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Running head: *H. pylori* and host DNA methylation

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Abstract (maximum 200 words)

Helicobacter pylori infection is associated with the development of gastric adenocarcinoma. Upstream stimulatory factors USF1 and USF2 regulate the transcription of genes related to immune response, cell cycle and cell proliferation. A decrease in their expression is observed in human gastric epithelial cells infected with *H. pylori,* associated to a lower binding to their DNA E-box recognition site as shown by electrophoretic mobility shift assay. DNA methylation leads to gene silencing. The treatment of cells with 5'-azacytidine, an inhibitor of DNA methylation, restored the USF1 and USF2 gene expression in the presence of infection. Using promoter PCR methylation assay, a DNA hypermethylation was shown in the promoter region of USF1 and USF2 genes, in infected cells. The inhibition of USF1 and USF2 expression by *H. pylori* and the DNA hypermethylation in their gene promoter region was confirmed in gastric tissues isolated from 12-18 months infected mice. Our study demonstrated the involvement of USF1 and USF2 as molecular targets of *H. pylori* and the key role of DNA methylation in their regulation. These mechanisms occurred in the context of metaplastic lesions, suggesting that alteration of USF1 and USF2 levels could participate in the promotion of neoplastic process during *H. pylori* infection.

Introduction

Helicobacter pylori infection is responsible for one of the most common worldwide infection in humans. It represents the major cause of gastric pathologies as chronic gastritis, gastric and duodenal ulcers. In some cases it also leads to gastric adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma (Parsonnet *et al*., 2004, Correa *et al*., 2007). The long-persistence of the bacteria and the induced chronic inflammation contribute to the development of malignancies by deregulating signaling pathways, cell proliferation and causing genetic instability (Atherton, 2006). Transcription factors are key regulators of the host response to infection at various stage of the disease (Naumann *et al*., 2004). For example, in *H. pylori* infected-Mongolian gerbils, Activating Protein-1 (AP-1) and Rel/Nuclear Factor κB (NF-κB) transcription factors act as early responders related to inflammation and late responders related to atrophy, respectively (Kudo *et al*., 2007).

Upstream stimulatory factors (USF1 and USF2) are pleiotropic transcriptional regulators of immune response, cell cycle and cell proliferation (Corre *et al*., 2005). USF1 and USF2 also modulate host functions in response to infection. For instance, *Chlamydia* inhibits interferon-γ-inducible MHC-II expression by breaking down USF1, thereby evading the host immune response (Zhong *et al*., 1999). Up to now, only one study has reported the involvement of USF2 as a positive regulator of the cyclooxygenase-2 transcription in *H. pylori* infection (Juttner *et al*., 2003). In addition, USF1 and USF2 have anti-proliferative properties (Luo *et al*., 1996). Their downregulation has been described in various cancers (Ismail *et al*., 1999; Jaiswal *et al*., 2001; Chang *et al*., 2005; Chen *et al*., 2006). These transcription factors are members of the basic helix-loop-helix leucine zipper family including c-Myc (Atchley *et al.*, 1997). They bind as homo or heterodimers to specific E-box consensus sites CANNTG (NN=GC or CG) *via* base-specific DNA-contact (Sirito *et al.*, 1994; Corre *et al*., 2005). Cytosine methylation at the central CpG of the E-box sequence or mutation can inhibit protein-DNA complex formation leading to gene deregulation (Griswold *et al*., 2001).

DNA methylation is an important mechanism that occurs predominantly in CpG islands located within the promoter region. It is generally associated with gene silencing and plays a critical role in the development of human cancer (Baylin *et al*., 2006). Aberrant methylation of CpG islands is exacerbated by chronic inflammation and has been reported as an early event during gastric carcinogenesis (Nardone *et al*., 2008). In the case of *H. pylori* infection, higher DNA hypermethylation has been described in infected patients (Maekita *et al*., 2006, Nakajima *et al*., 2006).

We previously analyzed the global host response to a chronic *H. pylori* infection and showed that *USF2* gene expression was downregulated in the gastric mucosa of 12 month-infected mice (Vivas *et al*., 2008). In the present study, we investigated the consequences of *H. pylori* infection on the level of USF1 and USF2 transcription factors. Our data highlight the involvement of DNA methylation in the regulation of *USF1* and *USF2* gene expression in the presence of *H. pylori*. Given the properties of *USF1* and *USF2* as tumor suppressor genes (Jaiswal *et al*., 2001), the mechanisms reported here may play an important role in the molecular events driving the infection towards the development of malignancies.

Results

H. pylori-infection leads to the downregulation of USF1 and USF2 transcription factors

The consequences of *H. pylori* strain B38, a clinical isolate from a MALT lymphoma patient, were investigated on USF1 and USF2 expression in gastric epithelial cell line (AGS). *USF1* gene expression decreased by a factor of two to three, after 12 h to 48 h infection. *USF2* transcript levels in cells infected for 48 h (MOI 100:1) were half those in uninfected cells (Fig 1A). The infection also decreased USF1 and USF2 protein levels (Fig 1B). These results were confirmed in other human gastric epithelial cell lines: KATOIII and MKN45 (Fig 1A). In addition, incubation of AGS cells with *H. pylori* B38 lysate (5, 10 and 50µg/ml) lead

to a decrease of USF1 and USF2 expression by a factor of two for both genes, suggesting a regulation by one or more soluble bacterial factors. It is to be noticed that an *E. coli* lysate had no effect (Fig 1C). An inhibition of USF1 and USF2 gene expression by a factor of four and three respectively, was also showed with other *H. pylori* MALT lymphoma derivative strains deficient for the cytotoxin-associated gene pathogenicity island (*cag* PAI^-), as B38: B49 and B25, and (*cag* PAI^+) strains: B60, B29 and B45 (kindly supplied by Pr. F. Mégraud, Bordeaux and Pr. JC Delchier, Créteil, France), thus demonstrating a Cag-independent mechanism (Fig 1D). This negative regulation of USF1 and USF2 gene expression was also confirmed with different *H. pylori* strains: SS1 harbouring a non-functional Cag PAI (Lee et al., 1997) and B128 (*cag*+) (Israel et al., 2001) (data not shown). The decrease of USF1 and USF2 was not attributable to cell apoptosis since the number of live cells negative for annexin V and 7-aminoactinomycin D labelling was 77.3% and 70% after infection with *H. pylori* B38, as compared to 79% and 75.5% in the controls, for 24 and 48h respectively. Furthermore, the induction of apoptosis with staurosporine (100 and 500 nmol/l) for 6 hours did not modify *USF1* and *USF2* gene expression, as measured by real-time PCR (data not shown). These data confirmed that the decrease of USF1 and USF2 levels in the presence of *H. pylori* infection was not due to a decline in cell viability.

