The *Helicobacter pylori* 5'ureB-sRNA, a cis-encoded Antisense Small RNA, Negatively Regulates ureAB Expression by Transcription Termination

Running title: *H. pylori* ureAB regulation by 5'ureB-sRNA

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Urease is an essential component of gastric acid acclimation by *H. pylori*. The increased level of urease in gastric acidity is due, in part, to acid activation of the two-component system consisting of the membrane sensor HP0165 (ArsS) and its response regulator HP0166 (ArsR), which regulates transcription of the seven genes in two separate operons (*ureAB*, and *ureIEFGH*) of the urease gene cluster. Recently, we identified a novel cis-encoded antisense small RNA, 5′*ureB*-sRNA, targeted at the 5′ end of *ureB*, which down regulates *ureAB* expression by truncation of the *ureAB* transcript at neutral pH. It is not known whether the truncated transcript is due to transcription termination or processing of the full-length mRNA by co-degradation of *ureAB* mRNA-sRNA hybrid complex. S1 nuclease mapping assays show that the truncated transcript is due to transcription termination. Further studies using an *in vitro* transcription assay found that 5′*ureB*-sRNA promotes premature termination of transcription of *ureAB* mRNA. These results suggest that the antisense small RNA, 5′*ureB*-sRNA, down-regulates *ureAB* expression by enhancing transcription termination at 5′ of *ureB*. With this mechanism, a limited amount of 5′*ureB*-sRNA is sufficient to regulate the relatively high level of *ureAB* transcript.
INTRODUCTION

Helicobacter pylori is a Gram-negative bacterial pathogen that colonizes the acidic environment of the human stomach and causes chronic superficial gastritis and peptic ulcer disease (10, 25). Persistent infection with *H. pylori* which, if untreated, lasts for the lifetime of the infected individual and predisposes to gastric malignancies like adenocarcinoma and mucosa-associated lymphoid tissue (MALT) lymphoma (16, 24). The *ureAB* gene of *H. pylori*, located in the urease gene cluster that consists of two operons (*ureAB* and *ureIEFGH*) (1), encodes the urease structural subunits UreA and UreB of the apoenzyme and is essential for the survival of *H. pylori* at low pH, together with the UreI that is a regulator of urea entry to the bacterial cytoplasmic urease (11, 12, 21, 38). UreA and UreB, are expressed at very high levels in *H. pylori* accounting for as much as 8% of the total bacterial protein (2, 20). Urease activity generates the buffers NH$_3$ and HCO$_3^-$ from the metabolism of ambient urea, thereby maintaining both cytoplasmic and periplasmic pH to enable the organism to survive and grow in the stomach (19, 31, 32).

As a key virulence and acid acclimation factor, urease gene expression in *H. pylori* is highly regulated in response to environmental pH changes. Transcription of the *ureAB* genes was found to be positively regulated by the NikR protein in response to increasing concentrations of Ni$^{2+}$ in the surrounding medium (40, 41), through which the environmental acidity might be indirectly sensed (39). Recent studies have shown that the transcriptional induction of urease genes (*ureAB* and *ureI*) in response to low pH is mediated mainly by the HP0165-0166 two-component system (ArsSR), since the pH-induced up-regulation was largely abolished in an ArsS-deficient mutant (26-28). Accordingly, the phosphorylated response regulator ArsR (ArsR–P) was found to bind to extended regions overlapping both the P$_{ureI}$ and P$_{ureA}$ promoters (28). However, although the up-regulatory response is suitable in gastric acidity, the homeostasis of urease expression needs...
to be tightly regulated, as uncontrolled expression at neutral pH leads to decreased fitness of the bacterium due to over alkalization (9, 19). In recent studies (43), we identified a novel urease regulatory mechanism that down-regulates \textit{ureB} expression, which is controlled primarily by the ArsSR two-component system. An antisense small RNA (5'\textit{ureB}-sRNA) encoded in the 5' region of the \textit{H. pylori} \textit{ureB} cistron was found to down regulate \textit{ureAB} expression by increasing the level of a 1.4 kb truncated \textit{ureAB} transcript lacking 3'\textit{ureB} under relatively neutral conditions, since the increased levels of the 5't\textit{ureB}-sRNA (transcribed by an intra-cistronic antisense promoter that may be induced by unphosphorylated ArsR) associate with increased levels of a truncated form (1.4 kb) of the \textit{ureAB} transcript and this truncated form is more abundant in cells growing at neutral pH. In contrast, at low pH the level of 5't\textit{ureB}-sRNA decreases, and then the intact form (2.7 kb) of the \textit{ureAB} transcript predominates allowing full expression of the urease apoenzyme.

