/tumor-related events.

NF-kB association to COX-2 promoter upon Hp infection

Because the COX-2 promoter contains potential binding sites for NF-kB transcription factor, we investigated by ChIP assays the possible association of NF-kB to the COX-2 gene during *Hp* infection. ChIP assays were performed at different times after *Hp* infection using anti-p65 antibodies and then semiquantitative PCR analyses were performed using specific primers amplifying the COX-2 promoter region (from −320 to +107) containing a NF-kB binding site (Fig. 3A). The maximal binding of p65 to the COX-2 promoter was observed at 30 min and at 4 h time points after *Hp* infection (Fig. 6A). Accordingly, Western blot analysis of IkB-a, a NF-kB inhibitor, demonstrated that the levels of this protein decreased starting from 60 min and were restored after 6 h of *Hp* infection (Fig. 6B). We then performed ChIP assays on the same samples using anti-RNA polymerase II antibodies. Interestingly, RNA polymerase II binding was detected at the greatest levels after 6 h and was undetectable later, at the 24-h time point (Fig. 6A). Altogether, these results suggest that the first peak of COX-2 mRNA levels (1 h) may be related

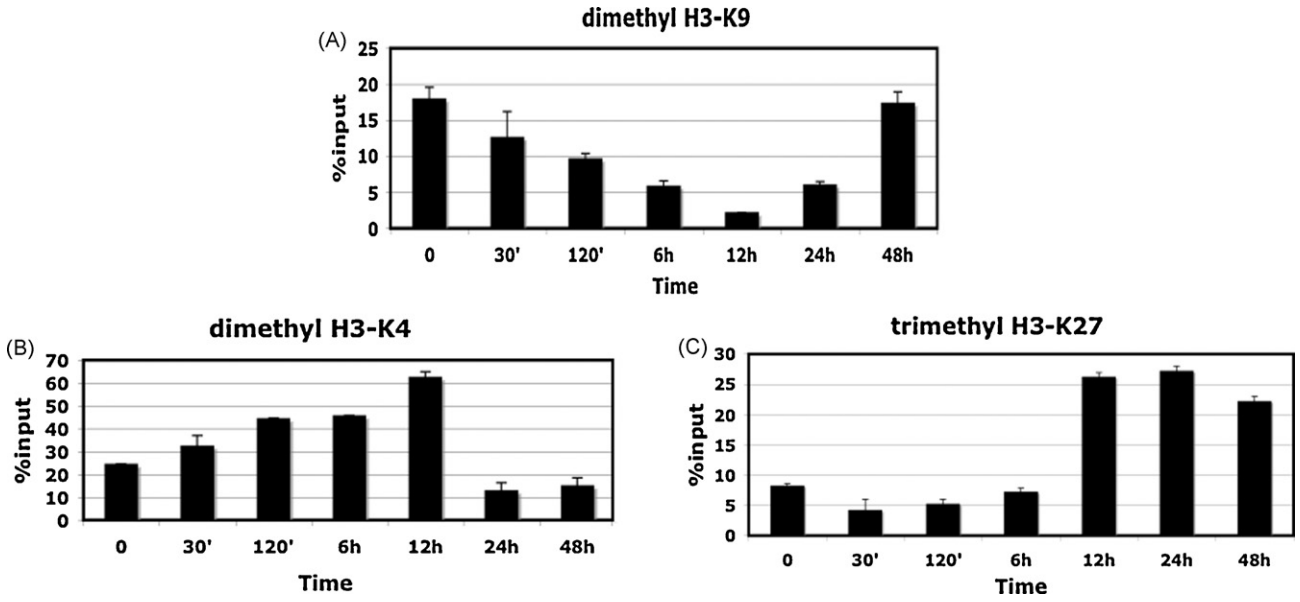

Fig. 4. *Hp* induces histone H3 methylation at the promoter region of COX-2. Chromatin from MKN28 cells was harvested at the indicated time points after infection with *Hp*. Shown are results of ChIP analyses using anti-dimethyl-H3K9 (A), anti-dimethyl-H3K4 (B), or anti-trimethyl-H3K27 (C) antibodies. Recovered DNA sequences were quantified by real-time PCR using primers described in (A). Average % input ± SD from 3 to 4 independent experiments is plotted.

to the above-described rapid dynamic DNA methylation changes while the later peak (12 h) was a consequence of the chromatin configuration changes after the observed histone modifications. Restoration of DNA methylation levels and repressive chromatin marks may lead to transcriptional silencing observed 24 h after *Hp* exposure. Interestingly, the H3K27me3-repressive mark appeared to be induced by *Hp* infection and to be still present after 48 h. This suggests a possible role of this modification as a negative modulator of COX-2 activation, possibly limiting the *Hp*-induced COX-2 expression levels or leaving the gene more refractory to eventual further *Hp* exposure-mediated activation.

