UNIVERSITÀ DEGLI STUDI DI NAPOLI **F**EDERICO II

FACOLTÀ DI MEDICINA E CHIRURGIA

SCUOLA DI DOTTORATO IN MEDICINA MOLECOLARE

DOTTORATO DI RICERCA IN GENETICA E MEDICINA MOLECOLARE XXII CICLO

TESI DI DOTTORATO

"Epigenetic dynamics of *Helicobacter pylori*-induced COX-2 activation"

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UNIVERSITÀ DEGLI STUDI DI NAPOLI **F**EDERICO II

DIPARTIMENTO DI BIOLOGIA E PATOLOGIA CELLULARE E MOLECOLARE "LUIGI CALIFANO"

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INTRODUCTION

The eukaryotic genome is assembled as a nucleoprotein complex known as chromatin which is a structural polymer consisting of positively charged histone proteins in addition to DNA. It provides a dynamic platform that controls all DNA-mediated processes within the nucleus. The basic unit of chromatin is the nucleosome that consists of 147 bp of DNA wrapping nearly twice around the octamer, containing two copies of each of core histones H2A, H2B, H3 and H4. Each nucleosome is separated by 10-16 bp linker DNA and this bead on a string arrangement constitutes a chromatin fiber of ~10 nm in diameter. Each core histone within the nucleosome contains a globular domain, which mediates histone-histone interactions and also bears a highly dynamic amino terminal tail approximately 20-35 residues in length and is rich in basic amino acids. These tails extend from the surface of the nucleosome. Histone H2A also has an additional ~37 amino acid, carboxy-terminal domain protruding from the nucleosome. All histone proteins are modified inside the nucleus of the cell, but so far only a few modifications have been studied. A fundamental tenet of biochemistry is that proteins are composed of 20 basic

building blocks. Given the limited range of chemical functionality present in the amino acid side chains, the diversity of protein structure and function is truly extraordinary. In reality, the situation is not very complex: nature uses several modifications of proteins to complement and expand its chemical repertoire. In fact, many distinct posttranslational modifications (PTMs) have been identified to date, and the number and variety of modifications increase continuously. Histones are subject to many PTMs including acetylation and methylation of lysines (K) and arginines (R), phosphorylation of serine (S) and threonines (T), ubiquitination, sumolyation and biotinylation of lysines as well as ADP ribosylation. These modifications are recognized by specific protein-protein modules and can regulate each other as well (Yap and Zhou, 2006). Recent findings have revealed that these histone tails do not contribute to the structure of individual nucleosomes or to their stability but play an important role in folding of nucleosomal arrays into higher order chromatin structure (Paterson and Laniel, 2004). Eukaryotes have developed many histone-based strategies to introduce variation into the chromatin fiber because the chromatin is the physiological template for all DNA-mediated processes, and

these strategies are likely to control the structure and/or function of the chromatin fiber. Two of the most common strategies employed are the PTMs of histones and replacement of major histone species by the variant isoforms (Taverna et al., 2006). Many studies have shown that the site-specific combinations of histone modifications correlate well with particular biological functions, such as transcription, silencing, heterochromatization, DNA repair and replication. These observations have led to the idea of a 'histone code', although the degree of specificity of these codes may vary as particular combinations of histone marks do not always dictate the same biological function. Moreover, there is a clear indication that mistakes in PTMs may be involved in many human diseases, especially cancer (Somech et al., 2004).

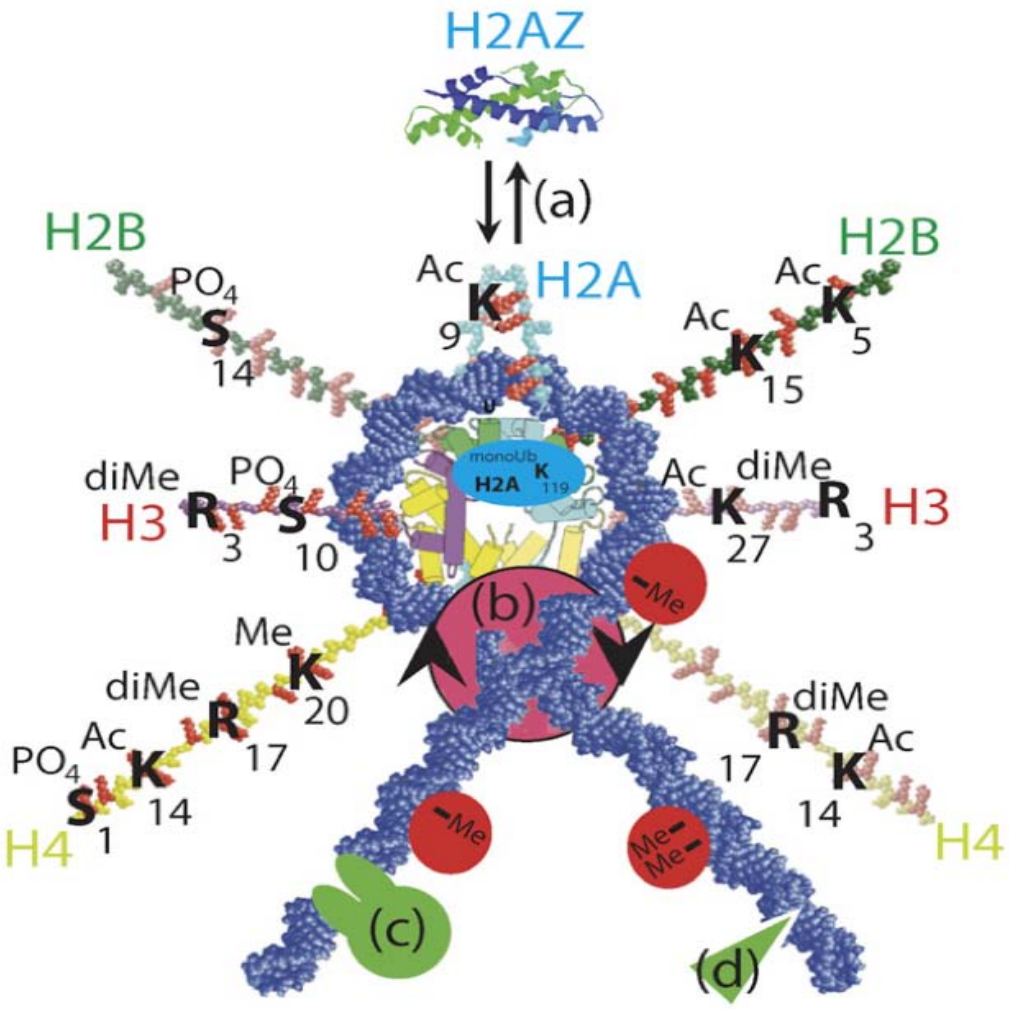

Figura 1 Covalent marks on chromatin: Chromatin consists of repeated units of 146 bp of DNA wrapped 1.7 times around a octamer composed of two copies each of the four core histones; H2a, H2b, H3 and H4. Chromatin provides a structural platform that is subject to extensive post-translational modification. These include methylation, acetylation, phosphorylation and ubiquitination of specific histone residues; methylation of CpG dinucleotides; exchange of histones (a); changes in the relative position of the nucleosome mediated by ATP-dependent remodelling complexes (b); induction of double-stranded DNA breaks by topoisomerase II (c) and the generation of single-stranded DNA breaks by topoisomerase I (d).

HISTONE MODIFICATIONS

More than 35 years ago, it was noticed that transcriptionally active histones are acetylated in vivo, and this led to the speculation that covalent modifications of histones might have a role in determining the states of gene activity (Allfrey et al., 1964). The histone N-terminal tails, exposed on the nucleosome surface, are subject to a variety of enzyme-catalyzed PTMs. As already mentioned, several histone-tail modifications have been identified. Moreover, each lysine residue can accept one, two or even three methyl groups, and similarly an arginine can be either mono- or dimethylated, which adds to the complexity. Although Strahl and Allis (2000) proposed the histone code, it's not clear how the code is established and maintained till now. Many modification sites are close enough to each other and it seems that modification of histone tails by one enzyme might influence the rate and efficiency at which other enzymes use the newly modified tails as a substrate (Chung, 2002). The model proposed by Allis has set a ground for researchers to discover post-translational writers, the enzymes that modify the histones post translationally, and also the readers, the modules or motifs that recognize the

histones based on their modification state (Strahl and Allis, 2000). Although the basic composition of the nucleosome is the same over long stretches of chromatin, the specific pattern of modifications on the nucleosome creates local structural and functional diversity, delimiting chromatin subdomains. Therefore, the challenge of histone PTMs has shifted from identifying sites of modification to identifying combinations of histone modification patterns that dictate specific biological readouts. Currently, the histone code is under a lot of investigation and is gaining experimental support as well. Methylation of DNA at cytosine residues as well as PTMs of histones, including phosphorylation, acetylation, methylation and ubiquitylation, contributes to the epigenetic information carried by chromatin. These changes play an important role in the regulation of gene expression by modulating the access of regulatory factors to the DNA. The use of a combination of biochemical, genetic and structural approaches has been allowed to demonstrate the role of chromatin structure in transcriptional control. The structure of nucleosomes has been elucidated and enzymes involved in DNA or histone modifications have been extensively characterized. Because deregulation of epigenetic marks has been reported in many

cancers, a better understanding of the underlying molecular mechanisms bears the promise that new drug targets may soon be found.

Acetylation

Although it has been known for many years that histones in eukaryotes are modified by acetylation, it is only in the past decade that the role of histone acetylation in transcription regulation has been focused (Grunstein 1997, Kou and Allis, 1998). Of all the histone modifications, acetylation is the most studied one. In the beginning, many of the enzymes responsible for acetylation of histones were known as transcriptional co-activators and later as enzymes. During specific biological processes, selected lysines such as lysine 9 and 14 are acetylated. Acetylation of lysine residues at core histone N-terminal is achieved through enzymes called histone acetyltranferases (HATs). However, the steady state balance of this modification is achieved using the orchestrated action of HATs and one more species of enzymes, namely histone deacetylases (HDACs) (Brownell and Allis, 1996; Kuo and Alis, 1998; Roth et al., 2000). There are two different types of HATs: type A-HATs, which are responsible for the acetylation of histones and are

directly involved in regulating chromatin assembly and gene transcription (Carrozza et al., 2003); type B-HATs, which are cytoplasmic proteins that catalyze histone acetylation in cytoplasm, particularly at lysine 5 and 12 of histone H4, prior histone incorporation into newly repli-cated chromatin (Parthun et al., 1996; Varreault et al., 1998). On the basis of sequence similarity, HATs can be organized into different families. These seem to display different mechanisms of histone substrate binding and catalysis. Some of the families are GNAT, PCAF, GCN5L, MYST etc. (Rosa and Caldas, 2005). Human HDACs are grouped into three classes, HDACs I, II and III, which are based on their primary homology to the three HDACs of yeast. Class I HDACs (HDAC-1, -2, -3, -8 and -11) are homologous to yRPD3 Class II HDACs are homologous to yHDA1 and are further subdivided into two subclasses IIa (HDAC-4, -5, -7 and -9) and its splice variant MITR and IIb (HDAC-6 and -10), which are based on their sequence homology and domain organization. Class III HDACs are homologous to ySIR2 of yeast and bear no homology to class I and II proteins (Verdin et al., 2003). HDACs are the components of large multiprotein complexes.