Small, non-coding RNAs (sRNAs) are increasingly recognized as being crucial for the regulatory network of all organisms including bacteria (42). In the last decade, there has been an explosion in the identification of regulatory sRNAs encoded on bacterial chromosomes. The majority of characterized sRNAs act by base pairing with target mRNAs. Bacterial base-pairing sRNAs fall into two categories (42): cis-encoded sRNAs are located in the same DNA region and are therefore fully complementary to their targets over a long sequence stretch, whereas trans-encoded sRNAs are located in another chromosomal location, and are only partially complementary to their target mRNAs. The regulatory mechanism of antisense sRNAs may be advantageous over other types of regulation since the antisense sRNAs could provide a specific mechanism whereby the levels of a particular protein need to be repressed or expressed under very select circumstances. In addition, many of the characterized antisense sRNA targets are
subject to extensive regulation, and these antisense sRNAs provide yet one more level of control
(36).

The currently known regulatory mechanisms employed by cis-encoded antisense sRNAs include
transcription attenuation/termination, translation inhibition, and promotion or inhibition of
mRNA degradation (4). Our previous studies (43) suggest that the 5’ureB-sRNA initiates the
down-regulation of ureB by base pairing with a coding region of ureB transcript which does not
include the Shine-Dalgarno sequence. Therefore, it eliminates the possibility that this sRNA
interferes with the initiation of translation through competition with 16S rRNA for the Shine-
Dalgarno sequence (3, 17). However, it is still not clear whether the 1.4 kb truncated ureB
transcript resulting from 5’ureB-sRNA is due to transcription termination or processing of the
full-length mRNA by co-degradation of ureAB-sRNA hybrid complex.

In the current study, we show that the antisense sRNA, 5’ureB-sRNA, down-regulates ureAB
expression by interacting with the ureAB mRNA resulting in premature termination of
transcription at 5’ of ureB through a transcriptional attenuation mechanism. We also provide in
vitro evidence suggesting that, with this mechanism, a limited amount of 5’ureB-sRNA is
sufficient to regulate the relatively high level of ureAB transcript.
MATERIALS AND METHODS

Bacterial strains and growth conditions. *H. pylori* strain 43504 was obtained from American Type Culture Collection (ATCC). A 5′*ureB*-sRNA overexpression strain [HP43504/pTM-P*cagA*-5′*ureB*-sRNA(+)] (43) was constructed by introducing plasmid pTM-P*cagA*-5′*ureB*-sRNA(+) into *H. pylori* strain 43504 via natural transformation as described below. Primary plate cultures of *H. pylori* were grown from glycerol stocks on TSA (trypticase soy agar) plates with 5% sheep blood (Fisher Scientific) for 2-3 days in a micro-aerobic environment (5% O₂, 10% CO₂, and 85% N₂) at 37 °C. In preparation for an experiment, bacteria were scraped from the plates, suspended in 1 mM phosphate HP buffer (138 mM NaCl, 5 mM KCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 10 mM glucose, 1 mM glutamine), pH 7.0, and transferred to fresh plates for 24 hours. For exposure to experimental low pH conditions, the overnight culture of *H. pylori* strain 43504 on TSA plates supplemented with 5% sheep blood were suspended in Brain Heart Infusion (BHI) medium (Difco) to an OD600 of 0.20-0.25. The pH of BHI was adjusted to pH 7.4 or pH 4.5 using concentrated HCl followed by filtration to remove any precipitate. *H. pylori* were then incubated in the presence of 5 mM urea with shaking (120 rpm) under microaerobic conditions at 37°C for 30 minutes. The media pH was measured after 30-min incubation to ensure that there was no pH change at the end of the experiment. *E. coli* strains were grown in Luria-Bertani (LB) broth. When necessary, antibiotics were added to the following final concentrations: ampicillin 100 µg/ml, kanamycin 50 µg/ml (for *E. coli*) or 20 µg/ml (for *H. pylori*).