Discussion

In this study, we described for the first time the chromatin and DNA methylation changes occurring at the COX-2 gene in gastric epithelial cells during the first phases, and are thus likely direct consequences, of *Hp* exposure. We show that *Hp* infection is followed by several expression, chromatin, and DNA modification events including: (i) biphasic activation of the COX-2 gene; (ii) rapid remodulation of HDACs expression and activity, increased acetylation, and release of HDAC-1 from the COX-2 promoter; (iii) transient gradual increase of H3 acetylation and H3K4 dimethylation and decrease of H3K9 dimethylation peaking in correspondence of the later (12 h) peak of COX-2 mRNA expression; (iv) long-lasting increase of H3K27 trimethylation; (v) rapid cyclical DNA methylation/demethylation events at specific CpG sites at COX-2 gene-regulatory regions mainly in concomitance with the early expression and NF-kB binding peaks (30–60 min).

The exciting recent emerging evidences of bacterial impact on host epigenetics emphasize a novel strategy used by bacterial pathogens to interfere with key cellular processes. Recent few, but illuminating studies demonstrate that bacteria are able to provoke histone modifications and chromatin remodelling in infected cells, thereby altering the host's transcriptional program and in most cases affecting the host innate immune response (Hamon and Cossart, 2008). The effects of lipopolysaccharide (LPS), *Mycobacteria*, *Shigella*, *Listeria*, and *Helicobacter* on some aspects of host epigenetics have been recently reported (Hamon and Cossart, 2008). For *Helicobacter*, it has been shown that the expression of IL-6 is modulated by *Hp* through activation of the ERK/p38/MSK1 cascade that provokes phosphorylation of H3S10 (Pathak et al., 2006), which in turn is necessary for IL-6 activation. Another important aspect linking epigenetic modification to *Hp* infection are the well-established alterations of chromatin and DNA methylation profiles described at several loci in gastrointestinal cancers (de Maat et al., 2007; Perri et al., 2007; Maekita et al., 2006). However, conflicting data are reported about the direct involvement of preexisting *Hp* infection of gastric mucosa as a determining factor for epigenetic alterations observed in gastric diseases. The limits of these kinds of studies are that many events, including inflammation, cancer progression, and stochastic epigenetic changes may occur between *Hp* infection and clinical diagnosis, and thus it is expected that a wide spectrum of epigenetic alterations may be observed in pathologic samples regardless of those directly provoked by the *Hp* infection.

In this work, we have investigated the epigenetic changes occurring at the COX-2 locus during the first 48 h after exposure of human MKN28 gastric cells to *Hp* and, thus directly attributable to host–parasite interaction. We show that MKN28 cells express COX-2 mRNA and proteins weakly under the unstimulated condition, and the expression levels increase dramatically upon *Hp* stimulation concomitantly with the occurrence of several chromatin and DNA methylation changes. Our data indicate that both the expression and chromatin changes at the COX-2 promoters are specific for *Hp* and are not dependent on the VacA/CagPAI status. Thus, it is likely that other bacterial components shared by all the *Hp* strains used in this study, but not by other Gram-negative bacteria, are responsible for the observed phenomena.

A role of histone acetylation in the regulation of the COX-2 gene was previously suggested in studies performed in macrophages or pulmonary cells under different kind of stimulation (Hamon and Cossart, 2008). In this work, we have shown that dramatic transient changes of the histone acetylation machinery, including HDACs expression levels and activity, histone H3 acetylation state, and displacement of HDAC-1 from the COX-2 promoter, accompany the first phases of *Hp* infection of gastric cells. The mechanisms by which *Hp* exposure modulates HDACs expression and activity during the first step of infection in gastric cells remain to be elucidated. The best-characterized link between a bacterial stimulus