Methylation

Histone methylation was first reported more than 40 years ago, yet this is among the least understood PTMs. In contrast to acetylation and phosphorylation, the lysine methylation relatively appears to be a stable histone modification. The most heavily methylated histone is H3 followed by H4. Histone K methylation occurs on lysine residues 4, 9, 27 and 36 on H3 and on position 20 on H4. H1 amino terminus also seems to possess K methylation. Therefore, lysine methylation might be providing an ideal epigenetic mark for more long-term maintenance of chromatin states (Jenuwein and Allis, 2001). Histone methyltransferases (HMTs) are the enzymes that regulate the site-specific methylation of lysine residues, for example lysine 9 and lysine 4 in amino terminus of histone H3. It has been reported that the standard HMTs contain the evolutionary conserved 130 amino acid SET-domain and thereby stimulate some of the biological processes such as gene activation or repression (Jenuwein, 2001). There are two classes of methyltranferases depending on their specificity to either lysine (K-HMT) or arginine (R-HMT) (Zhang and Reinberg, 2001). In addition a third class of

HMT has been described in yeast that affects the chromatin silencing, not by methylation of histone tails but instead targets a conserved lysine residue (K79) in the core domain of nucleosome (Khan and Hampsey, 2002). This is a product of DOT-1 (distrupter of telomeric silencing) gene, which is important for silencing (van Leeuwen et al., 2002). DOT-1 doesn't have a SET-domain and acts only when histone H3 is packaged into the nucleosome. The HMT activity of DOT-1 plays a direct role in telomeric silencing. As already mentioned, the extent of histone acetylation is determined by both acetyltransferases and deacetylases. It is unclear whether histone methylation is also regulated by enzymes with opposing activities or in other words demethylases. Shi et al. (2004) provided evidence that LSD1 (KIAA 0601), a nuclear homolog of amine oxidases func-tions as a histone demethylase and transcriptional corepressor. LSD 1 specifically causes demethylation of histone H3 lysine 4, which is linked to active transcription. Demethylases such as LSD1 and JHDM I mediate demethylation of di- and monomethylated histones (Whetsteinet al., 2006). However, it has been unclear whether demethylases that reverse lysine trimethylation exist. Whetstein et al. (2006) have shown that JmjC domain containing protein JMJD2A

reversed trimethylated H3-K9/K36 to dimethylated but not mono- or unmethylated products. In addition to this, JMJD2 subfamily members also functioned as trimethylation-specific demethylases, converting H3-K9Me3 to H3-K9Me2 and H3-K9Me1. So, this family of demethylases generates different methylated states at the same lysine, and thereby provides a mechanism for fine-tuning histone methylation. An increasing number of histone demethylases are being discovered which highlights the dynamic nature of regulation of histone methylation, which is a key chromatin modification. These recent studies have offered glimpses into the specific biological role of demethylases and their potential connections to human diseases (Shi, 2007). Although methylation of histones was initially thought to be an activating or repressing type of modification, it seems to be much more sophisticated than thought initially (Berger, 2007).

Ubiquitination

Histone ubiquitylation is emerging as one of the important modifications. Lys-123 within the carboxy terminal is a substrate for the Rad6 ubiquitin ligase in yeast. This modification has been found to be critical to mitotic and

meiotic growth. However, it is not clear whether it is involved in transcription (Robzyk et al., 2000).

Biotinylation

Recently, a novel PTM of histones has been identified, i.e, biotinylatin of lysine residue (Stanley et al., 2001). The biotinylation or the binding of biotin to histones is catalyzed by biotinidase and holocarboxylase synthetase (HCS) (Narang et al., 2004; Sarath et al., 2004). Biotinidase uses biocytin (biotinyl-E-lysine) as a substrate for biotinylation of histones (Hymes et al., 1995), whereas HCS uses biotin and ATP (Narang et al., 2004). K4, K9 and K18 in histone H3 and K8 and K12 in histone H4 have been identified as the biotinylation sites (Camporeale et al., 2004; Sarath et al., 2004).

DNA METHYLATION

DNA methylation is a covalent modification in which the 5' position of citosine is methylated in a reaction catalyzed by DNA methyltransferases (DNMTs) with S-adenosyl-methionine as the methyl donor. In mammals, this modification occurs at CpG dinucleotides and can be catalyzed by three different enzymes, DNMT1, DNMT3a and DNMT3b. DNA methylation plays a role in the long-term silencing of transcription and in heterochromatin formation. As an epigenetic modification, DNA methylation permits these silenced states to be inherited throughout cellular division.

in the context of DNA methylation, sequences within the genome can be classified into two different groups: CpG poor regions and CpG island. CpG island are defined as being longer than 500 bp and having a CG content grater than 55% and an observed CpG/expected CpG ratio of 0.65 (Takai and Jones, 2002).

CpG islands are often, but not always found in promoter region and about 40% of gene contains CpG islands that are situated at the end of the 5' region (promoter, untranslated region and exon I) (Jones and Baylin, 2002). The rest of the

genome, such as the intergenic and the intronic regions, is considered to be CpG poor. In healthy cells, CpG poor regions are usually methylated whereas CpG islands aregenerally hypomethylated, with a few exeptions including the inactive X chromosome. During the development of cancer, many CpG islands undergo hypermethylation while the CpG poor regions become hypomethylated. This alteration in DNA methylation patterns leads to change in chromatin structure causing the silencing of tumor suppressor genes and instability of the genome (Jones and Baylin, 2002). This change in methylation pattern during cancer is similar to the pattern observed on the inactive X chromosome. The inactive X chromosome is hypermethylated at CpG promoter, but contain less methylation than the active X cromosome at CpG sites located downstream of the promoter (Jones, 1999; Hellman and Chess, 2007).

Traditionally, DNA methylation has been divided into two tipes: de novo and maintenance methylation. De novo methylation is catalyzed by DNMT3a and DNMT3band is important for the establishment of methylation patterns in early embryos, during development and during carcinogenesis (Okano et al., 1999). In order to maintain

these methylation patterns set by de novo methylation, DNMT1 is localized to the replication fork during cellular division and conduct maintenance methylation (Leonhard et al., 1999; Liu et al., 1998). However, DNMT1 has been shown to be inefficient at maintaining the methylation of many CpG dense regions (Liang et al., 2002). therefore, de novo activities of DNMT3a and DNMT3b are also necessary in somatic cells in order to reestabilish the methylation patterns so that they are no lost due to the inefficient activity of DNMT1.

Although much progress has been made in understanding the role of DNA methylation plays in controlling cellular processes, there are still many details that are not fully understood.

GENE EXPRESSION AND SILENCING

There are evidences showing that the modifications of histones gives signals that are recognized by specific binding proteins which in turn influence gene expression and other chromatid functions. Although this idea, when proposed, was a hypothesis around 30 years ago, now it is regarded as an established scientific fact (Turner et al., 1992; Turner, 1993). The role of histone acetylation in transcription regulation has gained a lot of attention. The acetylation of lysine residues at core histone N-terminal weakens the interactions between core histones and DNA, which in turn destabilizes the nucleosomal structure and ultimately facilitates binding of transcription factors to DNA (Berger, 2002). However, such models, which suggest that acetylation of lysines will disrupt the histone DNA interactions and lead to open chromatin structure, do not have actually much evidence. For example, the histone H3 tail contains 13 positively charged amino acids, and only one to four residues get acetylated, which will decrease the positive charge by 10%-30% that is unlikely to perturb the ionic interactions with DNA. In fact, the current view is that the histone marks by themselves may not alter the

nucleosomal dynamics. There is a lot of evidence generating on the view that specific histone modifications control the binding of nonhistone proteins. In addition, several non-histone proteins such as HP1 or the PRC1 polycom complex, not only bind to methylated histone lysines but also recruit the methylase itself and thus provide means for new histone methylation events. So, modified histone tails provide recognition sites for factors involved in either the activation or repression of gene expression. H3 and H4 tails seem to be dominant players in chromatin fiber folding and also in intermolecular fiber-fiber interactions (Moore and Ausio, 1997; Tse and Hansen, 1997). Acetylation/deacetylation modifications of nucleosomal histones play a coordinated role in regulating gene expression. Hyperacetylation favors gene expression, whereas deacetylation by HDACs brings about silencing of the genes. Chen et al. (2002) have shown that histone acetylation significantly enhances the basal and the inducible expression of heat shock gene, hsp-70 in Drosophila melanogaster. It seems that when nucleosomes are at properly silenced genes, they may be stably hypoacetylated but there are several significant points of divergence from prevailing thinking. Clayton et al. (2006)

have found that for immediate early genes, which include many proto-oncogenes, it is not acetylation but turnover that seems to be necessary for efficient induction. HDACs have been implicated as regulators of gene expression, especially during cell-differentiation and development. HDACs do not bind directly to DNA but seem to be recruited to specific promoters through their interaction with DNA sequence-specific transcription factors. By virtue of their ability to interact with distinct transcription factors that each bind at multiple sites in the genome, the HDACs seem to be master regulators controlling differentiation pathways (Verdin et al., 2003). Researchers have used recombinant histone proteins to reconstitute the transcription system (reviewed by Lizuka and Smith, 2003). There is no doubt that the specific histones, templates, activators, and co-activators used in these experimental systems are quite different, and the results are generally consistent with the importance of co-activator mediated histone modification in transcriptional activation (Lizuka and Smith, 2003). Agalioti et al. (2002) have investigated the specificity of H3 and H4 lysine acetylation and the recruitment of specific co-activators for a specific gene Interferon-β (IFN-β). Nucleosomal template for this is reconstituted using

recombinant wild type and mutated histones as well as histone modifications in vitro. The tail lysines at the IFN-β promoter seem to be highly specific both in vivo and in vitro and marks three residues that are H4-K8, H3-K9 and H3-K14. Using mutated and recombinant modified histones, researchers have demonstrated that these acetylations provide distinct signals for the sequential recruitment of bromodomain containing proteins BRG1, a component of SWItch/Sucrose NonFermentable (SWI/SNF) complex (a family of chromatin modifying complexes which has BRG1 as the catalytic unit), and TAFII 250, a component of TFIID. Mutating H4-K8 lysine residue to alanine abolishes the ability to recruit BRG1 while mutating H3-K9 or K14 to alanine loses the ability to recruit TAFII 250. Therefore, the histone acetylation code for IFN-β promoter has been cracked (Agalioti et al., 2002). Early experiments have suggested that H3-K4 methylation is linked to active genes, whereas H3-K9 was linked to inactive genes (Lachner and Jenuwein, 2002). However, Briggs et al. (2001) and Bryk et al. (2002) have shown that SET-1-mediated methylation of H3-K4 is involved in rDNA silencing in budding yeast, and H3-K4 methylation is seen in both active and silenced regions. For some time, this was little confusing how H3-K4

methylation can be associated with both activation and silencing of transcription. Recently, it has been shown using antibodies (that could distinguish between di- and trimethylated H3-K4) that trimethylation is specific for active state of transcription, whereas dimethylated K4 exists in both active and repressive genes (Rosa et al., 2002). So, it is evident that not only the particular site but also the number of methyl groups in a modification appears to play an important role in the functional consequences of histone methylation. H3-K9 methylation characterizes repressed chromatin. G9a is considered to be the major eukaryotic HMT responsible for demethylation of H3-K9 (H3K9me2) at transcriptionally active sites (Rice et al., 2003). G9a targets specific genes where it functions to repress transcription (Chen et al., 2006). Methylated H3-K9 provides a binding platform for heterochromatin protein 1 (HP1). In turn, HP1 associates with a variety of other factors including suv39H1, HDACs, transcriptional repressors and chromatin remodeling enzymes (Hiragami and Festenstein, 2005). Smallwood et al. (2007) have tried to understand the relationship between HP1 family proteins and histone and DNA methyltransferases. In mammals, DNA methylation is controlled by DNMT1, DNMT3a and DNMT3b. DNMT3b

plays a role in the maintenance of methylation and DNMT1 is involved in de novo methylation in specific cases. Smallwood and his colleagues focused on HMT G9a and DNMT1 and found that the HP1 family members directly interact with DNMT1. This interaction results in a functional stimulation of DNMT1 methyltransferase activity. HP1 is sufficient to target DNMT1 activity in vivo, and HP1 dependently requires DNMT1. These researchers demonstrated that HP1 is recruited to the promoter of Survinin gene in a DNMT1-dependent manner. They support a model whereby HP1s mediate the cooperative silencing of DNA and HMTs. An emerging concept is that HATs and HDACs are housed in large multi-protein complexes. In addition, it seems that these complexes contain regulatory subunits that govern the enzyme-substrate specificity. Proteins that interact with HAT or HDAC may dictate whether the enzyme will either acetylate or deacetylate a specific site on a particular histone tail or on a nonhistone protein (Davie, 1998). Phosphorylation of histones H1 and H3 is known to play important roles in both transcriptional regulation and mitosis. Mutational dissection of phosphorylation sites in histone H1 provided the first evidence for functional regulation through a “charge patch”,

which has a localized concentration of positively or negatively charged amino acids (Dou and Gorovsky, 2000; Ren and Gorovsky, 2001). The phosphorylation of H1 charge patch increases dissociation from the chromatin. Dou and Gorovsky (2002) have demonstrated that phosphorylation of H1 certainly increases its dissociation rate from chromatin in vivo. The researchers measured the dissociation rates of green flouresent protein (GFP)-tagged H1 constructs by fluorescence redistribution after photobleaching (FRAP) with a variety of mutations in the charge patch. H1 phosphorylation is lower in the G1 phase of the cell cycle, increases continually when the cells enter S-phase, and becomes hyperphosphorylated when chromosome condensation is maximal during mitosis and meiosis (Hohmann, 1983). Serines 10 and 28 on the histone H3 tail are both preceded by the same three amino acids, Alanine-Arginine-Lysine. Both of the two phosphorylatable motifs have been conserved throughout the evolution being identical in yeast and man (Allison et al., 2003). Mitotic phosphorylation of histone H3 on serine 10 (highly phosphorylated on condensed chromosomes during mitosis) is mediated by members of the aurora kinase family (a conserved family of enzymes that have multiple

kinase functions during meiotic progression), such as Aurora-A in Drosophila (Giet and Glover, 2001) and Aurora-A and -B in mammals (Crosio et al., 2002). Aurora-B also seems to mediate a mitotic serine 28 phosphorylation. In addition to this, stimulus-induced phosphorylation has also been seen and this also uses the same serine residues. This is transient, affects a minute fraction of nucleosomes and is associated with active genes (Allison et al., 2003). Biotinylation of histones is a new emerging field of research. Therefore, we do not have much information on the biological roles for biotinylation of histones (Kothapalli et al., 2005).