Construction of overexpression strain for 5′*ureB*-sRNA. pTM117 that was reported as a transcriptional reporter and complementation vector in *H. pylori* (7) was used for the overexpression of 5′*ureB*-sRNA by insertion of a 593-bp DNA fragment containing the promoter region of *cagA* at KpnI/NcoI sites (pTM-P*cagA*). The *cagA* promoter fragment was prepared by...
PCR from the intergenic region between cagB and cagA (34) of H. pylori strain 26695 genome with a primer pair cagBA-IGR-5’P-KpnI/CagBA-IGR-3’P-NcoI (Table 1). For the plasmid used for overexpression of 5’ureB-sRNA [pTM-P_cagA-5’ureB-sRNA(+)], the fusion fragment of 5’ureB-sRNA and a transcriptional terminator identified from H. pylori (HP0092-T1) (8) was prepared in a two-step PCR process by amplification of 5’ureB-sRNA fragment with primer 5’-ureB sRNA(+)-5’P-NcoI and a fusion primer HP0092-T1-5’P-rev–5’-ureB sRNA(+)-3’P (Table 1), and HP0092-T1 fragment with a fusion primer 5’-ureB sRNA(+)-3’P–HP0092-T1-5’P and HP0092-T1-3’P-Psrl (Table 1). The two fragments were subsequently used together as templates in a PCR sewing with primer pair 5’-ureB sRNA(+)-5’P-NcoI/HP0092-T1-3’P-Psrl to enable the fusion, followed by cloning the fusion fragment into pTM-P_cagA at NcoI/Psrl sites. The plasmid pTM-P_cagA-5’ureB-sRNA(+) was introduced into H. pylori strain 43504 via natural transformation (29), and the transformants were selected on BHI plates containing kanamycin.

**RNA preparation.** Total RNA was isolated from H. pylori strains using TRIzol reagent (Invitrogen, CA) combined with RNeasy columns (QIagen, CA). The bacterial pellet was resuspended in 500 μl of TRIzol reagent (Invitrogen) and lysed at room temperature for 5 min before 100 μl of chloroform was added. After spinning at 12000 × g for 10 minutes at 4 °C, the supernatant was mixed with 250 μl ethanol and applied to an RNeasy spin column (QIagen), and RNA purification was processed following manufacturer’s instructions (beginning with the application to the column). RNA concentration was quantified by absorbance at 260 nm using NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies) and the quality was evaluated by capillary electrophoresis using an Agilent 2100 Bioanalyzer with RNA 6000 Nano Assay kit (Agilent Technologies).
Northern blot analysis. Total RNA (5 μg) was fractionated in 6% polyacrylamide-urea gels (Invitrogen) and electrically transferred to Zeta Probe GT membranes (Bio-Rad Laboratories). For verification of equal loading, the RNA on the gel was visualized by ethidium bromide staining and photographed to compare the rRNA band intensities before the RNA was transferred. For preparation of the oligonucleotide probes, the strand-specific sense oligonucleotides 5′ureB 2-1S (55 nt) and antisense oligonucleotides 5′ureB 2-1AS (55 nt) were synthesized (Eurofins MWG Operon) and 5′-end labeled radioactively by T4 polynucleotide kinase (Promega) and [γ-32P]-ATP. The blots were hybridized with 32P-labeled strand-specific oligonucleotide probes 5′-ureB 2-1AS (for detection of ureAB mRNA) or 2-1S (for detection of 5′ureB-sRNA) in ULTRAhyb-Oligo Hybridization Buffer (Ambion) at 42 °C overnight and then washed with 2 × SSC/0.5% SDS. The hybridized blots were autoradiographed using a Phosphor Imager 445 SI (Molecular Dynamics, Sunnyvale, CA). The bands representing each transcript in the hybridized blots and 23s and 16s rRNAs in ethidium bromide stained gels were quantified using ImageJ analysis system (available from rsb.info.nih.gov/ij/). The relative expression level for each transcript was normalized to its corresponding total intensity of 23S and 16S rRNA.