The *in vivo* relevance of these findings was assessed in chronically infected-mice with *H. pylori* SS1, known to colonize the mouse stomach for up to 12 and 18 months (Lee *et al*., 1997). As already reported (Touati *et al*., 2003), histology of the infected gastric mucosa showed an active gastritis with metaplasia associated with the presence of large lymphoid follicles after 18 months of infection (Fig 2A part b). Atypia in the antrum and the fundus are also observed. Consistent with our previous findings (Vivas *et al*., 2008), *USF1* and *USF2* gene expression levels were one third to one half and one sixth to one half those in uninfected mice at 12 and 18 months post-infection, respectively (Fig 2B).

***H. pylori* infection decreases USF1/USF2-DNA binding to the E-box promoter region**

USF1 and USF2 binds to the E-box element present in the promoter region of genes (Corre *et al.*, 2005). To assess the consequences of *H. pylori* infection on the E-box recognition by USF1 and USF2, a 20bp oligonucleotides including the E-box sequence CACGTG originated from the *hTERT* promoter region was used. A significant lower binding (62% and 40% less for the upper and lower complex, respectively) was observed with nuclear extracts from *H. pylori* B38-infected cells as compared to the control (Fig 3A and supplementary Fig 1). Competition with a cold probe corresponding to the AP2 consensus binding site had no effect, indicating that this binding is E-box specific (Fig 3A). As observed in Fig 3B (and in supplementary Fig 1), incubation of infected or non-infected cellular extracts with anti-USF1 or anti-USF2 antibodies decreased drastically the level of the protein-DNA complexes. It indicated that USF1 and USF2 are mainly involved in E-box recognition independently of the infection. The consequences of USF1 or USF2 overproduction *in vitro* were also assessed on E-box binding. The overproduction of USF1 and USF2 in AGS cells transfected with pSG5-USF1 and pSG5-USF2 (Luo *et al.*, 1996) was confirmed by western blot (Fig 3C). Both in infected and non-infected conditions, pSG5-USF1 or pSG5-USF2 led to a strong E-box binding, but at a lower extent in the presence of infection (Fig 3C and supplementary Fig 1). In the presence of pSG5-USF1 or pSG5-USF2, the respective levels of USF1 or USF2 are so high, that only USF1 or USF2 proteins contribute to the protein-DNA binding complexes leading to a single band in EMSA.

In the non-transfected conditions, the two protein-DNA complexes upper and lower, previously observed, (Fig. 3A and 3B) indicates the contribution of additional proteins. c-Myc transcription factor, another member of the basic helix-loop-helix leucine zipper family has been shown to bind to E-box sites (Atchley *et al.*, 1997). Accordingly, in control AGS cells, preincubation with a c-Myc antibody decreased the intensity of the lower complex (Fig. 4B

and supplementary Fig 2). Moreover, c-Myc expression is increased by *H. pylori* SS1 infection (Cheng *et al*., 2005) and *H. pylori* B38 as shown in Fig 4A. Therefore, we hypothesized that infection-induced c-Myc can compete with the USFs transcription factors at E-boxes. In the infected conditions, the incubation of cellular extracts with c-Myc antibody slumps both the upper and lower protein-DNA complexes (Fig. 4B and supplementary Fig 2), thus demonstrating a c-Myc binding at the E-box which may antagonize USF1 and USF2 action.

***H. pylori* infection leads to promoter methylation resulting in the downregulation of *USF1* and *USF2* gene expression**

Gene transcription can be inhibited by epigenetic mechanisms as DNA methylation responsible for tumor suppressor gene silencing (Baylin *et al*., 2006). DNA hypermethylation has already been described in patients infected with *H. pylori* (Kaise *et al*., 2008; Maekita *et al*., 2006; Nakajima *et al*., 2006). Then, we investigated the effects of a DNA methylation inhibitor, 5'-azacytidine, on *H. pylori*-mediated *USF1* and *USF2* gene downregulation. Pre-treatment of AGS cells with 5'-azacytidine followed by incubation with *H. pylori* B38 lysate led to a significant recovery of *USF1* and *USF2* gene expression which increased by two to three as compared to the control (Fig 5A). Using the promoter methylation PCR assay as described in the experimental procedures, the DNA methylation status of *USF1* and *USF2* gene promoter region including E-boxes and CpG islands (Fig 5B) was assessed on genomic DNA extracted from uninfected and *H. pylori* B38 infected AGS cells. As observed in Fig 5C, higher amount of methylated-DNA fragments are amplified from DNA isolated from infected cells as compared to the non-infected. Thus, DNA methylation at the promoter region of USF1 and USF2 gene is induced in the presence of *H. pylori* infection. The relevance of these data was also investigated *in vivo* on genomic DNA extracted from the stomach of 18 month-infected mice. The analysis of the DNA methylation status of mouse *USF1* and *USF2*

gene promoter region including E-boxes and CpG islands (Fig 6A), revealed a higher amount of methylated DNA in infected animals as observed by the intensity of the amplified methylated-DNA fragments (Fig 6B and Fig 6C). DNA methylation levels were higher at the *USF1* and *USF2* promoters for four and three of the six infected-mice analyzed respectively. These data confirmed that *H. pylori* infection increases DNA methylation in the promoter region of *USF1* and *USF2* genes, a mechanism involved in the downregulation of their expression.

Discussion

In the present study we demonstrate the inhibitory effect of *H. pylori* infection on the the pleïotropic transcription factors *USF1* and *USF2* gene expression in gastric epithelial cell lines as well as in the mouse model. A DNA hypermethylation is induced by the infection at the promoter region of *USF1* and *USF2* genes, responsible for their downregulation.

Despite a study reporting the MEK-ERK-dependent activation of USF1 and USF2 in the *H. pylori*-dependent host cyclooxygenase-2 transcription (Juttner *et al.*, 2003), their role in the development of gastric malignancies associated to the infection remains unexplored. Here, this negative regulation is demonstrated with the *H. pylori* strain B38 isolated from a patient with MALT lymphoma, and confirmed *in vivo* with the mouse-adapted strain SS1 harbouring a non functional *cag* pathogenicity island (*cag*PAI). The genome of B38 has been completely sequenced, showing the absence of genes coding for the *cag*PAI (Thiberge et al., *submitted*). Moreover, the infection of gastric epithelial cells with isogenic bacterial mutants and other MALT lymphoma derivative strains demonstrated the non-requirement of *cag*PAI-encoded virulence bacterial factors in this negative regulation. Even though the CagPAI is associated with the most severe diseases related to the infection, other studies have also reported a Cag-independent response of epithelial cells to *H. pylori* (Cox *et al.*, 2001; Wessler

et al., 2000). Moreover, the association of *cag*-deficient *H. pylori* strains is well documented in the case of MALT lymphoma (Lehours *et al.*, 2004, Farinha *et al.*, 2005). Lysates of the B38 strain had also a negative effect on USF1 and USF2 expression, indicating that a physical interaction of bacteria with gastric epithelial cells is not required. This regulation is rather mediated by bacterial soluble factor(s). The expression of USF1 and USF2 is also decreased in the stomach of mice chronically infected with *H. pylori* for 18 months. The gastric mucosa of these mice showed a severe active gastritis characterized by an antralization of the fundus, the presence of moderate dysplastic lesions, identified as preneoplastic steps in the malignant process (Correa *et al.*, 2007). Thus, low levels of USF1 and USF2 may have consequences on the transcription of key genes potentially involved in early events promoting the gastric neoplastic process.