The human genome project has revealed almost each gene within DNA but the regulation of gene expression in eukaryotes is still a matter of intensive research. Regulation of gene expression in eukaryotes is a multilevel hierarchical process. The cell has developed mechanisms to modify the chromatin organization and also to ensure that such organization is maintained through mitotic and meiotic cell divisions. There are four ways to achieve this: 1) chromatin remodeling factors; 2) posttranslational covalent modifications of histones; 3) replacement of canonical histones by histone variants; and 4) methylation at the C5

position of cytosine residues present in the CpG-islands by DNA methyl transferases (Rosa and Caldas, 2005). It is becoming quite evident that higher order chromatin structure, occurring via modifications of histones plays a crucial role in regulating gene expression. Inappropriate or altered gene expression might lead to disease states especially cancer. The acetylation of lysine residues creates new surface for protein association and give signals to many transcription factors and chromatin regulators to bind directly or indirectly to the acetylated histones. The acetylation of lysine residues seems to be crucial for cell proliferation. The HATs or HDACs have been found to be recruited by aberrant chimeric complexes to control abnormal gene expressions, for example, a fusion protein t(15;17) composed of PML-RAR in promyelocytic leukemia and another fusion protein AML1-ETD in acute myeloid leukemia aberrantly recruit HDACs to target genes leading to repression of otherwise active genes (Amann et al., 2001). HDACs may also function in cancer development by more than one mechanism. An abnormal increase in HDAC activity may result in the transcriptional inactivation of tumor suppressor genes such as p53. HDAC4, HDAC8 and HDAC9 have been shown to express to a greater extent

(human) in tumor tissues than in normal tissues. Cancer promoting mutations and chromosomal translocation repress transcription through abnormal recruitment and activation of HDACs leading to neoplastic transformation. This has led to the idea of using HDACs inhibitors as a new class in cancer therapy. Several classes of HDAC inhibitors have been classified such as short-chain fatty acids for butyric acid, hydroxamic acids such as trichostation A (TSA) and cyclic tetra peptides with or without 2-amino-8-oxo-9, 10 epoxy decanoyl (AOE) and Benzamides such as MS-27-275 (Neumeister et al., 2002).

Helicobacter pylori

H. pylori is a bacterium that chronically colonizes the gastric epithelium and infects approximately half of the human population worldwide. This pathogen is responsible for chronic gastritis and a high percentage of peptic ulcers, and its presence has been correlated to gastric cancer development (Marshall et al., 1988; Parsonnet et al., 1991).

Several factors have been associated with this germ's aggressiveness and hence implicated in epithelial damage, including vacuolizing cytotoxin (VacA), cytotoxin-associated gene A (cagA), surface lipopolysaccharide (LPS), bacterial urease, flagella, surface adhesins, oxidizing radicals, and citokines produced by leukocytes in response to infection. On the other hand, there is increasing evidence that *H. pylori* species are genetically diverse, that such diversity is associated with different aggresiveness degrees on the mucosa, and hence with gastric mucosal inflammation to different extents and a variety of clinical prognoses for infected patients. *H. pylori* genoma includes more than 1,000 preserved genes and strain-specific genes.

This bacterium may acquire or lose exogenous DNA, and thus follows an ongoing microevolution model allowing high genetic variability, which may result in strains adapted to

multiple adverse environments. Such variability also results from a high recombination rate during colonization of one host by non-related *H. pylori* species, in addition to a high frequency of mutation (Salama et al., 2000).

Over the last few years, it has become apparent that the most important single factor responsible for the development of gastric cancer is *H. pylori* infection which affects more than 50% of the world population. The risk of patients with *H. pylori* infection developing gastric cancer is in the order of two- to sixfold according to most retrospective, case-control, and prospective epidemiological studies (Montecucco et al., 2001). However, if the selection of patients and methodology is optimized the risk increases to 20 times (Evans et al., 2000). *H. pylori* colonizes the gastric epithelium inducing an inflammatory reaction that may persist throughout the patient's life despite a strong local immune reaction (Guruge et al., 1998). The extent and severity of gastric mucosal inflammation, as well as the clinical outcome of the infection depend on a number of factors including the virulence of the bacterium, host genetic susceptibility, immune response, age at the time of initial infection and environmental factors (Guruge et al., 1998). The complex interplay between these factors may explain why only a minority (<1%) of those

infected ultimately develop gastric cancer.

The multistep process starts with *H. pylori*-related inflammatory reaction, thereafter progressing through a cascade of molecular and morphological changes (Ilver et al., 1998). Histologically, gastric adenocarcinoma is prevalently divided into two types according to Lauren's classification (Mizushima et al., 2001). The intestinal type consists of a gland-like structure that mimics the intestinal glands, and recognizes a series of precancerous lesions. The diffuse type, more prevalent in females younger than 50 years, lacks any glandular structure and arises closer to the advancing border of inflammation but without any identifiable histological precursor lesion (Pride et al., 2001). The intestinal and the diffuse types of gastric cancer both show an equally strong association with *H. pylori* infection.

Exposure of gastric epithelial cells to *H. pylori* results in an inflammatory reaction with the generation of reactive oxygen species (ROS) and an increased level of nitric oxide (NO) synthase (Prinz et al., 2001;). NO synthase deaminates DNA and causes mutations, which may be the initial step in the genetic alterations of gastric epithelial cells (Ilver et al., 1998).

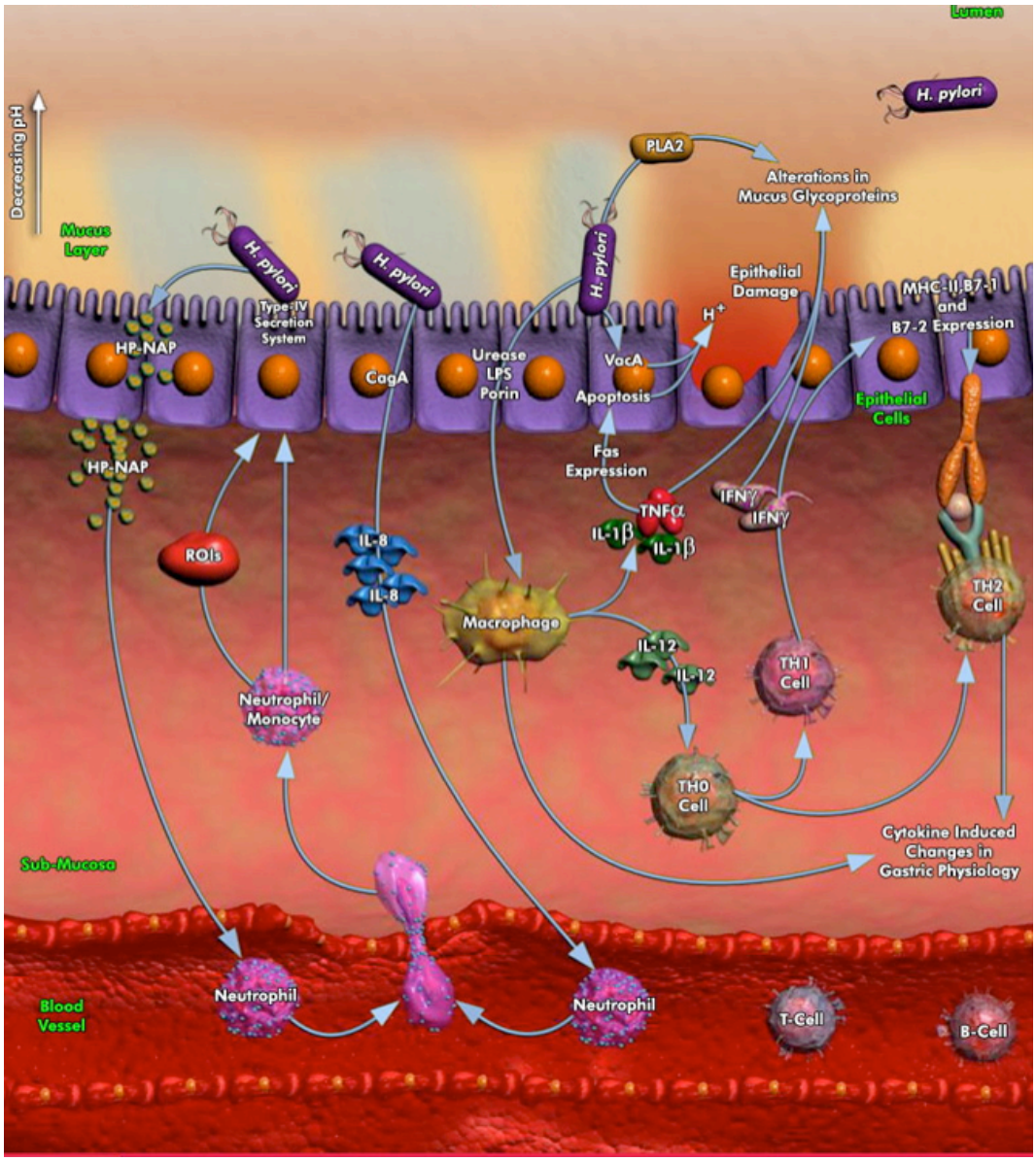

Figura 2 ***Model of H. pylori cell infection***

Higher concentrations of 8-hydroxydeoxy-guanosine (8-OH-dG), a wellknown marker of oxidative DNA stress, have been reported in *H. pylori*-positive patients with atrophic gastritis and intestinal metaplasia (IM). Furthermore, ROS and NO increase cell proliferation. The dynamic balance between cell proliferation and apoptosis is essential for maintaining normal mucosal integrity (Rad et al., 2002).

Sustained stimulation of apoptosis could ultimately result in excessive cell loss and ulcer development, while inhibition of apoptosis has been reported to be associated with the early phases of carcinogenesis. Prolonged survival of abnormal cells can favour the accumulation of sequential genetic mutations that would result in tumour promotion (Rad et al., 2002).

Moss et al. reported an increased rate of cell proliferation and a decrease in the apoptotic index in *H. pylori* infection (Rad et al., 2002). Increased proliferation of epithelial cells is an early event observed during *H. pylori* infection.