S1 nuclease mapping. S1 mapping of the mRNA in ureB coding region spanning the potential termination site was performed according to standard protocols (30). For the 5′-end labeled probe, the primer HP0072-3′P(1902-1923) was labeled using [γ-32P]ATP and T4-polynucleotide kinase, and then was used to amplify a DNA fragment with primer HP0072-5′P(1266-1290) and 26695 genomic DNA as template, followed by digestion with restriction enzyme AgeI (to generate the 625 bp probe). For the 3′-end labeled probe, a DNA fragment generated by PCR with primers HP0072-3′P(2653-2673) and HP0072-5′P(1266-1290) was digested with AgeI and the overhang was filled in using [α-32P]-dCTP and the Klenow fragment of DNA polymerase I,
followed by digestion with restriction enzyme BamHI (to generate the 718 bp probe). Single-
stranded DNA probes from both 5’-end labeled AgeI fragment (625 nt) and 3’-end labeled
BamHI fragment (718 nt) were separated by 6% polyacrylamide mini-gel containing 8M urea
and recovered from the gel by crash and soak procedure. Fifty µg of total RNA from wild-type
H. pylori and 25,000 cpm of 5’-end labeled or 3’-end labeled probe were hybridized at 37°C
overnight followed by digestion with the S1 nuclease at 45°C for 1 hour. The S1 digestion
products were examined by electrophoresis on a 5% polyacrylamide sequencing gel.

Production of 5’ureB-sRNA. Plasmid pCR4-5’ureB-sRNA (302) used for making in vitro
transcribed 5’ureB-sRNA was constructed by cloning a PCR-amplified fragment coding for
entire 5’ureB-sRNA with primers HP0072-5’P(1215-1234)/HP0072-3’P(1486-1516) into the
TA-cloning site of pCR4-TOPO (Invitrogen). The orientation of the insert was determined by
sequencing. The NotI linearized plasmid was used as template in in vitro transcription reaction
with T3 RNA polymerase (MAXIscript in vitro Transcription Kit, Ambion) to synthesize
5’ureB-sRNA. The in vitro transcribed sRNA was purified with RNA clean-up protocol for
RNeasy Mini Kit (Qiagen). RNA concentration was quantified by absorbance at 260 nm using
NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies).

In vitro transcription assays. An 813-bp 5’ureB fragment under cagA Promoter (PcagA) was
used as the template for in vitro transcription assay. The fusion fragment of PcagA and an 813-bp
ureB fragment (nucleotide 47-859 of ureB coding sequence) was constructed in a two-step PCR
process by amplification of a 286-bp PcagA fragment with primer pair HP0547-5’P(548-571)/
HP0547-3’P(1785-1806) (Table 1), and 5’ureB fragment with a fusion primer PcagA-HP0072-
5’P(1111-1133) and HP0072-3’P(1902-1923) (Table 1). The two fragments were subsequently
used together as templates in a PCR sewing with primer pair HP0547-5’(548-571)/HP0072-
3'P(1902-1923) to enable the fusion. For control template, a 515-bp 3'ureB fragment (nucleotide
1123-1608 of ureB coding sequence) was amplified with a fusion primer P_{cagA}-HP0072-5'P(2159-2182) and HP0072-3'P(2653-2673), and fused to the downstream of P_{cagA} fragment in
a PCR sewing with primer pair HP0547-5'(548-571)/ HP0072-3'P(2653-2673). For in vitro
transcription assays, gel-purified transcriptional fusion construct containing P_{cagA} (0.1 µg) was
transcribed in a volume of 5 µl containing 40mM Tris-HCl, pH 7.9, 10mM MgCl₂, 150mM KCl,
0.01% Triton X-100, 10mM dithiothreitol, 500µM ATP, 500µM CTP, 500µM GTP, 50µM UTP,
500nM [α^{32}P]-UTP (800 Ci/mmol), 2 U of RNasin ribonuclease inhibitor, and 0.5 U of E. coli
RNA polymerase (USB). Different amounts of purified 5'ureB-sRNA (100 ~ 550 fmoles) were
added to the reaction mixture before RNA polymerase. Rat liver mRNA (1µg) was used to
correct for specificity of 5'ureB-sRNA. After incubation for 30 min at 37°C, the reaction was
stopped by adding an equal volume of stop solution containing 95% formamide and 18mM
EDTA and heated at 90°C for 3 min. The samples were then separated by 6% urea-PAGE and
visualized by Phosphor Imager 445 SI (Molecular Dynamics, Sunnyvale, CA).