By modulating the expression of pleiotropic transcriptional regulators as USF1 and USF2, *H. pylori* infection influences upstream steps of the molecular mechanisms implicated in essential cellular functions, thus allowing a selective progression to cell transformation. As shown in this study, the downregulation of USF1 and USF2 led to a lower binding to their E-box elements that may have direct consequences on their target gene expression. We suggest that the decrease of USFs may also facilitate the access to other transcriptional regulators at E-boxes. The overexpression of USF1 and USF2 in transfected gastric epithelial cells increased the binding to the E-box, but at a lower extent in the infected conditions, suggesting that the E-box site was not accessible for further interaction. The binding of other transcriptional factors such as c-Myc, another member of the basic helix-loop-helix leucine zipper family (Atchley *et al.*, 1997), may antagonize USF-mediated regulation by affecting their E-box recognition. Accordingly, we demonstrated that c-Myc is increased in *H. pylori* infected gastric epithelial cells and also bound to E-box probes.

USFs transcription factors regulate genes implicated in cell cycle, cell proliferation,

and immune response (Corre *et al.*, 2005). In a previous analysis of the global host response to *H. pylori* in mice infected for 12 months, we found that the decrease in *USF2* expression was concomitant to an interferon γ-dependent immune response (Vivas *et al.*, 2008). The decline in USFs protein expression may have consequences on the immune response to *H. pylori,* thus contributing to the persistence of bacteria as shown with *Chlamydia* (Zhong *et al.*, 1999). However, in the case of *Chlamydia*, the decrease in USF1 levels is due to a proteolysis orchestrated by the infection. USFs have anti-proliferative properties and they have been suggested as tumor suppressor genes (Corre et al., 2005; Chang et al., 2005; Chen et al., 2006; Ismail et al., 1999; Jaiswal et al., 2001). Their downregulation may therefore promote cell proliferation and turnover (Luo *et al.*, 1996). Consistently, an exacerbation of the epithelial cell proliferation was observed with *H. pylori* infection as shown by an increase in the immunostaining for the proliferating cell nuclear antigen (PCNA) in the gastric mucosa of 12 months *H. pylori* infected-mice (unpublished data).

The epigenetic silencing of gene expression by CpG hypermethylation at the promoter sites is an alternative mechanism for tumor suppressor gene inactivation (Baylin *et al.*, 2006). It is thought to play a key role in the early steps of carcinogenesis also including gastric preneoplastic lesions (Kang *et al.*, 2001; Nardone *et al.*, 2008). The methylation index of many genes implicated in signal transduction, cell cycle, inflammation and angiogenesis has been reported to increase progressively from chronic gastritis to gastric cancer lesions (Sato *et al.*, 2006). Accordingly, high levels of CpG methylation have been described in *H. pylori*-infected subjects, associated with a higher risk of gastric cancer (Maekita *et al.*, 2006, Nakajima *et al.*, 2006). It is to be noticed that, when *H. pylori* infection is eradicated early, epigenetic alterations can be reversed and associated with a decrease in activity of chronic gastritis (Chan *et al.*, 2006). Interaction of *H. pylori* with gastric epithelial cells leads to the induction of inflammatory mediators, a potential sources of DNA hypermethylation at the

gastric level (Valinluck *et al.*, 2007). In the present study, we showed that both DNA isolated from AGS cells infected with *H. pylori* B38 and DNA isolated from stomach of chronically infected mice showed an hypermethylation in regions including CpG islands and E-boxes in the promoter of *USF1* and *USF2* genes. USF1 and USF2 activate their own transcription by binding to the E-box elements (Corre *et al.*, 2005). The methylation of CpG islands at their promoter sites and/or at CpG within the E-box may also inhibit their binding, resulting in their negative self-regulation during the infection. Such a regulation of *USF1* and *USF2* gene expression has never been reported so far in *H. pylori* infection. Other members of the helix-loop-helix family, the inhibitor DNA binding proteins (Id) are transcription factors involved in cell cycle regulation and neoangiogenesis (Israel *et al.*, 1999). Id4 has been observed downregulated by DNA hypermethylation in *H. pylori* infected mongolian gerbils and more particularly during the progression from gastritis to hyperplasia (Manzo *et al.*, 2006). Accordingly, in our previous transcriptome analysis (Vivas *et al.*, 2008) *Id2* and *Id4* were 4-fold decreased in mice infected with *H. pylori* SS1 for 6 and 12 months (unpublished data). In human cancer, a decrease in Id proteins level occurs at an early stage and is associated with an unfavorable prognosis (Umetani *et al.*, 2004).

In summary, the striking results of this study demonstrate the induction of DNA methylation during *H. pylori* infection and its implication in the decrease of *USF1* and *USF2* transcription factors gene expression. This mechanism is also observed in the mouse model of chronic infection and gastric inflammation at the stage of metaplasia known as pre-neoplastic events. Thus, *H. pylori* exerts cell deleterious effects through induction of promoter methylation resulting in downregulation of key transcriptional regulators as USF1 and USF2. Taken together, these findings strongly suggest that this mechanism plays an important role in the exacerbation and severity of gastric lesions associated to *H. pylori* infection. The development of a clinical approach will further clarify whether USF1 and USF2 expression

levels may be proposed as detection/prognosis markers of a severe clinical issue of the *H. pylori* infection.

Experimental procedures

Bacteria, Cell culture and infection

H. pylori B38 isolated from a patient with MALT lymphoma (Lehours *et al.*, 2004) and SS1 (Lee *et al.*, 1997) were grown on 10% blood agar under microaerophilic conditions. Bacterial lysates were obtained by passage through a French press and protein concentrations were determined by Dc Protein assay (Biorad, Hercules, CA).

Human gastric epithelial cells (AGS: gastric adenocarcinoma, CRL-1739, ATCC-LGC®, MKN45 and KATOIII, gift from Dr C.Figueiredo, Porto, Portugal) were grown in DMEM medium with 10% fetal bovine serum and 1% penicillin-streptomycin for AGS and MKN45 cells and with 20% fetal bovine serum for the KATOIII cell line (Life Technologies Corporation, Carlsbad, CA). Bacteria were added at a multiplicity of infection (MOI) of 100 or 250 bacteria per cell for 12, 24 and 48 hours. USF1 or USF2 were overexpressed by transient transfection of cells with plasmids — pSG5-USF1, pSG5-USF2 or pSG5 as control (Luo *et al.*, 1996) (kindly provided by Dr N.Chen, Houston, TX). DNA methylation was inhibited by treating cells with 5'-azacytidine (1 μmol/l) (Sigma Chemical Co., St. Louis, MO) for three days prior 48 h infection. As similar results were obtained with live bacteria and lysate, and to avoid any effect of 5'-azacytidine on bacteria, we used *H. pylori* B38 lysate (20 μg/ml).