The expression of c-fos (mRNA and protein), which regulates the transcription of genes related to cell cycle control, was significantly higher in the *H. pylori*-infected mucosa than in normal mucosa and in precancerous lesions (Cover et al., 1994). Cyclooxygenase-2 (COX-2) interferes with the balance between cell proliferation and apoptosis and is also

abnormally expressed in the *H. pylori*-infected mucosa (Atherton et al., 1997). Overexpression of COX-2 has been detected in *H. pylori*-positive gastritis, in precancerous lesions and in gastric cancer, suggesting an early role of COX-2 in gastric carcinogenesis (Atherton et al., 1997). A positive correlation between COX-2 expression and the Ki-67 labelling index, as well as an inverse correlation between COX-2 expression and the apoptosis index have been demonstrated (Boquet et al., 2003).

Human stomach carcinogenesis occurs after a multistep process originating from epithelial stem cells which is driven by the accumulation of molecular alterations involving either the suppressor pathway (defect in tumour suppressor genes) or mutator pathway (defect in DNA mismatch repair genes) (Kuniyasu t al., 2000; 385: 69–74). The scenario of these alterations shows different genetic pathways for well-differentiated or intestinal type and poorly differentiated or diffuse type gastric cancers.

Two mechanisms have been implicated in the molecular alterations: genetic and epigenetic.

The former includes changes in the DNA sequence, the latter involves methylation of CpG islands which occurs without DNA sequence changes. The most important difference between

genetic and epigenetic alterations is that epigenetic changes are potentially reversible by eliminating the toxic agents or with the use of therapeutic interventions and chemical agents (Lengauer et al., 1998).

The stomach is one of the organs, the epithelial cells of which frequently undergo aberrant methylation of CpG islands because of direct contact with the environment (Kang et al., 2003). Furthermore, *H. pylori*, infecting the gastric mucosa, may induce methylation of promoters containing CpG islands by release of ROS and NO and by activation of DNA methyltransferase (Tamura et al., 2004). Methylation of CpG islands of multiple genes including APC, COX-2, DAP-kinase, E-cadherin, GSTP1, hMLH1, MGMT, p16, p14, RASSF1A, THBS1, and TIMP3, in precancerous gastric lesions have been investigated and it was shown that aberrant CpG island methylation tends to accumulate along the multistep process of gastric carcinogenesis (Kang et al., 2003). Elimination of *H. pylori* infection has the potential to induce regression of epigenetic alterations and restore normal phenotype (Tamura et al., 2004). An intense survey of the literature revealed that precancerous lesions, such as atrophy and even intestinal metaplasia or non-invasive neoplasia, may undergo regression after eradication of *H.*

pylori infection.

In fact, intestinal metaplasia and lesions so-called indefinite for dysplasia are typical abnormalities of differentiation which dependent upon epigenetic changes (El-Zimaity et al., 1999).

Induction of enzymes and cytokines by H. pylori

Cox enzymes catalize the conversion of arachidonic acid to prostanoids such as prostaglandin E2, which protect the gastric mucosa from apoptosis by increasing cell proliferation (Gisbert et al., 2003). Two isoenzymes exist: COX-1 and COX-2; the former is constituent and the latter inducible in case of injury, and mediates inflammation among other processes (Halter et al., 2001). *H. pylori*-related gastritis has been shown to induce COX-2 expression according to type of bacterial strain, which may in part account for differential pathogenicity (Guo et al., 2003). Infection by *H. pylori* cagA + strains has been seen to overexpress COX-2 in patients with gastric cancer (Guo et al., 2003).

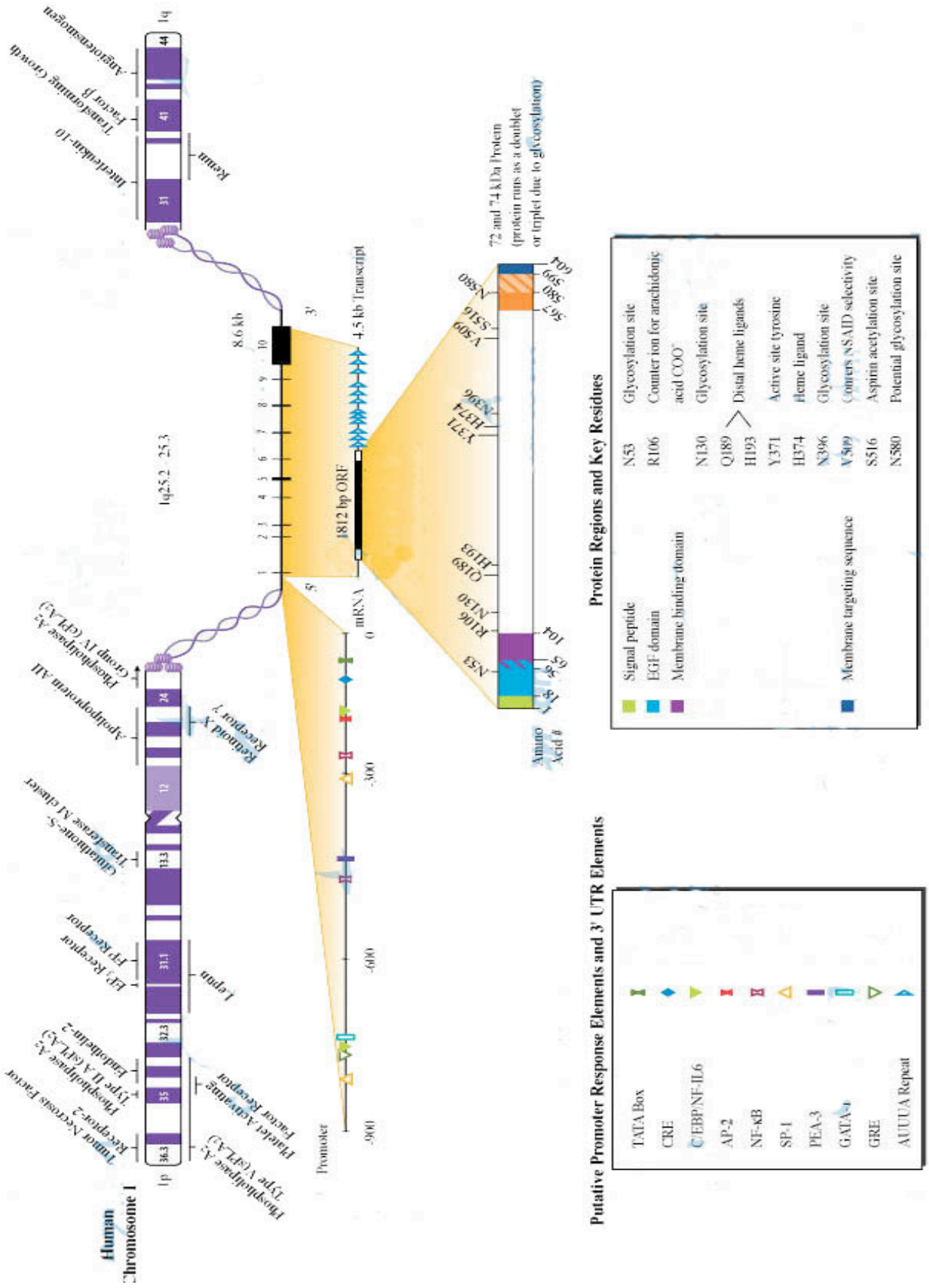

Figura 3 ***COX-2 gene map.***

In addition, some studies have demonstrated that organism eradication is associated with a decreased gastric COX-2 expression (Konturek et al., 2003). Another molecule, nitric oxyde (NO), has been documented to contribute to gastric mucosal protection by increasing blood flow and inhibiting leukocyte adhesion to the endothelium. There is no inducible NO synthase (iNOS) in the normal gastric mucosa, but its expression increases in patients with *H. pylori*-related gastritis (Fu et al., 1999). Both iNOS and COX-2 are induced by cytokines, including IL-1β, tumor necrosis factor α, interferon γ, phorbol esters, and growth factors, as well as bacterial lipopolysaccharides (Arias-Negrete et al., 1995). Epithelial gastric cells substantially contribute to the cytokine-induced proinflammatory response to *H. pylori* infection, both through active production and the capture of cytokines derived from the lamina propria and intraepithelial leukocytes. Epithelial IL-1β, IL-6, IL-8, and tumor necrosis factor αlevels have been shown to be significantly higher in infected patients versus healthy subjects. There is also interferon γ, but not IL-4, overexpression in infected patients, which suggests a Th1 lymphocyte-mediated response. Finally, increased interferon γlevels may contribute to gastric inflammation no

only through phagocyte and neutrophil activation, but also the induction of MHC II overexpression in epithelial cells, with an ensuing increase in *H. pylori* adhesion.

AIM OF THIS STUDY

In this study, we show that histone modifications play a pivotal role in *H. pylori*-induced expression of COX-2 promoter in the human gastric epithelial cell line NKN28.

To provide a novel molecular mechanism of COX-2 epigenetic regulation in *H. pylori* infected cells, we whant to demonstrate, by modification of chromatin analyses, that the induction of COX-2 expression is associated with both methylation and acetylation of histones and methylation of DNA at the COX-2 promoter,

MATERIALS AND METHODS

Bacterial Strain and Growth Conditions

We used the urease-positive Tox+ (type s1a/m1 *vacA* allele) CagA+ wildtype *H. pylori* strain 60190 (American Type Culture Collection, Rockville, MD, USA, 49503) and wild-type *H. pylori* strain CCUG 17874 (Tox^+ $CagA^+$) (National Collection of Type Cultures, London, England, 11638). 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 5x 10^7 CFU/ml in DMEM supplemented with 10% FCS. Cells were incubated in the absence (controls) or in the presence of bacteria for indicated times. 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, in all experiments gastric cells were serum starved for 16 h before and throughout the period of treatment.

Human Gastric Epithelial Cells in Culture

Since a normal human gastric epithelial cell line is not available, we used the following human gastric epithelial cell lines: MKN 28 cell line, derived from a human gastric tubular adenocarcinoma and showing moderate gastric-type differentiation (Taplick et al., 2001; Grozinger et al., 2002); this cell line has proven to be a suitable in vitro model for the study of interactions between *H. pylori* and gastric epithelium (Ricci at al., 1993a; Ricci et al., 1993b; Sommi et al., 1998). MKN 28 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% CO2.

Preparation of cell extracts and Western Blot Analysis

After incubation with *H. pylori*, cells were rapidly washed twice with PBS to remove bacteria and lysed in RIPA buffer (PBS containing 1% Nonidet P 40, 0.5% sodium deoxycholate, 0.1% SDS, 100 ng/mL phenylmethyl sulfonyl fluoride and 10µg/mL aprotinin). The samples, containing 50 µg protein per lane, were resolved by electrophoresis using 12 % SDS-PAGE precast gels (Bio-Rad Laboratories, Milan, Italy) as appropriate, then transferred to BA 85 0.45 mm PROTAN

nitrocellulose filters (Schleicher & Schnell, Inc., Dassel, Germany).

The blots were pretreated in tris-buffered saline containing 5% non-fat dry milk and 0.1% Tween 20, and then incubated with antibody for COX-2 or IkB-alfa (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Filters were washed three times and then incubated with a horseradish peroxidase-conjugated secondary antibody against goat or rabbit IgG (Amersham Pharmacia Biotech, Buckinghamshire, UK), developed using a commercial enhanced chemiluminescence system (ECL, Amersham Pharmacia Biotech, Buckinghamshire, UK), and exposed to films (Hyperfilm, Amersham Pharmacia Biotech). Immunoblot analysis using anti γ-tubulin antisera was performed as a control for protein loading. Western blot analyses of each sample were performed more than three times.