For mutation of YUNR U-turn motif in the stem-loop structure II, the fusion fragment P_{cagA}-5'ureB
(813) was cloned into the TA-cloning site of pCR4-TOPO (Invitrogen) to generate
pCR4- P_{cagA}-5'ureB (813). Substitution of three bases on 5'ureB sequence (positions 322, 323, and 325 of ureB coding sequence), to produce the Mut-YUNR mutant [pCR4- P_{cagA}-5'ureB
(813)M], was carried out by the QuickChange Site-Directed Mutagenesis Kit (Stratagene) using
pCR4- P_{cagA}-5'ureB (813) as template and the mutagenic oligo primers Mut-YUAN-up and Mut-
YUAN-dn (Table 1). The EcoRI fragment (containing the mutated P_{cagA}-5'ureB template) from
the resulting plasmid pCR4- P_{cagA}-5'ureB (813)M was used in in vitro transcription assay. The
mutation was confirmed by DNA sequencing.
RESULTS

5’ureB-sRNA regulates ureAB expression by controlling the level of intact 2.7 kb/truncated 1.4 kb of ureAB transcript in response to environmental pH change. In order to examine the regulatory role of the cis-encoded 5’ureB-sRNA on ureAB expression, an overnight culture of wild-type H. pylori (strain 43504) and 5’ureB-sRNA overexpression strain [HP43504/pTM-P_cagA-5’ureB-sRNA(+)] (43) was treated at different pH conditions (pH 7.4 and 4.5) for 30 min, and Northern analysis was performed on equal amounts of RNA fractionated in 6% polyacrylamide-urea gels with a strand-specific deoxyoligonucleotide sense probe (5’ureB 2-1S) detecting 5’ureB-sRNA and an antisense probe (5’ureB 2-1AS) detecting ureAB transcripts. As shown in Fig. 1, in the H. pylori wild-type strain, the level of the 2.7 kb intact ureAB transcript increases at pH 4.5 corresponding to the decrease of the truncated 1.4 kb transcript, compared to the level of transcripts at pH 7.4 (Fig. 1C and D). Meanwhile, at pH 4.5 the expression of the 5’ureB-sRNA is significantly decreased compared to pH 7.4 (Fig. 1A and B). In the 5’ureB-sRNA overexpression strain, while the level of 5’ureB-sRNA is highly increased at both pH conditions compared to the wild-strain (Fig. 1A and B), the level of the truncated 1.4 kb ureAB transcript significantly increases corresponding to the decrease of the intact 2.7 kb intact transcript, under both pH conditions (Fig. 1C and D). However, the pH 4.5 induced increase of the intact 2.7 kb ureAB transcript is still detectable in 5’ureB-sRNA overexpression strain, even though it is significantly weaker compared to wild-type strain (Fig. 1C and D). These results suggest that the acid responsive expression of ureAB is controlled by the cis-encoded antisense RNA 5’ureB-sRNA, which provides fine tuning of ureAB expression by regulating the level of 1.4 kb truncated ureAB transcript at the expense of the 2.7 kb full length transcript.
The level of 5’ureB-sRNA even at neutral pH is very low compared to that of ureAB (the Northern results were only detectable for 5’ureB-sRNA after overnight exposure to be comparable with that of ureAB which only needed 30 min exposure, as shown in Fig. 1).