Animal infection

Six week-old specific pathogen-free C57BL/6 male mice (Charles Rivers, France) were administered by gavage either with *H. pylori* SS1 (n=12; cfu=10^7) or peptone broth (n=12).

After 12 and 18 months, stomachs were removed for histology and RNA isolation (Touati *et al.*, 2003). Tissue sections were stained with hemalun and eosin (Prophet EB, 1992).

All experiments were performed accordingly to the guidelines of the Central Animal Facility Committee (Institut Pasteur) and the French Ministry of Agriculture.

Analysis of gene expression

RNA was isolated from cell culture and mouse stomach using TRIzol extraction (Life Technologies) (Vivas *et al.*, 2008). Primers for *USF1, USF2, GAPDH* and *18S* genes were used for RT-PCR (supplementary data Table 1) and real-time PCR (TaqMan® Gene Expression Assays, Applied Biosystems, Foster City, CA; supplementary data Table 2).

Analysis of protein levels by western blot

After coculture with *H. pylori* B38, AGS cells were lysed in RIPA buffer containing protease inhibitors; 25 μg/lane was separated on a 12% SDS-PAGE and transferred onto Immobilon-P membranes (Millipore, Bedford, MA) by electroblotting. USF1 and USF2 antibodies (Santa Cruz Biotechnology Inc., CA; 1/200) and GAPDH antibody (Santa Cruz Biotechnology Inc., CA; 1/500) were used followed by a donkey anti-rabbit antibody conjugated to horseradish peroxidase (Amersham Biosciences, Pittsburg, PA, USA; 1/2000). Detection was performed using the SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL) and using a ChemiDoc™ XRS (Biorad).

Electrophoretic mobility shift assay (EMSA)

Nuclear protein cell extracts were prepared from non-infected and *H. pylori*-infected AGS cells (Pierce Biotechnology Inc., Rockford, IL). EMSA (Gel Shift Assay Systems, Promega, Madison, WI) was performed using a double-stranded oligonucleotide probe (20 bp) 5'-TCCTGCTGCG*CACGTG*GGAAGCCCTGG-3' including an E-box consensus binding site (underlined) present in the *hTERT* promoter (Takakura *et al.*, 1999). Detection was performed using PharosFX Plus System, Biorad.

Determination of DNA methylation status

DNA methylation status of *USF1, USF2* promoter was analyzed by Promoter Methylation PCR assay (Panomics, Redwood City, CA). Genomic DNA was extracted from both *H. pylori* B38 infected and uninfected AGS cells, and from stomachs of uninfected and 18 month-infected mice as described previously (Touati *et al.*, 2003). The genomic DNA was digested either with *Ban*II or *Mse*I (New England Biolabs, Ipswich, MA) and the methylated DNA was isolated according to the manufacturer's instructions, amplified by PCR and detected by electrophoresis on agarose gel followed by UV detection (Gel Doc System, Biorad, supplementary data Table 3). Quantitation was perfomed by using Quantity One® software (Biorad).

Statistical analysis

Statistical analyses were performed using a 2-tailed *t* test. A *P* value ≤ 0.05 was considered significant.

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Figures

Figure 1: *In vitro* inhibitory effect of *H. pylori* on *USF1* and *USF2* expression.

A. AGS cells were infected with *H. pylori* B38 for 12 h, 24 h and 48 h (MOI 1:100 and 1:250). MKN45 and KATOIII cells were infected with *H. pylori* B38 for 24 h and 48 h (MOI 1:100 and 1:250). Quantitation of *USF1, USF2* gene expression was performed by real-time PCR (Taqman). B. Analysis of USF1, USF2 and GAPDH protein levels by western blot on AGS cells extracts. C. AGS cells were incubated for 48 h with *H. pylori* B38 or *E. coli* lysate. *USF1* and *USF2* gene expression was quantitated by real-time PCR and protein level analyzed by western blot. D. AGS cells incubated for 48h with various *H. pylori* strains isolated from patients with MALT lymphomas (MOI 1:250). Results are expressed as means ± SEM of two independent experiments in duplicate (infected *versus* control $*P<0.05$; $**P<0.01$; $***P<0.001$).

Figure 2. Downregulation of USF1 and USF2 gene expression in chronic gastritis.

A. Histology of the gastric mucosa of mice infected with *H. pylori* SS1 for 18 months stained with hemalun and eosin. In contrast to the normal glandular architecture of uninfected tissues (a) the infection leads to an atrophic gastritis with the presence of metaplasia, atypia and inflammatory infiltrates in the *lamina propria* and within the submucosa (b). Note the large follicle of lymphocytes (arrow) and polymorphonuclear cells observed after 18 months of *H. pylori* infection (Magnification ×100). B. RNA was isolated from gastric tissues of 12 and 18 months infected mice. *USF1* and *USF2* expression was assessed by RT-PCR and quantitated (infected *versus* non-infected: $*P<0.05$; $**P<0.01$). Results are expressed as means ± SEM of three independent experiments.

Figure 3: Binding of USF1 and USF2 to the E-box element. A. Representative EMSA. Nuclear cell extracts from non-infected or *H. pylori*-infected AGS cells for 48 h (MOI 1:250)

were incubated with a 20bp ^{32}P-radiolabeled probe as described in materials and methods. For competition experiments the same cold probe was added. B. Identification of transcription factors in the protein-DNA complexes by incubation of nuclear cell extracts with USF1 and USF2 antibodies. C. AGS cells were transfected with pSG5-vector or plasmids overexpressing the *USF1* or *USF2* gene (pSG5-USF1, pSG5-USF2), and then activated with *H. pylori* B38 lysate (20 μg/ml) for 48 h. The upper panel is a representative western blot confirming the overproduction of USF1 and USF2 in transfected cells. The lower panel shows an EMSA representative gel of the USF1 and USF2 E-box binding in these conditions. These data are representative of two independent experiments.

Figure 4: *H. pylori* induced c-Myc expression leading to its E-box binding. A. AGS cells were infected with *H. pylori* B38 (MOI 1:250) for 12, 24 and 48h. c-Myc gene expression and protein level were assessed by RT-PCR and western blot, respectively. B. Representative gel of EMSA on nuclear cell extracts from non-infected or *H. pylori*-infected cells for 48 h (MOI 1:250) incubated with a 20bp ^{32}P-radiolabeled probe containing an E-box site. The identification of c-Myc in the protein-DNA complexes was performed by incubation of the nuclear cell extracts with a c-Myc antibody.