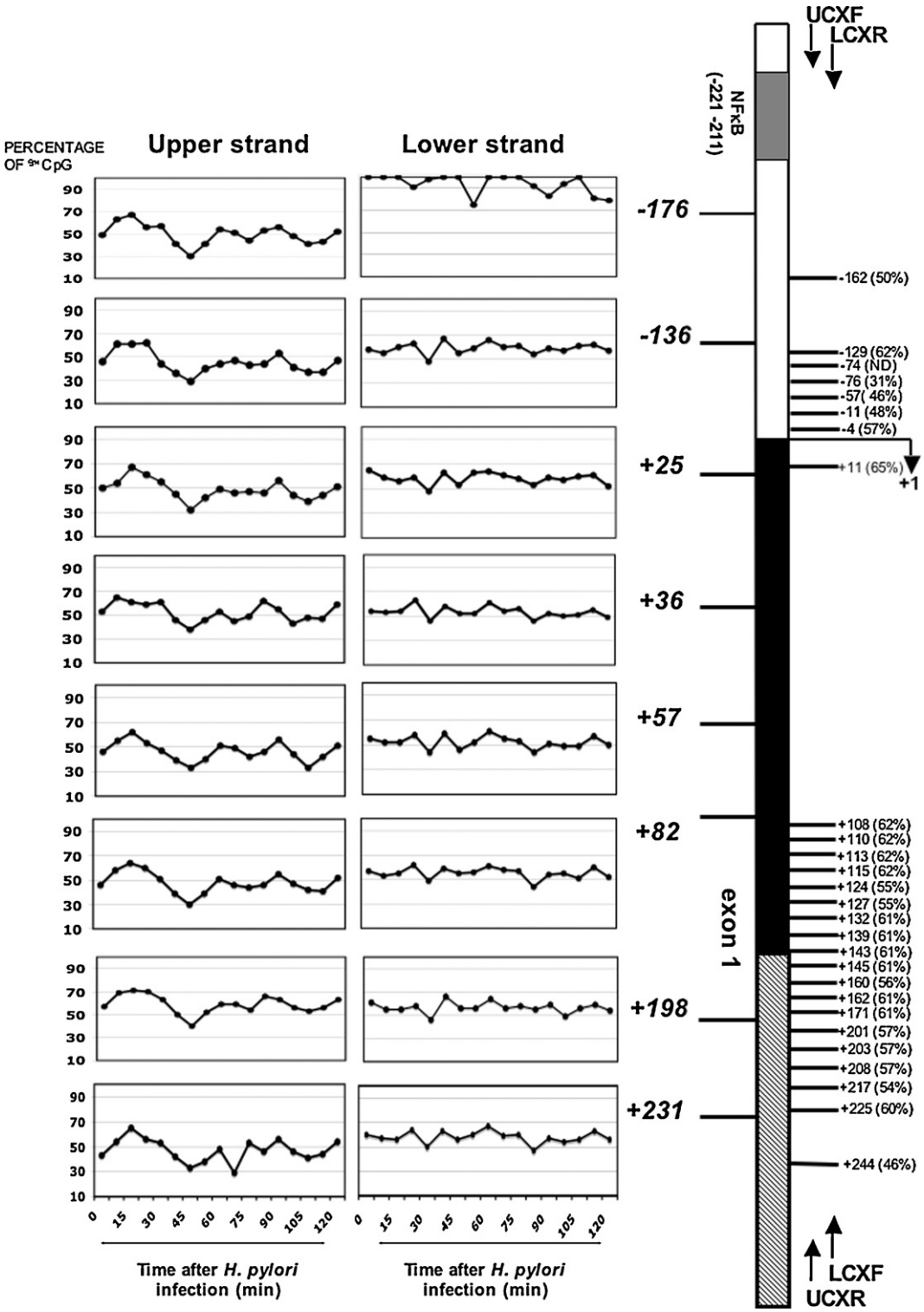

Fig. 5. DNA methylation dynamics at the COX-2 promoter upon *Hp* exposure. Left: Methylation status of individual CpG sites on upper and lower strands as determined by MALDI-TOF methylation analysis. 'Time 0' indicates the initial methylation status before *Hp* infection. The methylation degree was measured at intervals of 7.5 min for 2 h after infection. Right: Diagrammatic representation of the human COX-2 gene. Regulatory upstream region (open box), proximal NF-kB binding site (gray box), exon I (black box), and intron I (striped box) are indicated. Horizontal bars represent the relative positions of each CpG site present in the analyzed region. CpG sites indicated on the left side of the bar represent those showing significant changes in DNA methylation and correspond to the graphs shown in the figure. CpG sites indicated on the right side of the bar represent those showing constant methylation levels (indicated in parentheses). TSS is indicated by an arrow. The relative positions of the primers utilized for amplification are indicated.

and its effect on histones is the MAPK (mitogen-activated protein kinase) cascade, which upon activation leads to phosphorylation of histone H3 on serine 10 (H3S10) which in turn may modulate histone acetylation status (Weinmann et al., 2001; Saccani et al., 2002). Interestingly, it has been previously shown that different *Hp* factors interacting with Toll-like receptor 4 (TLR4) may activate a phosphoacetylation pathway leading to chromatin modifications in host cells (Pathak et al., 2006). TLR4 stimulation may induce his-