The truncated 1.4 kb ureAB transcript results from transcription termination. The Northern blot analysis presented in Fig. 1 showed a higher level of truncated 1.4 kb ureAB transcript at neutral pH compared to acidic condition. The results from the 5’ureB-sRNA overexpression strain suggested that the 5’ureB-sRNA is needed for these increased levels of the truncated ureAB transcript and concomitantly the decreased levels of the full-length ureAB mRNA. However, these studies cannot determine whether the truncated ureAB transcript is due to termination of transcription or processing of the full-length mRNA followed by rapid degradation of the ureAB mRNA. In order to discriminate between these two possibilities, both 3’-end and 5’-end S1 nuclease mapping were performed. As shown in Figure 2, S1 mapping using 5’-end labeled probe detected only the band (625 nt) representing the full-length ureB transcripts, without detection of smaller degraded bands. The smaller band that appeared in the 5’-end S1 mapping is not a specific S1 digested product, since a similar band was also found in the probe control experiment in which no S1 nuclease was included in the reaction (Fig. 2C). Using the 3’-end labeled probe, while a band (718 nt) representing full-length transcript corresponding to the unterminated ureB mRNA was detected, two major smaller bands (~220 nt and ~195 nt) were also detected after S1 nuclease treatment. These two specific S1 digested products may represent the 3’-end of the ureAB gene up to the termination site. Therefore, the results are consistent with a termination mechanism, but not degradation of the processed RNA in which low-molecular weight bands corresponding to protection of the labeled probe by
transcript representing both the 3’ and 5’ ends of the processed mRNAs should be detected along with a band corresponding to the full-length ureAB transcript resulting from protection by the unprocessed mRNAs (Fig. 2B).

The transcription termination of ureAB is induced by 5’ureB-sRNA. Although the S1 mapping results suggested a transcription termination mechanism, the possibility of the co-degradation still cannot be completely eliminated, especially in the case when the co-degradation happens in an extremely rapid manner. To provide further evidence for transcription termination and to confirm the possible effect of 5’ureB-sRNA on ureAB transcription, we cloned the DNA sequence coding for this antisense sRNA into pCR4-TOPO. The recombinant plasmid was used to synthesize 5’ureB-sRNA by in vitro transcription with T3 RNA polymerase. After purification, 5’ureB-sRNA was added to an in vitro transcription assay in which an 813-bp 5’ureB DNA fragment that spans the potential termination site was fused downstream of a cagA promoter (P_{cagA}) and used as template for E. coli RNA polymerase. As shown in Fig. 3A, a smaller band (~400 nt) representing the terminated transcript is formed even in the presence of low amount of 5’ureB-sRNA (150 femoles) and its level progressively increases with the increasing amounts of 5’ureB-sRNA, becoming the only transcript detectable at higher 5’ureB-sRNA concentrations (350 femoles), while the band (~800 nt) representing the full-length run-off transcript of ureB gradually disappears with increasing amounts of 5’ureB-sRNA. When no 5’ureB-sRNA is added (lane 3) or if rat liver mRNA (lane 2) is added to the reaction mixture, only the full-length transcript is detected. Under the same experimental conditions, the transcription product of a control template (3’ureB) is not affected by 5’ureB-sRNA with only the band representing full-length transcript (~500 nt) detectable (Fig. 3B). These results suggest
that the antisense small RNA (5’ureB-sRNA) regulates the transcription of the target gene (ureAB) by a specific transcription termination mechanism.

The amount of the ~800-nt transcript representing the full-length ureB from an in vitro transcription reaction under the same conditions except for no addition of 5’ureB-sRNA was estimated on an ethidium bromide-stained gel by comparison with known amounts of purified ~800-nt transcript from a separate reaction without radioactive labeling. About 475 fmoles of ~800-nt transcript was produced in a 5 µl transcription reaction (30 min) without 5’ureB-sRNA. This suggests that a relatively small amount of 5’ureB-sRNA (150 ~ 300 fmoles) is enough to start terminating the higher level transcription of its target mRNA.

In our in vitro transcription system, the E. coli RNA polymerase was used due to the unavailability of H. pylori RNA polymerase. Previous study has reported that the PcagA from H. pylori can be activated in vitro by purified E. coli RNA polymerase which shows similar activity to H. pylori RNA polymerase (34). We assume that the RNA polymerase from both E. coli and H. pylori also share a similar property in interpreting terminator signals.

The potential secondary structure of the 5’ureB-sRNA. To better understand how this cis-encoded antisense 5’ureB-sRNA regulates the expression of ureAB, the analysis of the secondary structure of full-length 5’ureB-sRNA and prediction of the sense mRNA (ureAB) in the complementary region were performed with the Vienna RNA Package program RNAfold web server (http://www.tbi.univie.ac.at/). The sequences of both sRNA and mRNA predict several stem-loop structures and three of these structures have sequence complementarities in the loops between the sRNA and mRNA (Fig 4). These structures are designated as I, II, and III in sense
mRNA and I*, II*, and III* in 5’ureB-sRNA. A YUNR U-turn motif (UUAA) is found in the stem-loops II and II*. The YUNR U-turn motif [a motif containing a pyrimidine (Y) followed by a uracil (U), any nucleotide (N), and a purine (R)] is important for RNA-RNA interactions by serving as an initial point of contact between the antisense sRNA and the mRNA target and leading to complete duplex formation (13).