Real-Time PCR

Total RNA was isolated from MKN28 cells using an RNeasy Mini kit (Qiagen) according to manufacturer's instructions. A reverse transcription-PCR (RT-PCR) procedure was used to determine relative quantities of mRNA (QuantiTect Reverse Transcription kit, Qiagen). Negative control samples (no first-

strand synthesis) were prepared by performing reverse transcription reactions in the absence of reverse transcriptase. Quantitative real-time PCR was carried out with cDNA using the QuantiTect SYBR Green (Qiagen), gene-specific primers and an Chromo4 Real Time thermocycler (Biorad). The primers for all genes tested were described as follows: Glucose-6-Phosphate Dehydrogenase (G6PD), 5'-GATCTACCGCATCGACCACT-3' (sense) and 5'-AGATCCTGTTGGCAAATCTCA-3' (antisense); Cyclooxygenase-2 (COX-2) 5'- TCACGCATCAGTTTTTCAAGA-3' (sense), 5'-TCACCGTAAATATGATTTAAGTCCAC -3' (antisense); Histone deacetylase 1 (HDAC-1) 5'-AGCTCCACATCAGTCCTTCCA-3' (sense), 5'-GTGCGGCAGCATTCTAAGGT-3' (antisense); Histone deacetylase 2 (HDAC-2) 5'-AAACTGCATATTAGTCCTTCAA-3' (sense) 5' -TGAGGTAACATGCGCAAATTTT-3' (antisense) and Histone deacetylase 3 (HDAC-3) 5'-GGAGCTGGACACCCTATGAA-3' (sense) 5'-TATTGGTGGGGCTGACTCTC-3' (antisense). Gene-specific primer pairs were designed using Roche Applied Science software (http://www.roche-applied-science.com/sis/rtpcr/upl/adc.jsp). The reaction mixture contained 2 µl cDNA, 0.3 µM of primers, and 10 ml of Sybr green Mastermix (Qiagen), in a total volume of 20 ml. PCR

cycles were as follows: 95°C for 15 min followed by 40 cycles of 95°C for 15 s, 58°C for 30 s, and 72°C for 30 s. Each reaction was performed in triplicates. Melting curve analysis was performed to verify the specificity of products. The comparative method of relative quantification (2^{-DDCt}) method (Livak *et al.,* 2001) was used to calculate expression levels of each target gene, normalized to the housekeeping gene G6PD. The data are presented as fold changes in gene expression. At least three independent experiments were performed and results are expressed as averages ± S.M.

HDAC activity assay

Assays were performed using the colorimetric HDAC activity assay (Upstate) according to manufacturer's instructions. Briefly, 50 µg of nuclear extracts from MKN28 cells, infected with Hp at different times, were diluted in 85 µL of ddH_2O; then, 10 µL of 10× HDAC assay buffer were added followed by addition of 5 µL of colorimetric substrate; samples were incubated at 37°C for 1 h. Subsequently, the reaction was stopped by adding 10 µL of lysine developer and left for additional 30 min at 37°C. Samples were then read in an ELISA plate reader at 405 nm. HDAC activity was expressed as relative OD values per µg of protein sample. The kit contains

negative and positive controls, that is, nuclear extract of HeLa treated and untreated with trichostatin A, respectively.

Chromatin immunoprecipitation (ChIP) assay

Cross-linking was performed by adding 1% formaldehyde directly into tissue culture dishes containing 3 X 10^6 MKN28 at different time points after Hp infection, followed by incubation at room temperature for 10 min. Cells were washed twice with PBS, collected, and pelleted by centrifugation at 400 *g* for 5 min. The pellets were resuspended in sonication buffer (containing 50 mM Tris-HCl, pH 8.1, 10 mM EDTA, 1% SDS, and protease inhibitors) and incubated in ice for 10 min to lyse the nuclei. Nuclear extracts were then sonicated to obtain 400-800 bp fragments of chromatin using a 3 mm tip equipped Bandelin Sonoplus UW-2070 sonicator (small size). Immunoprecipitation was carried out according to the protocol provided by Upstate Biotechnology (Lake Placid, NY, USA). Briefly, chromatin was diluted10-fold in ChIP dilution buffer. A small amount of chromatin was kept aside at this step to be used as input control in subsequent PCR reactions. Antibodies anti-dimethyl-histone H3K4, anti-dimethyl-histone H3K9, anti-tri-methyl-histone-H3K27, antiacetyl-histone-H3, antiacetyl-histone-H4 (Abcam), anti-HDAC1, anti-HDAC2,

anti-RNA-polymerase II, and anti-NFKB (p65) (Santa Cruz) were incubated with diluted chromatin at 4°C overnight. Immunoprecipitations were also carried out with no Ab controls. Protein A Sepharose (Amersham Biosciences), blocked with sheared salmon sperm DNA, was used to collect Ab-chromatin complexes. Immune complexes were then washed once with low salt immune complex wash buffer (containing 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1, 150 mM NaCl), once with high salt immune complex wash buffer (containing 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl, pH 8.1500 mM NaCl), once with LiCl immune complex wash buffer (0.25M LiCl, 1% IGEPAL-CA630, 1% deoxycholic acid, 1 mM EDTA, 10 mM Tris-HCl, pH 8.1), and twice with sterile TE buffer.

The chromatin (histone-DNA complexes) were eluted with freshly prepared elution buffer (containing 1% SDS and 0.1M NaHCO3), followed by reverse cross-linking with 0.3M NaCl at 65°C for 4–5 h. DNA fragments were then purified with QIAquick spin column followed by semiquantitative PCR and Real Time PCR amplification. The primers for the COX-2 promoter, used for conventional PCR were: 5'GGCAAAGACTGCGAAGAAGA3' and 5'GGGTAGGCTTTGCTGTCTGA 3'. The PCR product covers DNA

sequence from -320 to +107 and contains NF-kB, cyclic AMP response element (CRE), CCAAT-enhancer binding protein (C/EBP), and TATA *cis*-elements in the promoter region. Initially, PCR was performed with different numbers of cycles or dilutions of input DNA to determine the linear range of the amplification; all results described fall within this range. The PCR conditions used were: initial denaturation at 95°C for 2 min followed by 30 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. The PCR products were separated on a 1.4% agarose gel and stained with ethidium bromide. Alternatively, recovered DNA was subjected to quantitative real-time PCR as described above.

Quantification of ChIP DNA by real-time PCR.

Immunoprecipitated DNA recovered from ChIP were quantizated by real-time PCR using a 7500 Chromo4 Real Time thermocycler (Biorad). The primers for the COX-2 promoter, used for real-time PCR were: 5' AAGGGGAGAGGAGGGAAAAATTTGTG 3' (sense) and 5' GAGGCGCTGCTGAGGAGTTCCTG 3' (antisense). The reaction mixture contained 2 µl of ChIP or input DNA, 0.3 µM of primers, and 10 ml of Sybr green Mastermix (Qiagen), in a total volume of 20 ml. PCR cycles were as follows: 95°C for 15

min followed by 40 cycles at 95°C for 15 s, 62°C for 30 s, and 72°C for 30 s. Input DNA was the unbound fraction of the non-immunoprecipitated sample. Melting curve analysis revealed a single PCR product. Serial dilutions of input DNA revealed that PCR results were linear from 100 ng to 0.1 ng and were used to calculate absolute amounts of PCR products. Results are represented as input percentage and calculations take into account the values of minimum three independent experiments.

***Methylation DNA analysis with MALDI-TOF MS*.**

The modification with sodium bisulfite was followed by the amplification COX-2 promoter region with the application of primers containing a fragment of T7 promoter. The product, including CpG sites, was transcribed in vitro, creating a mosaic DNA/RNAstructure and cut with RNaseAin the way depending on U or C bases. Mass signals transmitted by DNA molecule was analyzed in Matrix-assisted laserdesorption/ionization time-toflight mass spectrometry (MALDI-TOF MS). The relation between the methylated and unmethylated DNA was established by comparing the intensity of signals sequences, which is achieved by means of using different fluorescent dyes (VIC and FAM)

Statistics analysis

Three separate experiments carried out in duplicate were performed and average values and standard errors (S.M) were calculated.

RESULTS

H. pylori infection induces activation of COX-2 gene

H. pylori infection of gastric mucosa leads to activation of COX-2 gene (Takahashu et al. 2000).

In order to investigate the early transcriptional events occurring at the human COX-2 promoter gene upon induction by *H. pylori*, the human gastric epithelial cell line MKN28 has been taken as a model. MKN28 cells were grown to confluence, incubated with bacterial suspension of the *H. pylori* 60190 (wild-type) strain and then the levels of COX-2 mRNA were measured. As expected, a time-dependent increase of COX-2 mRNA in response to *H. pylori* infection was found (Figure 4A). The increase in COX-2 mRNA expression showed a first peak after 60 minutes of infection and a second more pronounced peak at 12 hours, before declining at 24 hours (Figure 4A). Similar experiments using a different strain of *H. pylori* (strain CCUG 17874) gave comparable results (data not shown) showing that the observed pattern of COX-2 induction was not related to a specific strain of *H. pylori*. Western Blot analyses showed that levels of COX-2 protein increased following a pattern

consistent with the one predicted by the mRNA levels over the same time course (Figure 4B).

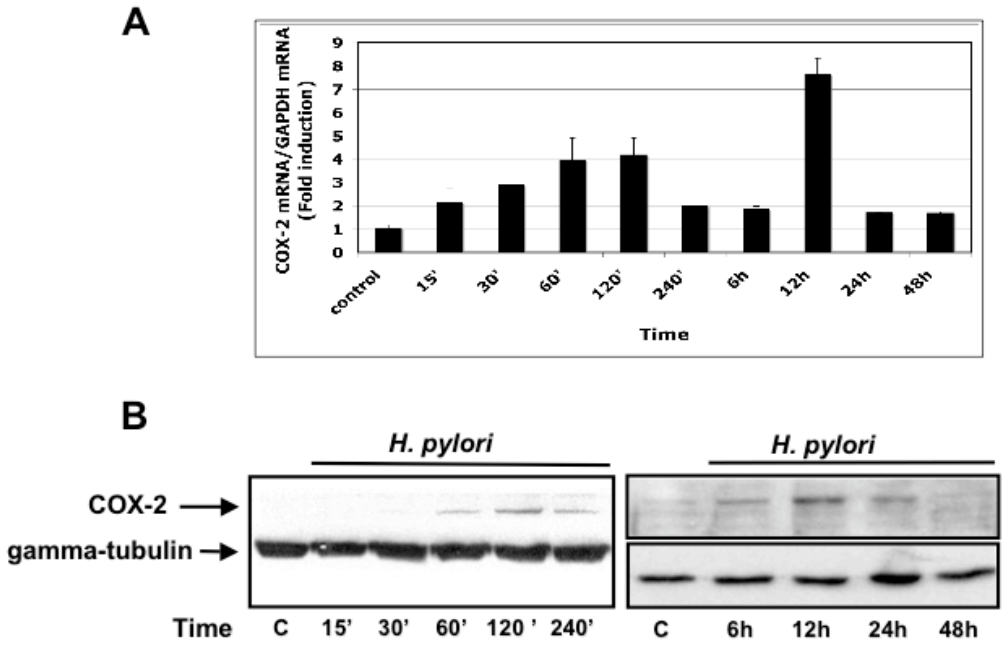

Figure 4 *H. pylori* induces expression of the COX-2 gene. MKN28 cells were grown to confluence and infected with *H. pylori* for 15, 30, 60 min and 2, 4, 6, 12, 24 and 48 hours. (A) Total RNA was isolated and used in real-time PCR reactions which were evaluated for COX-2 and GAPDH levels. The COX-2 levels were evaluated relative to time point 0 (Control) and normalized to GAPDH levels. Data points represent the average of triplicate determinations +/- SD. Similiar results were obtained in 3 independent experiments. (B) Lysate were collected in RIPA buffer and 50 mg of protein samples were loaded for electrophoresis. The expression levels of COX-2 were detected using antibody against COX-2. The levels of γ-tubulin were used to demonstrate equal loading. Relative values represent the average of three independent experiments +/- SD.

Role of histone acetylation on H. pylori-induced COX-2 expression

In order to test the involvement of histone acetylation on the *H. pylori*-induced COX-2 expression, we pretreated MKN28 cells with an inhibitor of histone deacetylases (thricostatin, TSA) and measured COX-2 mRNA levels by real time PCR (Figure 5). Both basal and *H. pylori*-induced COX-2 mRNA levels showed a two-fold increase upon TSA treatment indicating a role of histone acetylation state in COX-2 gene regulation. We then measured the levels of HDAC1, -2, and -3 mRNAs after *H. pylori* infection. Constitutive expression of HDAC1, -2, and -3 mRNA was detectable in untreated MKN28 cells (Figure 6A-C). Interestingly, *H. pylori* infection induced a marked time-dependent reduction in the level of HDAC1 and -3 in these cells, which was evident after 4 hours and progressed at 12 hours post-infection. HDAC2 mRNA levels reduced after 6 hours and then slightly rose after 12 hours of *H. pylori* infection. Then, we analyzed the global HDAC activity in nuclear extracts of MKN28 cells exposed or not to *H. pylori*. We found a time-dependent decrease of HDAC activity in exposed cells compared to non-exposed MKN28 (Fig. 6D). Taken together, these data show that *H. pylori*-induced transcriptional activation of COX-2 gene expression in gastric

epithelial cells is accompanied by reduction of HDAC1, -2 and -3 expression and of global histone deacetylase nuclear activity.