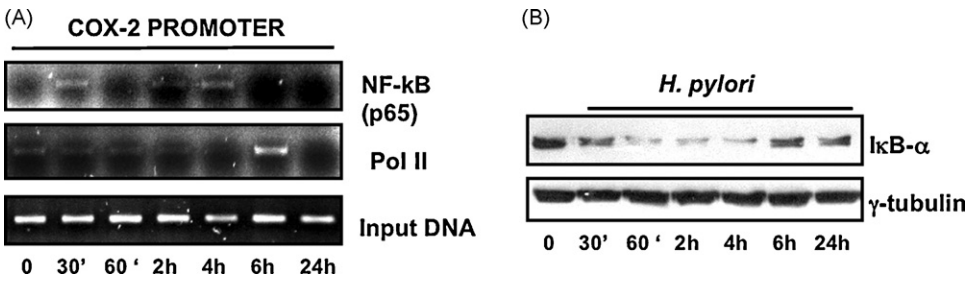

Fig. 6. Time course of *Hp*-induced binding of NF-kB (p65) and RNA polymerase II at the COX-2 promoter. MKN28 cells were grown to confluence and infected with *Hp* at the indicated time points. (A) Chromatin was harvested and precipitated with anti-NF-kB (p65) or anti-RNA-Pol II antibodies, as indicated. After DNA recovery, the precipitates were evaluated by conventional semiquantitative PCR using the COX-2 promoter primers shown in Fig. 3A. PCR products were separated by agarose gel electrophoresis and detected by ethidium bromide staining. PCR amplifications of the COX-2 promoter (without prior immunoprecipitation) were used as a control (Input DNA). (B) IkB-alfa protein levels were detected with a specific antibody in Western blot and simultaneous detection of γ-tubulin demonstrated equal protein loading. Representative gels of 3 separate experiments are shown (A and B).

tone acetylation and H3S10 phosphorylation allowing for NF-kB to gain access to specific inflammatory gene promoters.

Then, we investigated the modifications of the histone methylation state at the COX-2 gene following *Hp* exposure. As a general rule, most reports indicate that the increase in H3K4me2 as well as the decrease in H3K9me2 and H3K27me3 levels mark transcriptionally active chromatin structure (Kouzarides, 2007; Jenuwein and Allis, 2001; Turner, 2007; Berger, 2007). Accordingly, our results indicate that the main drivers of COX-2 activation upon *Hp* exposure are the increase in H3K4me2 levels and the decrease in the H3K9 methylation state. Interestingly, both modifications are transient occurring gradually during the first 12 h, and then the initial histone modification status, low H3K4 and high H3K9 methylation, is restored concomitantly with the decrease of COX-2 transcription. Conversely, the modification status of H3K27 during COX-2 activation appears to be non-canonical. In fact, H3K27me3 levels increase consistently at the COX-2 gene after 12 h of *Hp* exposure despite the concomitant detection of the highest COX-2 mRNA levels. Thus, conflicting chromatin modification signals are present at 12 h since we detected a simultaneous presence of histone modifications associated with transcriptional repression (high H3K27me3) and activation (high H3K4me2 and low H3K9me2). Noticeably, this transient condition resembles the so-called 'bivalent chromatin domain' that has been recently shown to be associated in particular with developmental genes in embryonic stem cells (Bernstein et al., 2006). Most interestingly, while the methylation state of H3K4 and H3K9 is restored at the initial degree within 24 h, the high H3K27me3 levels remain high at least up to 48 h after *Hp* infection possibly underlying a more refractory state of the COX-2 gene in cells exposed to *Hp* with respect to unexposed cells. A similar phenomenon, called LPS tolerance, has been described in macrophages exposed to LPS (Hamon and Cossart, 2008; Chan et al., 2005; El Gazzar et al., 2007). In fact, when macrophages are exposed to LPS for a short period, a TLR4-mediated chromatin remodelling on LPS target genes occurs allowing NF-kB to bind and activate target promoters. A prolonged exposure to LPS is accompanied by long-lasting H3K9 hypermethylation and a subsequent decreased response of inflammatory