To investigate the role of the YUNR U-turn motif in 5’ureB-sRNA mediated transcription termination, we mutagenized the pCR4-Pcag-t-5’ureB (813) creating pCR4-Pcag-t-5’ureB (813)M which carries a three-base substitution in the stem-loop structure II of 5’ureB mRNA (at positions 322, 323, and 325 of ureB coding sequence). This mutation was designed to eliminate the YUNR U-turn motif that may facilitate the interaction between 5’ureB-sRNA and its target mRNA. As shown in Fig. 5, 5’ureB-sRNA is not able to cause transcription termination when the mutated 5’ureB mRNA (Mut-YUNR) is synthesized, except when higher amount of 5’ureB-sRNA was added (450 fmoles or more). These results suggested that the YUNR U-turn motif in the stem-loop structure II may play a role in the initial pairing between 5’ureB-sRNA and ureB mRNA.
Emerging evidence suggests that the regulation of gene expression through cis-encoded asRNAs constitutes a distinct level of control in bacteria (14). Different levels of regulation are especially important for genes that must be tightly controlled or are critical in multiple cellular responses.

In *H. pylori*, UreA and UreB are the enzymatically active subunits of the urease enzyme necessary for cytoplasmic and periplasmic pH homeostasis and are present as inactive apoenzyme until nickel insertion by UreE, F, G and H. Transcription of *ureAB* operon is positively regulated by the ArsRS two-component system (26, 28). The periplasmic histidine kinase sensor ArsS perceives lowering of medium pH via protonation of its histidine residue 94 (H94) in the periplasmic input domain (22) and triggers autophosphorylation and subsequent phosphorylation of its cognate response regulator ArsR. The phosphorylated response regulator ArsR (ArsR−P) then binds to extended regions overlapping the *P*<sub>ureA</sub> promoters (28) to activate the transcription of *ureAB* into a 2.7-kb mRNA coding for functional UreA and UreB. However, the activity of the urease could be lethal at a relatively neutral pH due to over-alkalization of the medium (9, 19). Thus, an additional level of regulation of urease activity in response to relatively neutral pH is needed. We have identified a cis-encoded asRNA (5′*ureB*-sRNA) (43) that negatively regulates *ureAB* transcription by leading to the accumulation of a truncated 1.4-kb *ureAB* transcript at neutral pH. A direct interaction between the promoter for 5′*ureB*-sRNA and unphosphorylated ArsR was also found (43) suggesting that unphosphorylated ArsR may activate the expression of the 5′*ureB*-sRNA. In the current study, we show that overexpression of 5′*ureB*-sRNA significantly increases the level of truncated 1.4-kb *ureAB* transcript at both pH 7.4 and pH 4.5 (Fig. 1), confirming that this cis-encoded asRNA (5′*ureB*-sRNA) provides an
additional point of control for urease activity by regulating the level of 1.4 kb truncated ureAB transcript.

The chromosomally encoded cis-asRNAs can regulate transcription of the genes encoded on the opposite strand in different ways, including transcription termination, co-degradation, control of translation, transcriptional interference, and enhanced stability of their respective target transcripts (14). The fact that the truncated 1.4-kb ureAB transcript is longer than the distance (1247 bp) between the two convergent promoters P_{ureA} and P_{5’ureB-sRNA} eliminates the possibility that the negative regulation of ureAB is caused by transcriptional interference, which refers to a direct negative influence of one transcriptional process on a second transcriptional process occurring in cis (33). It was speculated that the transient formation of 5’ureB-sRNA/ureAB-mRNA duplexes makes them a target for selective co-degradation, which would result in a 1.4 kb truncated ureAB transcript that lacks most of the 3’ ureB transcript (43). A previous study (1) had shown that several species of mRNA including 2.7-kb ureAB transcript from urease gene cluster of H. pylori are more stable at lower pH condition than at neutral pH and therefore suggested a pH-dependent post-transcriptional regulatory mechanism for urease gene expression by mRNA decay. However, no further studies have been done to show how ureAB mRNA decay is facilitated, and we could not rule out transcription termination that may be responsible for the ureAB transcript truncation.