Figure 5: Promoter methylation downregulates *USF* transcription factor gene expression in *H. pylori* infection. Cells were treated with 5'-azacytidine (1μM) for 3 days before incubation with *H. pylori* B38 lysate (20 μg/ml) for 48 h. A. Quantitation of *USF1* and *USF2* gene expression was done by real-time PCR. B. Structure of the human promoter region of *USF1* and *USF2* genes, containing CpG islands and E-box elements. Primers designed for PCR include CpG islands and E-boxes (supplementary data Table 3). Grey arrows indicate the amplified regions. C. DNA methylation status of *USF1* and *USF2* promoter regions analyzed on genomic DNA isolated from uninfected and *H. pylori* B38 infected AGS cells and assessed by promoter methylation PCR assay as described in the

experimental procedures. A representative gel of amplified methylated DNA is reported. Quantitations correspond to means ± SEM of three independent experiments in duplicate (infected *versus* control ***P<0.001; *P<0.05, infected and treated *versus* infected only §P<0.05; §§P<0.01).

Figure 6: DNA methylation status of the promoter of *USF1* and *USF2* gene is increased during gastritis. A. Structure of the murine promoter region of *USF1* and *USF2* genes, containing CpG islands and E-box elements. Primers used for PCR were designed to include CpG islands and E-boxes (supplementary data Table 3). Grey arrows indicate the amplified regions. B. DNA methylation status of *USF1* and C. *USF2* promoter regions analyzed on genomic DNA isolated from the stomach of uninfected and 18 months *H. pylori* infected mice and assessed by promoter methylation PCR assay as described in materials and methods. A representative gel of amplified methylated DNA is reported with each well corresponding to one mouse.

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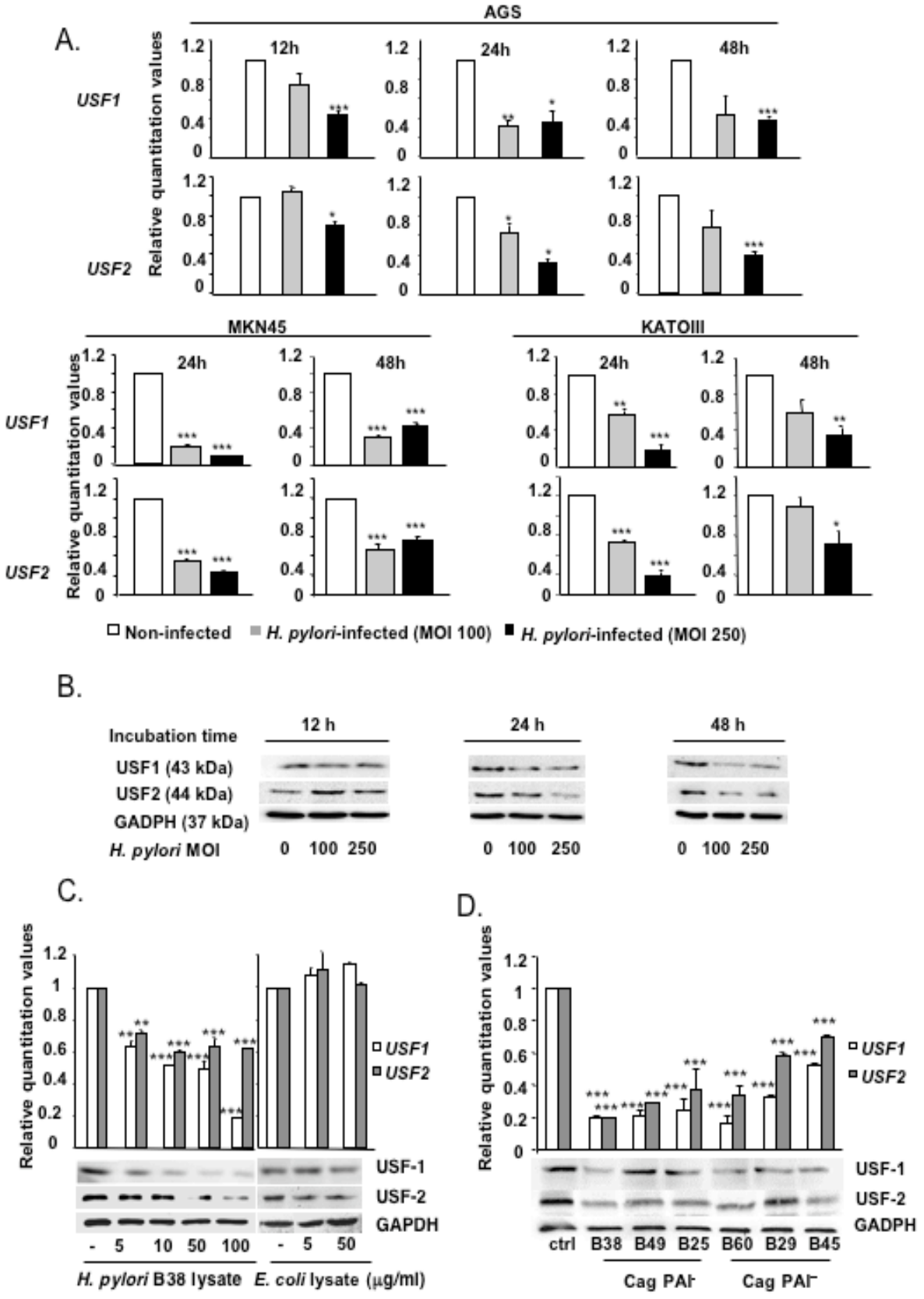
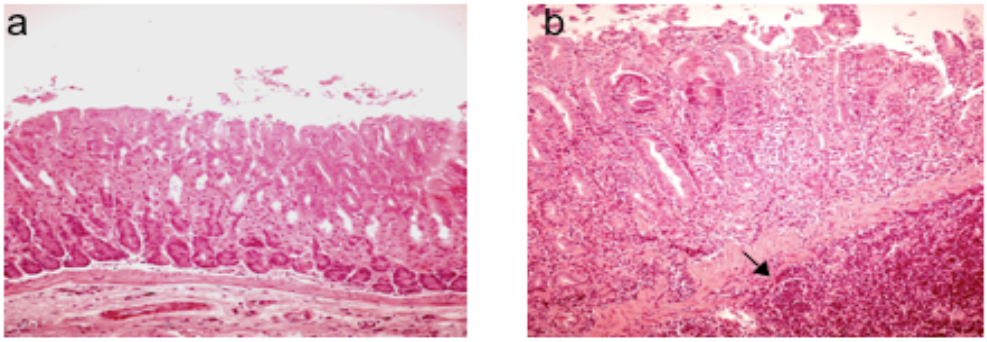
Fig 1


A.
AGS
12h
24h
48h
USF1
USF2
Relative quantitation values
MKN45
KATOIII
Non-infected
H. pylori-infected (MOI 100)
H. pylori-infected (MOI 250)
B.
Incubation time
12 h
24 h
48 h
USF1 (43 kDa)
USF2 (44 kDa)
GADPH (37 kDa)
H. pylori MOI
0 100 250
C.
USF-1
USF-2
GAPDH
- 5 10 50 100
- 5 50
H. pylori B38 lysate
E. coli lysate (μg/ml)
D.
ctrl B38 B49 B25 B60 B29 B45
Cag PAI+
Cag PAI-

A.


a
b

B.