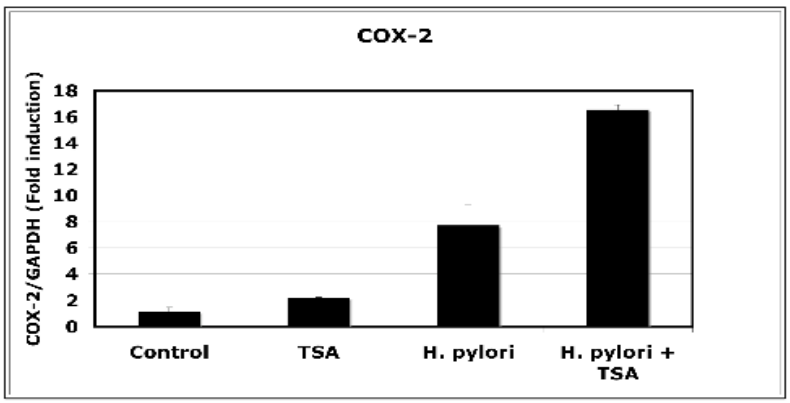

Figure 5 Prevention of histone deacetylation with TSA increased *H. pylori*-induced COX-2 expression. MKN28 cells were treated with TSA (100ng/ml), *H. pylori*, or *H. pylori* + TSA for 12 hours. Total RNA was isolated and used in real-time PCR reactions which were evaluated for COX-2 and GAPDH levels. The COX-2 levels were evaluated relative to control and normalized to GAPDH levels. Data points represent the average of triplicate experiments +/- SD.

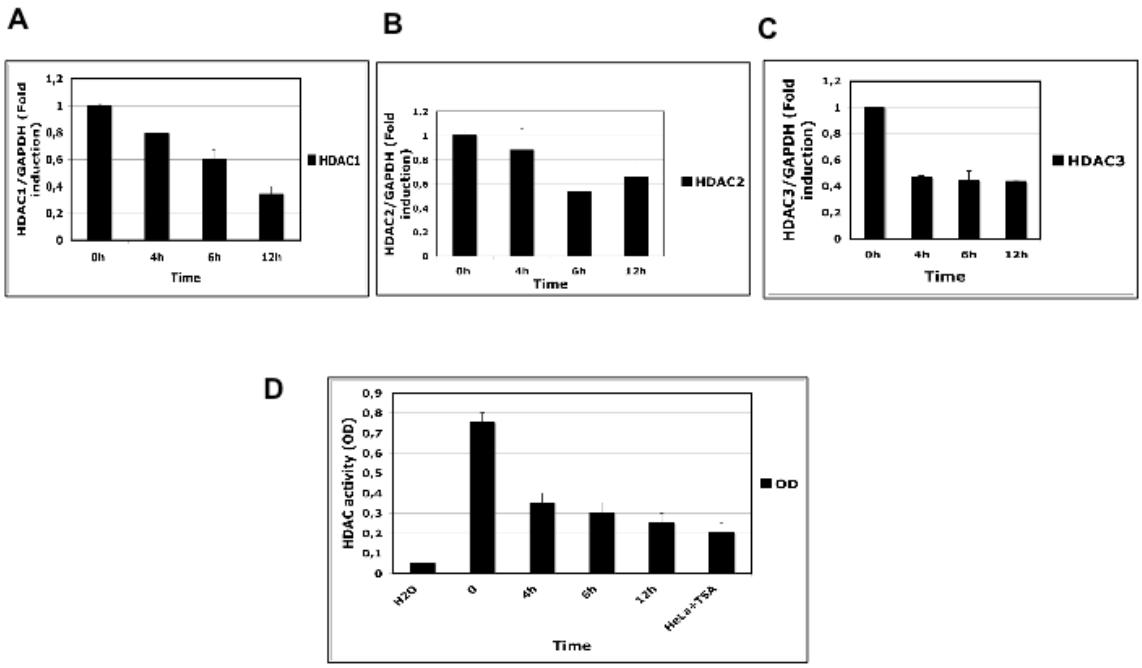

Figure 6 *H. pylori* exposure down-regulate HDAC1, 2, and 3 expression and global HDAC activity. (A-C) MKN28 cells were grown to confluence and exposed to *H. pylori* for 0, 4, 6, and 12 hours. Total RNA was isolated and used in real-time PCR reactions which were evaluated for HDAC1 (A), HDAC2 (B), HDAC3 (C) and GAPDH mRNA levels. 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 *H. pylori* for 0, 4, 6 and 12 hours. The HDAC activity was measured in triplicate with a HDAC colorimetric histone deacetylase activity assay kit (Upstate Biotechnology). H_20 was used as negative control. TSA-treated Hela cells served as positive control. Data are expressed as relative OD values per µg of protein sample and are presented as average of three independent experiments +/- SD.

Role of histone acetylation induced by H. pylori

In order to determine whether decreased HDAC activity and expression correlated with an increase in acetylated histones H3 or H4, in the promoter region of COX-2 induced by *H. pylori* in MKN28 cells, ChIP analysis was performed. Cells were incubated with *H. pylori* for different time points and chromatin was then immunoprecipitated with an antibody raised against acetyl H3 and H4. We found that upon infection of MKN28 with *H. pylori*, the acetylation in histone H3 reveal a transient pattern at the promoter of COX-2 gene. In fact, the histone H3 is acetylated at 6h time point with maximum detection at 12h, loses this modification after 24h, but then regains it at 48h (Figure 7A). Hyper-acetylation of histone H3 is in agreement with expression of the COX-2 gene. This pattern reflects the one of H3-K4 dimethylation (Figure 9A), and these two modifications are likely to act together to form a post-activation signal. In contrast, histone H4 was constitutively acetylated and remained almost unchanged following *H. pylori* infection (Figure 7B). These two types of positive histone modifications, H3 acetylation and H3-K4 dimethylation, are selectively present at the COX-2 promoter, activating its gene expression in gastric epithelial cells after infection with *H. pylori*.

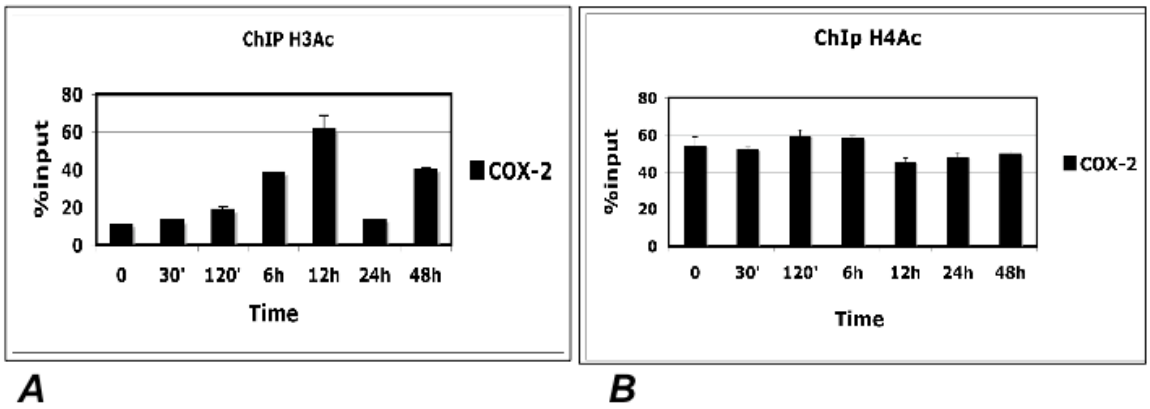

Figure 7 *H. pylori* induces acetylation of histone H3 in the promoter region of COX-2. Chromatin from MKN28 cells was harvested 0, 30, 120 min, and 6, 12, 24 and 48 h after infection with *H. pylori*. Results are shown from ChIP analyis using anti-acetyl-H3 (A) or anti-acetyl-H4 antibodies (B). Recovered DNA sequences were quantified by real-time PCR using primers described in Fig. 4A. Average % input recoveries and +/- S.D. from 3-4 independent experiments are plotted.

Effect of H. pylori on the recruitment of HDAC-1 and HDAC-2 to the COX-2 promoter

Since the expression of COX-2 induced by *H. pylori*, in MKN28 cells correlates with increased histone H3 acetylation, we hypothesized that *H. pylori* infection might mediate this effect by reducing recruitment of HDACs to the COX-2 promoter. To test this hypothesis, we performed ChIP assays, and the immunoprecipitated DNA was quantified by semi-quantitative PCR. Results reveal that the 2 HDACs were readily detected at the COX-2 promoter (Figure 8B). 6h after infection by *H. pylori*, we observed a release of HDAC1 from the COX-2 promoter, but no effect on the HDAC2 levels. These results show that the increase in histone H3 acetylation and the induction of COX-2 transcription by *H. pylori* involve release of HDAC-1 from COX-2 promoter.

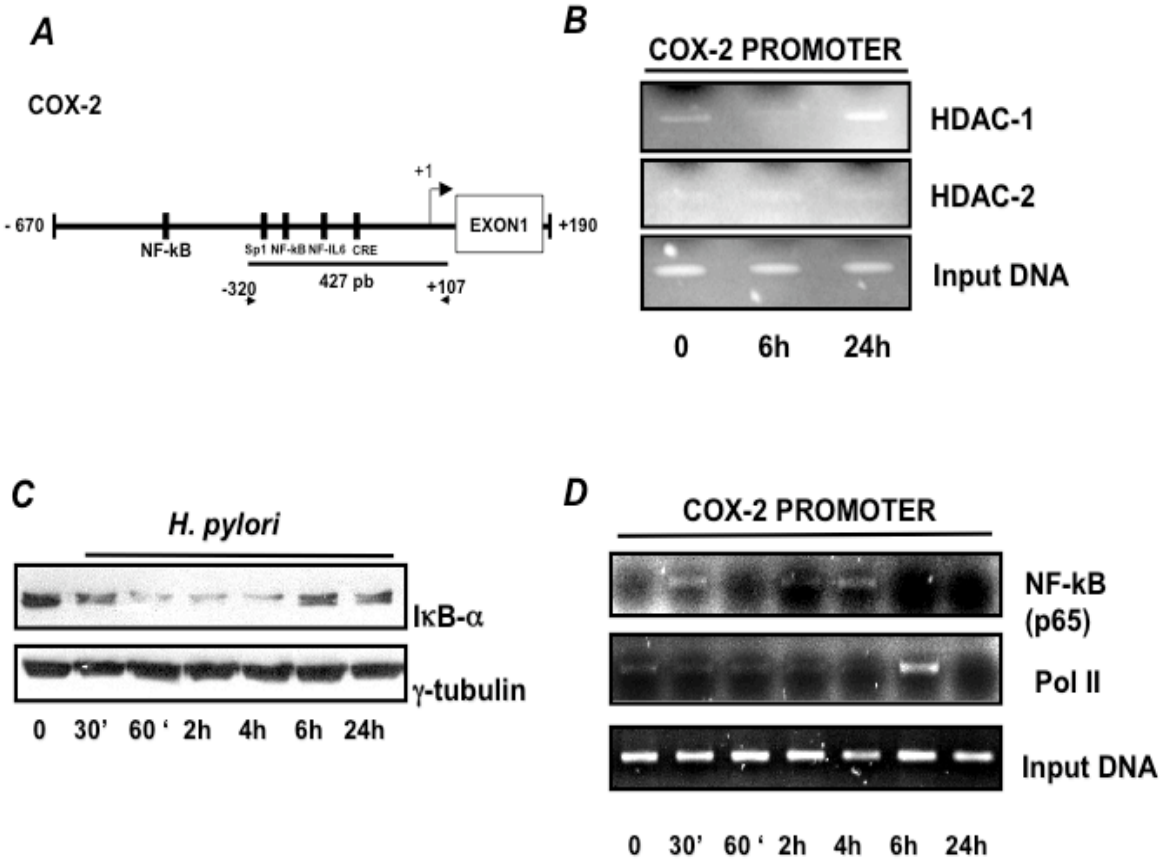

Figure 8 Time course of *H. pylori*-induced binding of HDAC-1 and -2, NFk-B (p65) and RNA Polymerase II at the COX-2 promoter. (A) Chart of the COX-2 promoter showing the locations of PCR primer used for ChIP assay. (B and D) MKN28 cells were grown to confluence and infected with *H. pylori* at indicated times. Chromatin was harvested and precipitated with anti-HDAC-1, anti-HDAC-2 (B), anti-NFk-B(p65) or anti-RNA-Pol II antibodies (D). After DNA recovery the precipitates were evaluated by conventional semi-quantitative PCR with COX-2 promoter primers (A). 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 control (Input DNA).(C) IkB-alfa protein levels were detected with a specific antibody in Western blot and simultaneous detection of γ-tubulin demonstrated equal protein load. Representative gels of three separate experiments are shown in B-D.