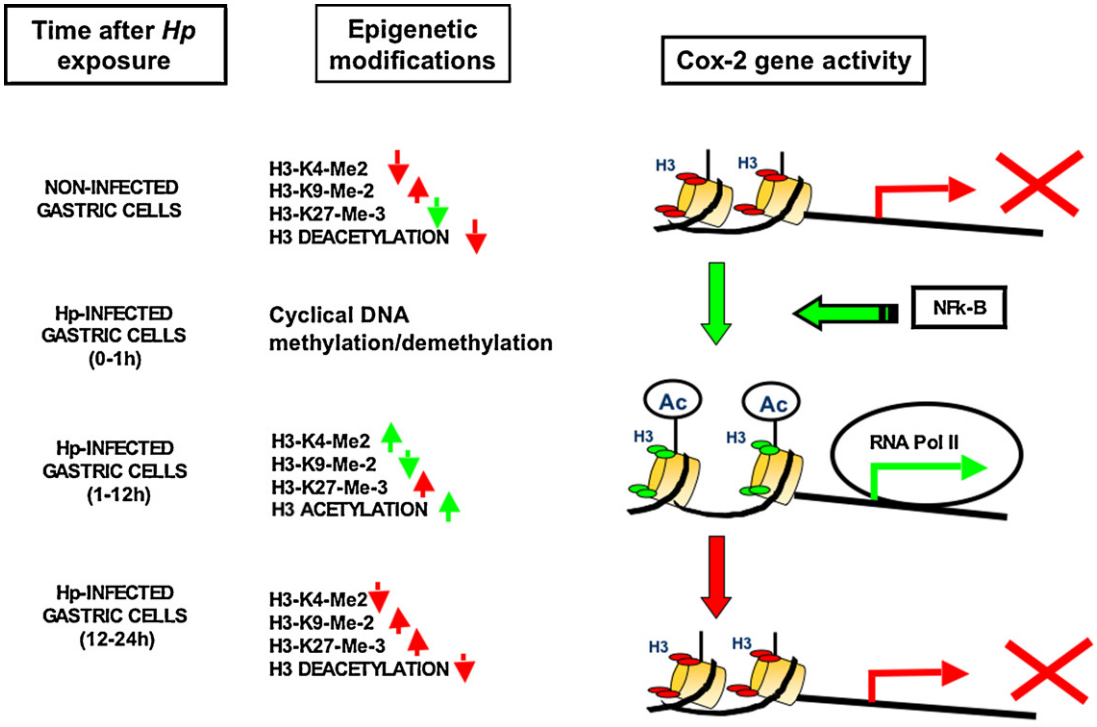

Fig. 7. A schematic model illustrating a possible sequence of events at the COX-2 gene during *Hp*-induced activation. Green arrows represent chromatin modifications associated with open configuration. Red arrows represent chromatin modifications associated with a repressive condensed state. See text for further explanation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

genes to LPS stimulation (El Gazzar et al., 2007). In the present study, the stable repressive mark that we have observed after 48 h of *Hp* infection, H3K27me3, is associated to the Polycomb repressor complex (PrC). Interestingly, PrC may include DNA methyltransferase activity (Viré et al., 2006) and, thus, could make the COX-2 gene more susceptible to possible alterations of DNA methylation profiles.

Finally, one of the most striking findings of our study is that gene activation and most of the chromatin events are preceded by rapid strand-specific DNA methylation/demethylation events at specific CpG sites of the COX-2 gene-regulatory region.

DNA methylation has been considered for long time a simple epigenetic mark generally associated with transcriptional silent, condensed chromatin. Only very recently, evidence of an unanticipated dynamic role of DNA methylation in gene regulation in human cells has been reported for estrogen responsive genes (Kangaspeska et al., 2008; Métivier et al., 2008). The mechanisms underlying the methylation/demethylation cycles at ER-responsive genes have been investigated in-depth and revealed that the demethylation process is initiated by the same enzymes that establish the methylation mark, the DNA methyltransferases DNMT3A and DNMT3B (Kangaspeska et al., 2008; Métivier et al., 2008). The data presented here suggest that a similar phenomenon may be directly initiated at the COX-2 gene promoter upon *Hp* infection of gastric cells. However, it will be very interesting in the near future to investigate whether the precise molecular mechanisms underlying these phenomena may resemble those observed for the estrogen response. Because in MKN28 cells as well as in normal gastric mucosa most of CpG sites at the COX-2 promoter display about 70% methylation degree, the observed transient changes in DNA methylation could be critical for COX-2 derepression. Our data are compatible with a model in which the DNA methylation/demethylation events are triggered by the binding of NF-kB to the COX-2 promoter allowing chromatin to reach an open conformation through modification of histone acetylation and methylation (Fig. 7). Most of DNA methylation and chromatin marks are then restored in a short time after *Hp* infection with the exception of H3K27me3 which remains high, possibly underlying a more condensed chromatin state at the COX-2 gene.

Overall, our data show that specific epigenetic events occur in the first phases of *Hp* exposure in cultured gastric cells as a primary response to host–parasite interaction.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijmm.2010.06.009.

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