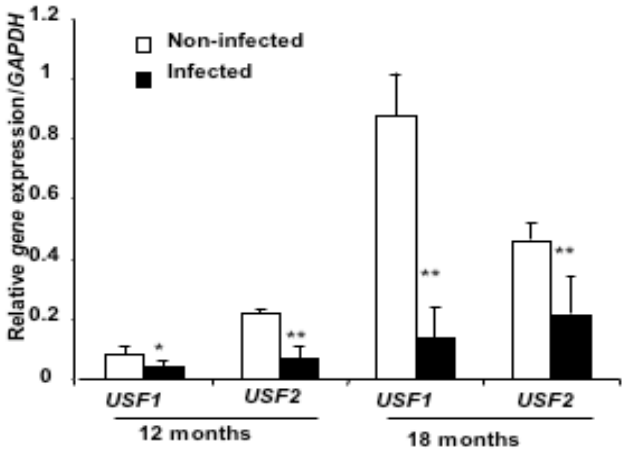

Relative gene expression/GAPDH
1.2
1
0.8
0.6
0.4
0.2
0
Non-infected
Infected
*
**
**
**
USF1
USF2
USF1
USF2
12 months
18 months

Fig2

Fig3

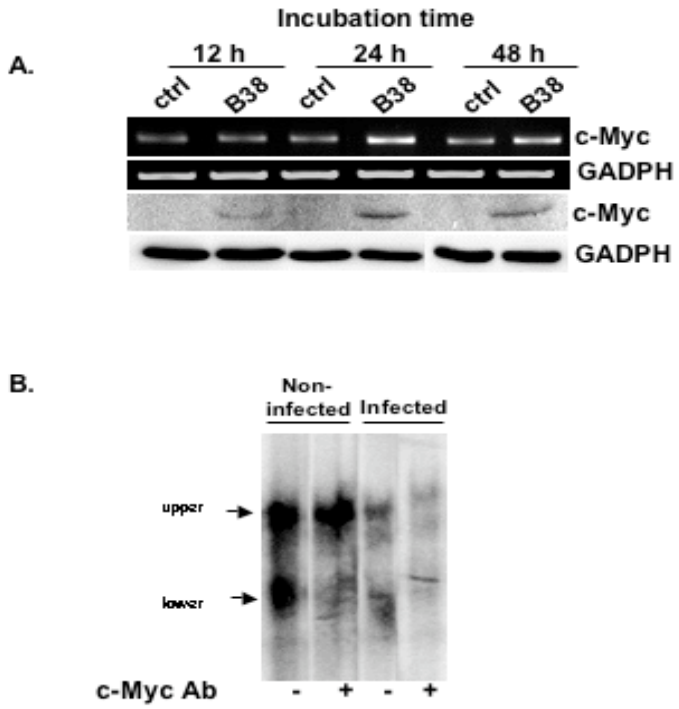

A.
Incubation time
12 h
24 h
48 h
ctrl
B38
ctrl
B38
ctrl
B38
c-Myc
GADPH
c-Myc
GADPH
B.
Non-infected
Infected
upper
lower
c-Myc Ab
-
+
-
+

Fig 4

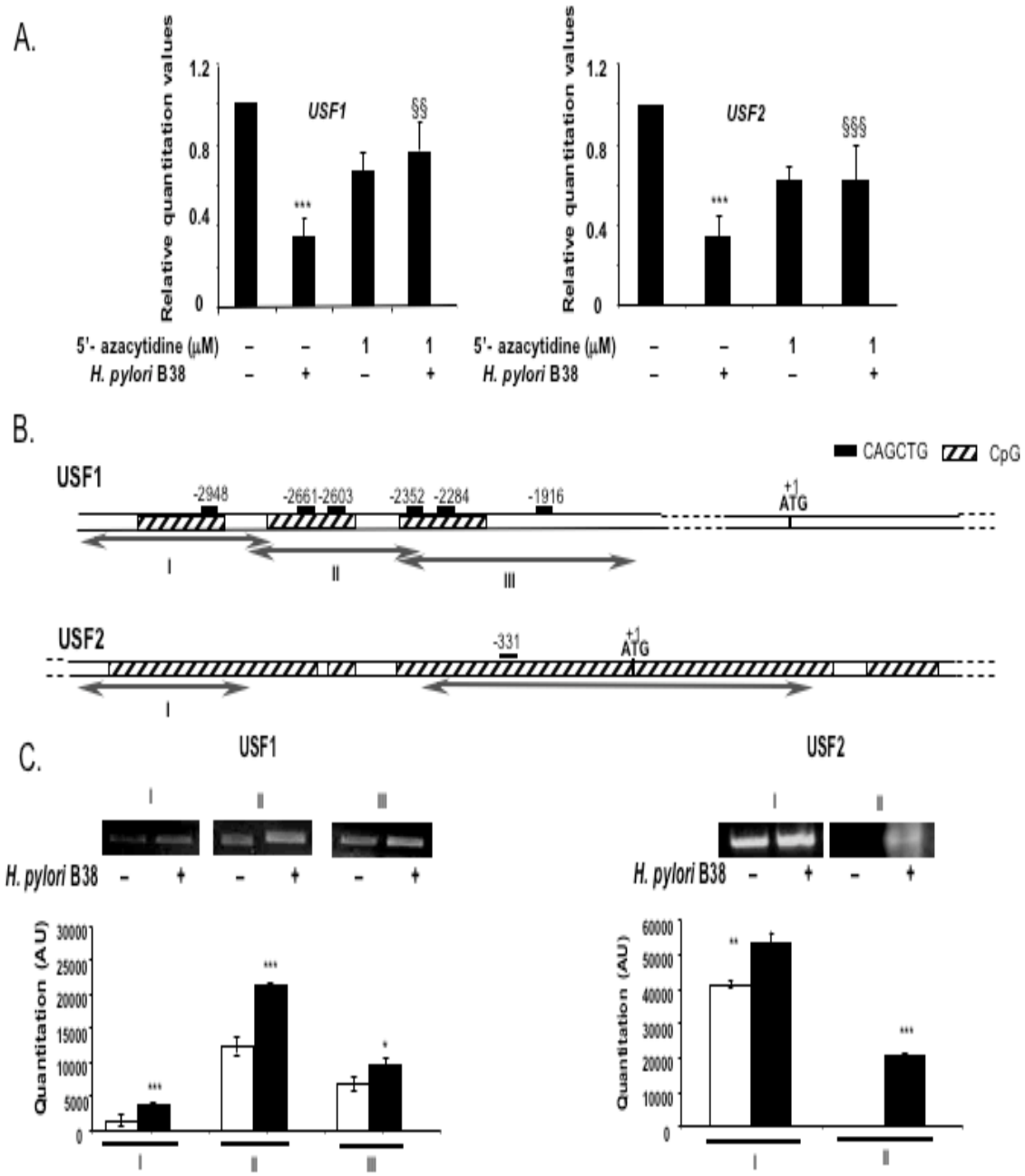

A.
Relative quantitation values
USF1
USF2
§§
§§§
***
5'- azacytidine (μM) – – 1 1
H. pylori B38 – + – +
B.
CAGCTG
CpG
USF1
-2948
-2661-2603
-2352 -2284
-1916
+1 ATG
I
II
III
USF2
-331
+1 ATG
I
C.
USF1
USF2
H. pylori B38 – + – + – +
Quantitation (AU)