Histone Methylation at H. pylori induced COX-2 gene

To determine whether induction of the COX-2 gene upon Hp infection is accompanied by modification 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. For this purpose, MKN28 were infected with *H. pylori* at different time points and chromatin was prepared for ChIP analysis. COX-2 promoter region examined in these assays is shown in Figure 8A. We found that H3K9me2 had significantly decreased in gastric epithelial cells 12 hours after *H. pylori* infection and gradually returned to its basal state at 48 hours (Fig. 9B). H3-K9 methylation serves as a signal for chromatin silencing by recruiting the HP1 protein (heterochromatin protein 1) and these results suggest that the increased COX-2 expression seen in the *H. pylori* -infected MKN28 may be due to a loss of this repressive epigenetic histone modification. In contrast, we found that H3K4me2 levels were low in non-infected gastric cells, while significantly increased at 12 hours after *H. pylori* infection returning to a nearly basal level after 48 hours (Figure 9C). These results are in agreement with the repressive role of H3K9me2 and with the positive role described for H3K4me2 in

gene transcription (Lachner and Jenuwein, 2002).

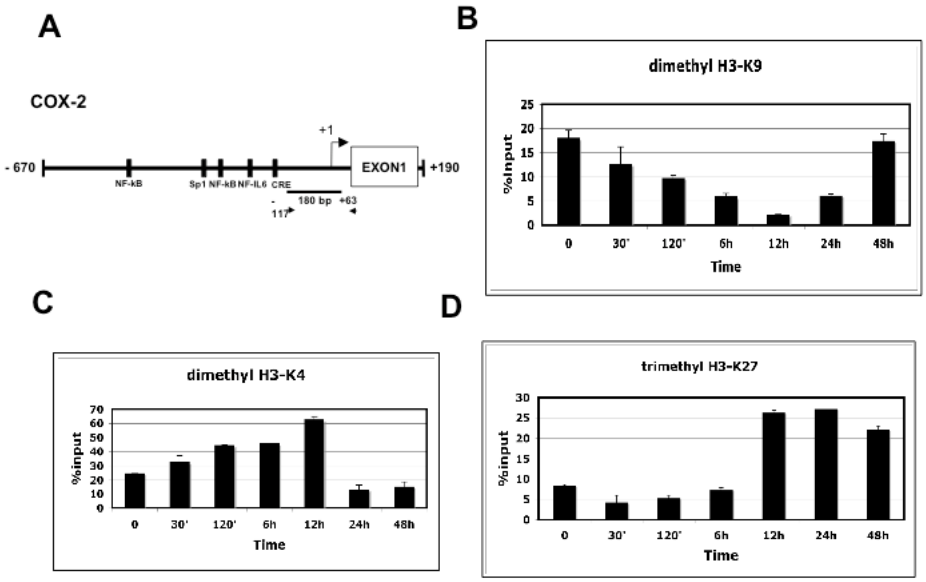

Figure 9 *H. pylori* induces methylation of histone H3 in the promoter region of COX-2. (A) Chart of the COX-2 promoter. Locations of the primers used for evaluating anti-histone modification ChIPs are shown below the line. Primer positions shown indicate 5' position of the forward primer relative to the transcription start site. Chromatin from MKN28 cells was harvested 0, 30, 120 min, and 6, 12, 24 and 48 h after infection with *H. pylori*. Results are shown from ChIP analyis using anti-dimethyl-H3-K9 (B), anti-dimethyl-H3-K4 (B) or anti-trimethyl-H3-K27 (B), antibodies. Recovered DNA sequences were quantified by real-time PCR. Average % input recoveries and +/- S.D. from 3-4 independent experiments are plotted.

Methylation of H3-K27 has been associated to active processes leading to gene silencing. Surprisingly, we found that upon infection of MKN28 with *H. pylori*, the H3-K27 residues remained almost unchanged at 6h, but then significantly increased at 12 h, suggesting an inverse correlation with COX-2 expression.

CpG methylation of COX-2 promoter

Dynamic variations in the methyaltion status of some CpGs was evaluated trough MALDI-TOF MS technique (Sequenom) before (time 0) and after infection with Hp for 2 h. About 50 CpGs have been analyzed. Ten more representatives are shown in Figure 10. Within the 8 CpGs located between -176/+244 of the COX-2 promoter, all exhibit kinetic changes of the methylation status. All the cytosines located on the upper strand are methylated at 20-25 min, undergo demetylation at 50-55 min and then remethylate. The methylation status of the CpGs on the bottom strand is more variable. All the CpGs examined are continually demetylated and remethylated.

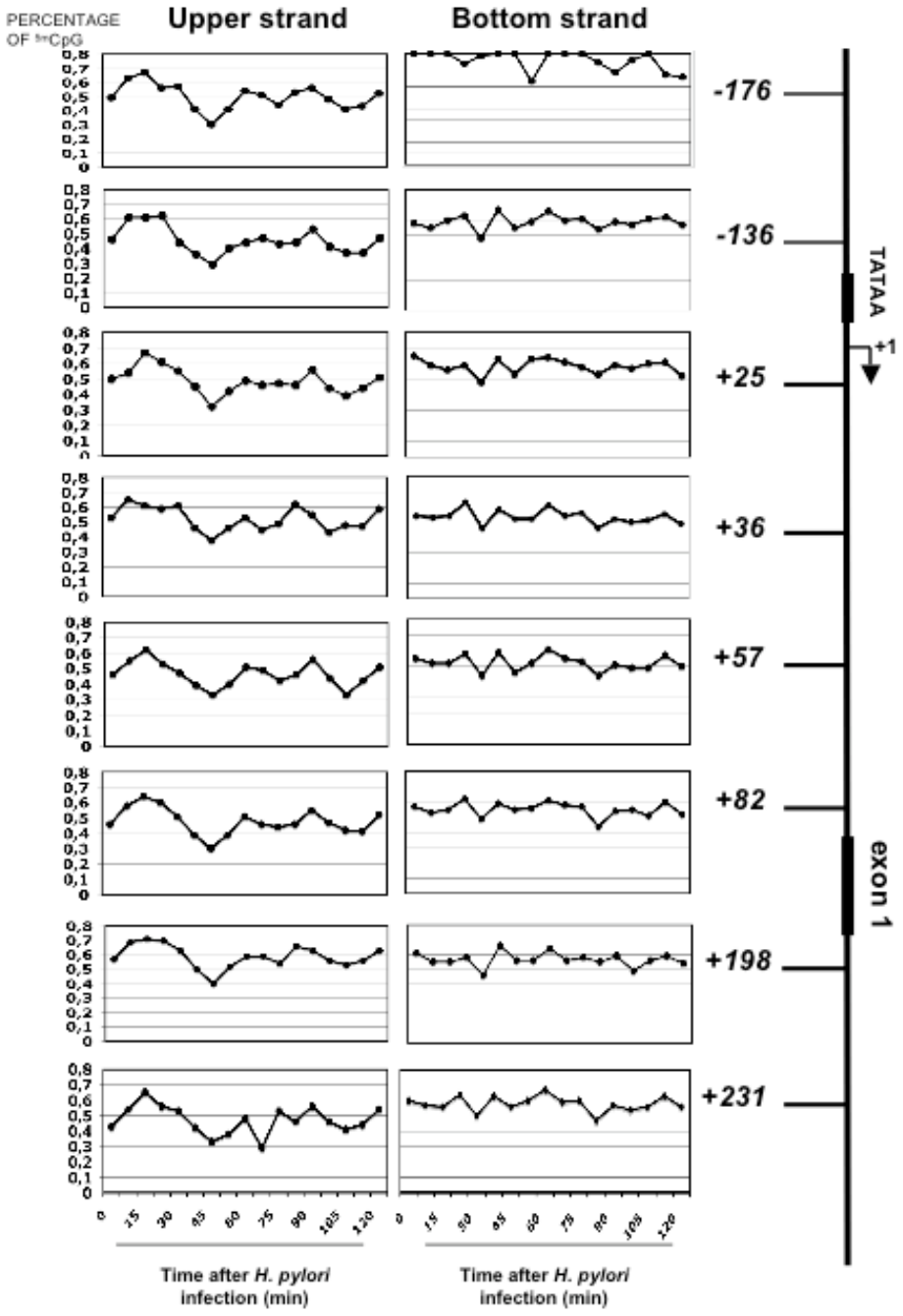

Figure 10 Methylation status of the the COX-2 promoter. Methylation status of individual CpG on upper (coding) and bottom (transcribed) strands as determined by MALDI-TOF MS technique. "Time 0" refers to initial methylation status before *H. pylori* infection.

NF-kB associates with COX-2 promoter upon H. pylori infection

Because COX-2 promoter contains potential binding sites for NF-kB transcription factor (Appleby et al., 1994), possible association of NF-kB with COX-2 gene during Hp infection has been investigated through ChIP assays.

ChIP assays were performed at different times after Hp infection using anti-p65 antibodies, followed by semi-quantitative PCR analyses with specific primers amplifying the COX-2 promoter region (from -320 to +107) containing a NF-kB binding sites (Figure 7A). We observed that the binding of p65 to COX-2 promoter occurred starting 30 min after *H. pylori* infection (Figure 7B). Detection of p65 peaked before maximal COX-2 mRNA accumulation (12 hours) and appeared to be transient, with enrichment levels falling at 4 hours. Accordingly, Western blot analysis of IkB-a, a NF-kB inhibitor, proved that the levels of such protein decreased starting from 60 minutes and were restored after 6 hours of *H. pylori* infection (Fig. 7C). ChIP assays were then performed on the same samples using anti Pol II antibodies. Interestingly, RNA polymerase II loaded onto the COX-2 promoter after 6 hours and disappeared after 24 hours (Figure 7B). These results suggest that the first peak of COX-2 mRNA levels detected at

1 hour following hp infection (Fig. 4), may be due to post-transcriptional events while the later peak (12 hours) was primarily transcriptional.

DISCUSSION

Emerging data suggest that bacteria are able to provoke histone modifications and chromatin remodelling in infected cells, thereby altering the host's transcriptional program (Hamon et al 2008). In this study, for the first time the effect of *H. pylori* is described on chromatin modification of the COX-2 promoter in the human gastric epithelial cell line MKN28.

Firstly, we examined mRNA and protein expression levels of COX-2 and the effects of *H. pylori* stimulation on the MKN28 cells. The MKN28 cells weakly expressed COX-2 mRNA and proteins under the unstimulated condition with *H pylori,* and these expression levels increased dramatically with *H pylori* stimulation (Figure 4A and B). The COX-2 expression has been demonstrated in *H pylori*-associated acute and chronic gastritis, and is correlated with chronic inflammatory cell infiltration (Fu et al., 1999; McCarthy et al., 1999; Romano et al., 1998). Our results concerning induction of COX-2 by *H pylori* are in line with these reports.