Fig 5

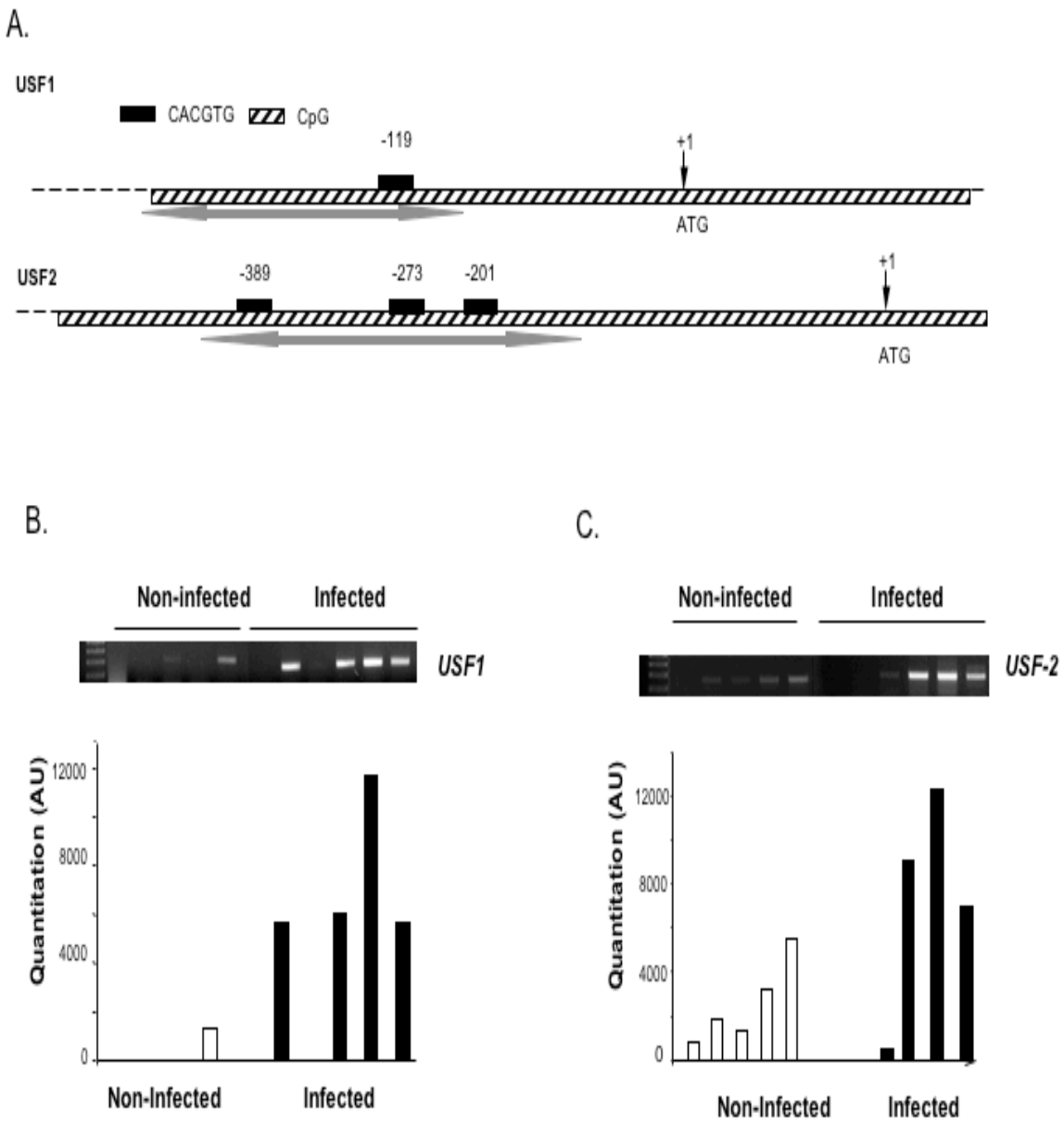
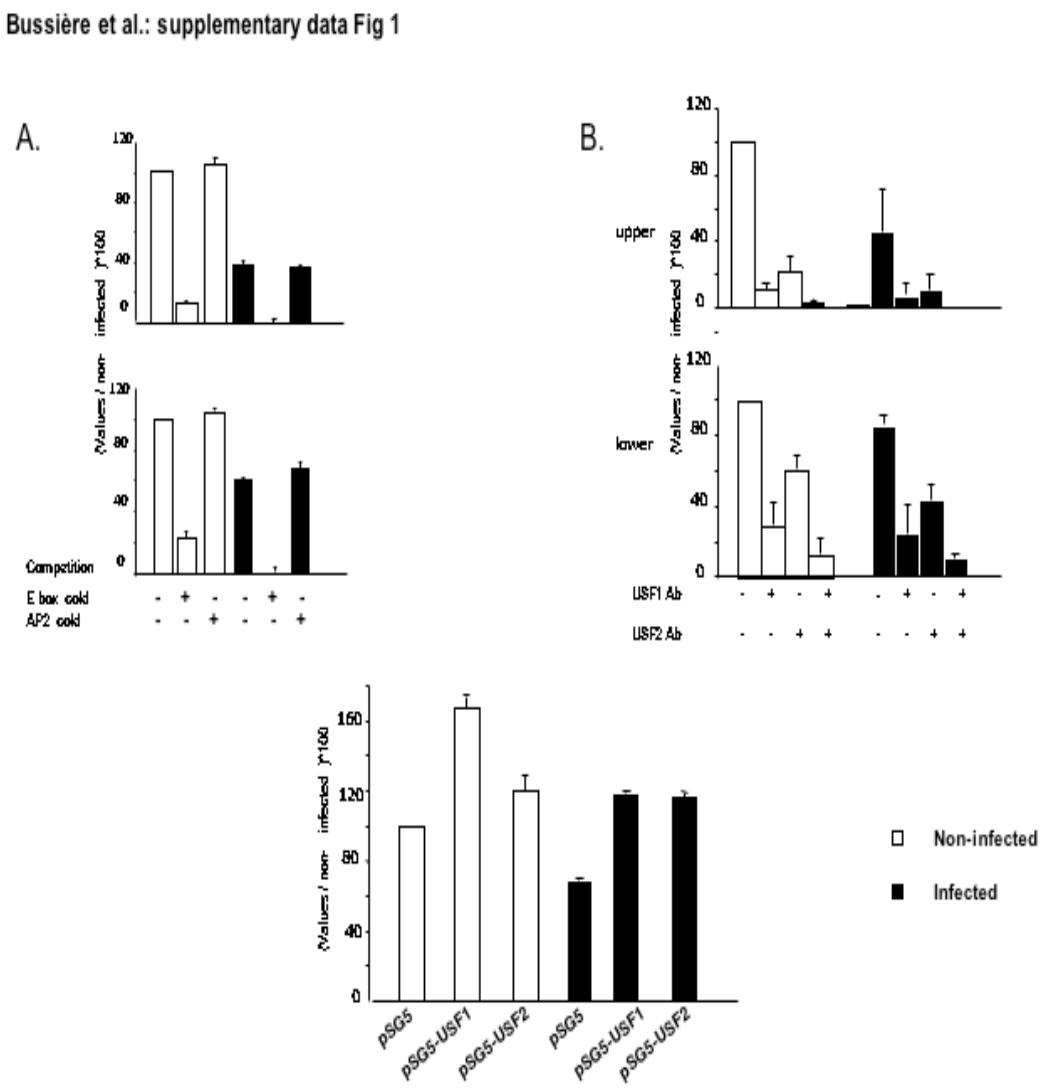
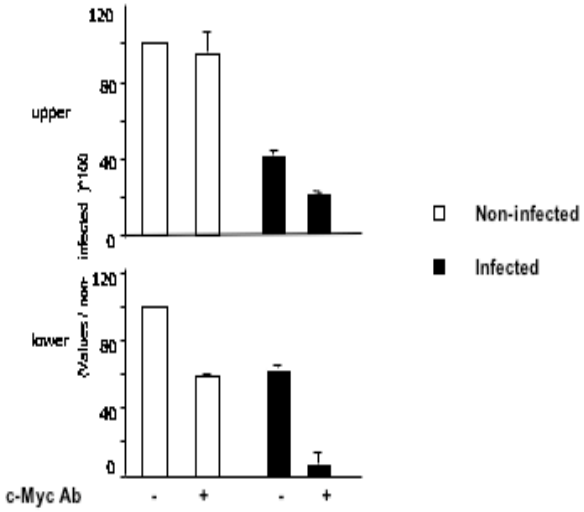