Next, we provided evidence that *H. pylori* strain 60190-induced expression of the COX-2 gene is controlled by

histone modifications in the MKN28 cells. *H. pylori* induced methylation of H3-K4-me2, H3-K9-me2, H3-K27-me3 and acetylation of histone H3 specifically at the COX-2 gene promoter. These changes are accompanied by coactivator recruitment and chromatin remodelling. This is indicative of dynamic chromatin remodelling being triggered by *H. pylori.* Chromatin alteration is shown to control the expression of COX-2, which might explain the known effects of *H. pylori* on inflammation, tissue damages, and gastric tumorigenesis. *H. pylori* infection induces inflammation in gastric mucosa, which causes chronic gastritis (Blaser MJ et al., 1998). Gastritis may progress to gastric atrophy (GA), intestinal metaplasia (IM), and gastric cancer (GC). It may also result in monoclonal lymphocyte proliferation, lymphoid follicle (LF) development, and later primary gastric lymphoma (PGL). These precancerous lesions develop only in a proportion of subjects affected by gastritis because of multifactorial effects of bacterial virulence and host factors that vary among different ethnic groups (Lee et al., 2005). Among host factors, several inflammatory proteins including cytokines, growth factors, and chemokines have been known to control adaptive and innate immune response against *H. pylori* infection (Rad et al., 2004; Macarthur et al., 2004).

COX-2 is an inducible enzyme, catalyzing conversion of arachidonic acid to prostaglandins in response to various inflammatory stimuli (Herschman 1996). Up-regulation of COX-2 plays a central role in the inflammatory changes and tissue damage associated with chronic *H. pylori* infection and is also involved in gastric tumorigenesis (Harris et al., 2005; Willhite et al., 2003). Understanding the mechanisms by which Cox-2 expression is regulated could provide insight into how its expression increases in diseased tissue, and may also suggest targets for novel pharmacological agents aimed at suppressing its expression. The regulation of Cox-2 gene expression has been studied by several groups, and there have been reports of regulation at a number of different steps (Ramsay et al., 2003). In addition to the transcriptional regulation of the gene, increasing evidence shows that histone modifications may be important for the transcriptional activity state of genes by loosening the DNA-histone interaction and unmasking of transcription factor binding sites. Transcription repression or gene activation is regulated by specific covalent modifications of accessible N-terminal histone tails (Strahl et al., 2000; Nightingale et al., 2006), including acetylation (mostly lysine), phosphorylation (serine/threonine), and methylation (lysine) (Claus et al., 2003, Jenuwein et al., 2001).

The N-terminal tails of H3 and H4 can be methylated at several lysine and arginine residues that are linked to either transcriptional activation or repression. For example, methylated H3-K9 provides a specific interaction site for the heterochromatin protein 1 (HP1) proteins, which are implicated in transcriptional repression and in establishing and maintaining heterochromatin (Bannister et al., 2001; Lachner et al., 2002; Hwang et al., 2001). Interestingly, a common pattern of H3-K9 methylation occurs in a subset of inducible inflammatory genes (Saccani and Natoli, 2002). This pattern is characterized by a loss of constitutive levels of H3-K9 methylation after activation followed by a restoration of methylation concurrent with post-induction transcriptional repression. Dynamic changes in the methylation of the COX-2 promoter did occur after induction of the gene. In the COX-2 promoter there are constitutive levels of H3-K9 methylation, which significantly decreased 12 h after Hp infection and returned to its basal at 48h (Figure 4A). Lysine 4 of histone H3 can be di- or trimethylated. Trimethylation is associated with actively transcribed genes, whereas dimethylation is associated with both silent and active genes. Our results show that histone H3-K4 dimethylation occurs at the promoter region of COX-2 and persists at the 12-time point. This is

consistent with studies suggesting that H3-K4 methylation is a marker of recent transcription. Trimethylation of H3K27 is a facultative heterochromatin mark that promotes recruitment of Polycomb group proteins for gene silencing (Czermin *et al.*, 2002; Orlando, 2003). Interestingly, in MKN28 cells, H3K27me3 is almost unchanged at 6h and significantly increases at 12h. Several loci in both ES and adult stem cells, including those encoding key developmental regulators, are characterized by the simultaneous presence of H3-K4me3 and H3-K27me3, a configuration described as "bivalent domain" (Bernstein et al., 2006). In undifferentiated ES cells this unusual combination of marks is thought to keep genes repressed or expressed at very low levels though ready for later activation (Bernstein et al., 2006), which implies a functional dominance of the inhibitory mark (H3K27me3) over the active one (H3K4me3). In differentiated cells bivalent domains may have a more complex function in fine-tuning gene expression, as suggested by a genome-wide analysis in T lymphocytes (Roh et al., 2006). The same model has been proposed for the *Bmp-2* gene promoter in macrophages.

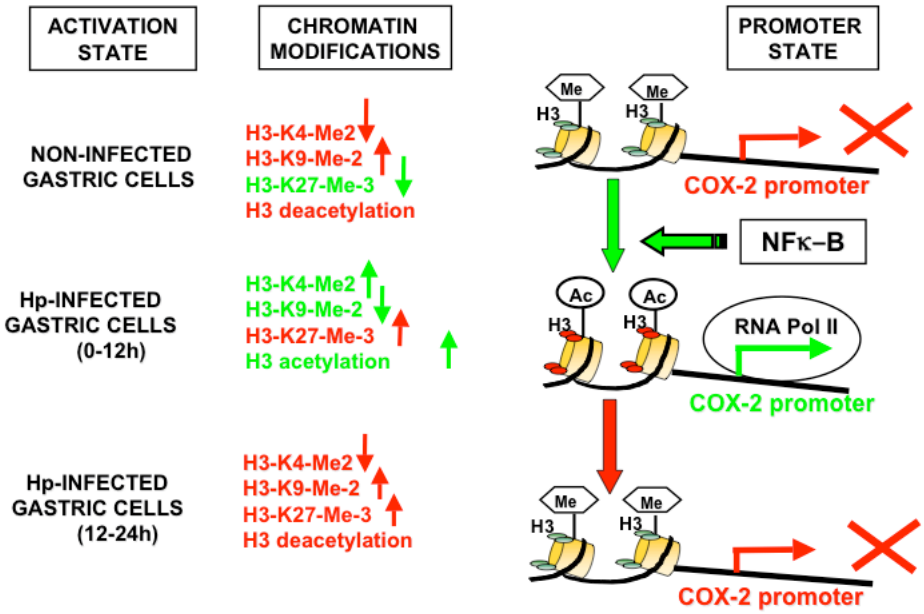

Figura 11 Schematic representation of the model for epigenetic regulation of COX-2 promoter in gastric epithelial cells upon infection with *H. pylori*. See "Discussion" for details.

Hypoacetylation and deacetylation of histones, respectively, lead to tighter winding of DNA and reduced gene transcription. HDACs are known to decrease acetylation of individual lysines of histones H3 or H4 relevant in gene transcription (Ito et al., 2002). The proposed relation between *H. pylori*-infection and HDACs was supported by studies that made use of TSA, a specific inhibitor of HDAC activity, for COX-2 expression. Preventing histone deacetylation clearly increased the COX-2 mRNA expression in *H. pylori*-infected

gastric cells (Figure 5). Moreover, we demonstrated that *H. pylori* reduced the global expression and activity of class I HDAC1, 2 and -3 in gastric epithelial cells, indicating an important role of HDACs in regulation of COX-2 expression (Figure 4A, B, C and D). Another well-described mark, acetylation, which is also a modification found on lysine residues, has been mostly shown as associated with active transcription. It is thought to render chromosomal domains more accessible to the transcriptional machinery. Recent studies demonstrate that histone acetylation is implicated in the induction of COX-2 transcription (Soloff et al., 2004; Nie et al., 2003; Park et al., 2004; Miao et al., 2004). Interestingly, in the present study we found that *H. pylory* increased histone H3 acetylation at the COX-2 promoter, but had no significant effect on the level of histone H4 acetylation (Figure 7A and B). In particular, the ChIP studies of the pattern of histone acetylation across the COX-2 promoter revealed a rapidly inducible acetylation of histone H3, followed by deacetylation as the level of mRNA declined (Figure 7A). To our knowledge, this is the first study to prove that *H. pylori* selectively modulates the level of histone acetylation at the COX-2 promoter. These results also suggest that the pattern of histone H3, rather than histone H4, acetylation at the COX-2

promoter may be more indicative of the changes in COX-2 transcription. In order to focus on the molecular mechanisms responsible for the increase of histone H3 acetylation by *H. pylori*, we hypothesized that *H. pylori* determines COX-2-induced histone acetylation by inhibiting recruitment of HDACs to the COX-2 promoter. Such a regulatory mechanism has been demonstrated for the $p21^{WAF1}$ promoter. As a matter of fact, our ChIP analysis reveal a release of HDAC-1 from the COX-2 promoter that might explain the induction of histone H3 acetylation by *H. pylori* (Figure 8B). Though further investigation is recommended, these results strongly suggest that the effect of *H. pylori* on induced COX-2 expression is mainly mediated by an HDAC-dependent mechanism. The COX-2 promoter contains *cis*-elements for multiple transcription activators including NFk-B, NF-IL-6, AP-1, CRE-binding protein and C/EBP (Appleby et al., 1994). Different external stimuli use a combination of different *cis*-elements in the COX-2 gene promoter to activate its expression by activation of a specific group of transcription factors (Deng et al., 2003; Inoue et al., 1995). The *cis*-elements used to drive COX-2 gene expression in response to *H. pylori* infection are not yet determined. Nonetheless, it has been suggested that the increased COX-2 production in *H. pylori*-infected gastric

mucosa is mediated through a NF-κB pathway (Wu et al., 2005). In resting cells, IB molecules sequester NFk-B in the cytosol. After cell activation, a complex signalling cascade containing IKK kinase results in degradation of IB, thus allowing transfer of NFk-B into the nucleus (Chun et al., 2004). Thus, in order to analyze more directly the influence of *H. pylori* on NFk-B dependent COX-2 transcription, we performed ChIP assays. We found degradation of IB and an increased recruitment of the NFKB subunit p65/RelA as well as of RNA polymerase II to the COX-2 promoter in *H. pylori*-infected epithelium, thus enhancing COX-2 gene transcription (Figure 8C and D). These data imply a crucial involvement of NFk-B in *H. pylori*-caused COX-2 induction. Of great interest is the observation that the histone H3 residues are acetylated at 12 h post-infection (Figure 7A). At this time point, RNA polymerase II is no longer detectable on the promoter, suggesting that its release occurs regardless extensive histone deacetylation. Moreover, RNA polymerase II recruitment, hence gene transcription in general, starts only when NFk-B/RelA approaches the promoter. A proposed model of action for *H. pylori* in regulation of COX-2 is outlined in Figure 11. In non-infected gastric cells there are low levels of H3-K4-me2, H3-K27-me-3 and H3 acetylation at the COX-2 promoter,

due to the action of demethylases and histone deacetylases. Infection with Hp transiently down-regulates demethylases and HDACs activity and causes an increase in histone methyltransferases (MLL1, SUV39H1,-2, G9a, GLP..) and acetyltransferares (p300/CBP, PCAF). Consequently, histone methylation (H3-K4 and H3-K27) and acetylation (H3) increase, NFk-B and Pol II are recruited and this, in turn, facilitates transcriptional activation. Subsequently, methtylation (H3-K4) is diminished, recruitment of HDAC-1 is increased, and HDAC-1 acts to deacetylate histone H3 and shut down gene expression with consequent release of NFk-b and Pol II. In this model, methylation of H3-K27 may have a role in keeping COX-2 gene repressed or expressed at very low levels though ready for later activation (Bernstein et al., 2006).

In conclusion, our study provides novel data on important molecular mechanisms, that were used by *H. pylori* to activate gastric epithelial cells to affect COX-2 transcription, resulting in a strong inflammatory response. After *H. pylori* infection, we found alterations in histone pattern and modification, reduction of global HDAC expression and activity as well as release of HDAC-1 from the COX-2 promoter. Concurrently, histone modifications is accompanied by increased

recruitment of NFk-B and RNA Pol II towards the promoter. A better understanding of the mechanisms involved in *H. pylori*-mediated COX-2 induction may help to develop new strategies to modify the pathophysiology of inflammatory and tumoral diseases caused by COX-2 overexpression.

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