A.
USF1
CACGTG
CpG
-119
+1
ATG
USF2
-389
-273
-201
+1
ATG
B.
Non-infected
Infected
USF1
Quantitation (AU)
12000
8000
4000
0
Non-Infected
Infected
C.
Non-infected
Infected
USF-2
Quantitation (AU)
12000
8000
4000
0
Non-Infected
Infected

Fig 6

Supplementary data



Bussière et al.: supplementary data Fig 2



Supplementary data

Table 1: Primers for RT-PCR analysis

Gene name	Primer	Product size (bp)
human USF1	F: 5'-CACCACGGATTAGAGGTCGT-3' R: 5'-GAGCATCACCTGTCAGCAAA-3'	397
human USF2	F: 5'-GATCCAAAATCCCTTCAGCA-3' R: 5'-CTTTACTCGCTCCCGTCTTG-3'	395
human GAPDH	F: 5'-TTCATTGACCTCAACTACAT-3' R: 5'-GTGGCAGTGCTGGCATGGAC-3'	443
mouse USF1	F: 5'-CTTAGCATTCAGGCCTTTGAGT-3' R: 5'-TCAGTTCGGAAGACGTACTTGA-3',	244
mouse USF2	F: 5'-GACACACCCCTATTCTCCGAAA-3' R: 5'-TCCAGGTTGTGCTGCTGTAG-3'	361
mouse GAPDH	F: 5'-GATGACATCAAGAAGGTGGTGA-3' R: 5'-TGCTGTAGCCGTATTCATTGTC-3'	199

Table 2: Primers for TaqMan® Gene Expression Assays (Applied Biosystems, Foster City, CA)

Gene name	Primer
human USF1	Hs00982868_m1
human USF2	Hs00231528_m1
18S	Hs99999901_s1

Relative expression levels were calculated from the threshold Ct values. Fold-changes for *USF1, USF2* mRNA levels were determined from the threshold cycle values normalized with respect to the values obtained for *18S* and then with respect to the values for the uninfected control.

Table 3: Primers used for studying DNA methylation status by PCR in genomic DNA mouse samples and on genomic DNA obtained from AGS cells

Gene promoter region	Location ATG -> +1	Regulatory elements	Primers	Product size (bp)
Mouse USF1	- 301 - 82	E box (CACGTG): -119 CpG islands: -307-> 193	F: 5'-CCGTCTGTTTCCCTTCAGAG-3' R: 5'-AGCCTTCTCCGTCTTTGGAT-3'	218
Mouse USF2	- 392 - 141	E-boxes (CACGTG): -201, -273, -389 CpG islands: -941->-510; -495-> 37;	F: 5'-GGCAGCTGCAGCCACATTC-3' R: 5'-GGGGTCATGTGAGGAGGAGA-3'	252
Human USF1	-3242 -2778	E-box (CAGCTG): -2948 CpG islands: -3072->-2925	F: 5'-TTCCCATTATACCACACTACTCC-3' R: 5'-GTTGGTGTTTGTGGGTTATTTGT-3'	464
	-2801 -2425	E-box (CAGCTG): -2661; -2603 CpG islands: -2778->-2585	F: 5'-ACAAATAACCCACAAACACCAAC-3' R: 5'-CAGGGTATGAGATAAAGAACCAG-3'	376
	-2448 -1947	E-box (CAGCTG): -2352; -2284;-1916 CpG islands: -2410->-2155	F: 5'-CTGGTTCTTTATCTCATACCCTG-3' R: 5'-AACGGGGTCAGGGTACTTATTC-3'	501
Human USF2	-1990 -1432	CpG islands: -1845->-1520; -1475->1265	F: 5'-ATGAGTGAAGTCACCTCCCT-3' R: 5'-GTAGTAGGCCTCTTCCTCCTC-3'	558
	-532 332	E-box (CAGCTG): -331 CpG islands: -806-> 473	F: 5'-AAAAGGGGGAAAAATAGAAAGC-3' R: 5'-AGGAGGGAGCAGGGTCAG-3'	864

Figure legend for supplementary figures:

Supplementary Fig 1: Quantification of the binding of USF1 and USF2 to the E box promoter region analyzed by EMSA. The protein complex formation at the E-box was quantified using Quantity One software®. Values were expressed as a percentage of the control and correspond to the means of two independent experiments. These data are related to gels reported in Fig 3A ,B and C.

Supplementary Fig 2: Quantification of the binding of c-Myc to the E box promoter region analyzed by EMSA. The protein complex formation at the E-box was quantified using Quantity One software®. Values were expressed as a percentage of the control and correspond to the means of two independent experiments. These data are related to gels reported in Fig 4B.