In this study, we show that this truncation is indeed caused by transcription termination at the 5’-one third of ureB resulting in truncated 1.4 kb ureAB transcript that lacks 3’-two thirds of ureB. The transcription was investigated in vitro as function of increasing amounts of purified 5’ureB-sRNA using a cagA promoter driven 813-bp 5’ureB DNA fragment that spans the potential termination site as template. A terminated transcript (~400 nt) appeared only in the presence of
the 5’ureB-sRNA in place of the full-length transcript (~800 nt) (Fig. 3A), and the transcription of the control gene (3’ureB) is not affected in vitro even at high concentrations of 5’ureB-sRNA (Fig. 3B), suggesting that the transcription termination of 5’ureB is highly specific to 5’ureB-sRNA.

Antisense-RNA-mediated transcriptional attenuation is a replication control mechanism first discovered in Gram-positive bacteria for the staphylococcal plasmid pT18 (23) and later for the streptococcal plasmids pIP501 (5) and pAMβ1 (18), in which the antisense sRNA can regulate transcription by binding and folding the target RNA so that a Rho-independent terminator structure forms (6). Recently, some evidence has shown that in Gram-negative bacteria an antisense sRNA can cause premature termination of transcription of the target gene by a transcriptional attenuation mechanism (15, 35). The 427-nt antisense sRNA, RNAβ, encoded opposite the fatDCBA-angRT iron transport-biosynthesis operon in the fish pathogen Vibrio anguillarum, is complementary to the 3’ region of fatA and the 5’ end of angR. The interaction of the asRNA to the growing fatDCBA transcript leads to transcription termination at a potential hairpin close to the fatA stop codon, which results in increased levels of the fatA portion of the mRNA compared to the downstream angRT portion providing a mechanism for dis-coordinate expression within an operon (35). Another example is the Shigella flexneri virulence gene icsA, which is down-regulated by the antisense sRNA RnaG with both transcriptional interference and transcription attenuation (15). The 5’ part of the nascent icsA RNA forms two long hairpin motifs that seemingly are similar to an anti-terminator structure. The binding of RnaG to the actively transcribed icsA mRNA prevents the formation of the anti-terminator and promotes the formation of a terminator hairpin that leads to transcription termination (15).
The physical interaction of the asRNA with its target is an essential prerequisite for any asRNA regulatory mechanism. Antisense RNAs can initiate base pairing with their target mRNAs via stem loops containing YUNR (where Y = pyrimidine, U = uracil, N = any nucleoside, R = purine) U-turn motif that mediates fast RNA pairing in the majority of the RNA controlled systems (13). Using computational analysis of the secondary structure of full-length 5′ureB-sRNA and sense mRNA in its complementary region, we found three stem-loop structures (Fig. 4) that have sequence complementarities in the loops between the antisense sRNA and the sense mRNA, of which the stem-loop II contains a YUNR U-turn motif (UUAA). The in vitro transcription assay (Fig. 5) with a mutated 5′ureB mRNA (Mut-YUNR) suggests that this structure may play an important role in facilitating rapid bi-molecular interaction between 5′ureB-sRNA and its target mRNA.

Although S1 nuclease mapping and in vitro transcription assays have suggested that the 5′ureB-sRNA down-regulates ureAB expression by enhancing transcription termination at 5′ of ureB, the computational search around the potential termination site on ureB did not reveal any typical transcription terminator structures with Rho-independent stem-loop or Rho-binding site (3). This appears to be similar to a report by Stork, et al (35), in which a novel mechanism for transcription termination by an antisense sRNA has been suggested without Rho-independent terminator structures or Rho binding sites. It has been speculated that, as indicated by the in vitro study with the complementary oligoribonucleotides (44), the antisense sRNA that hybridizes to the nascent RNA strand may form part of the terminator and lead to destabilization of the RNA polymerase-template-transcript elongation complex and subsequently termination.

Given that the level of the target mRNA ureAB is significantly higher than the regulatory asRNA, 5′ureB-sRNA, under any circumstances, and the active termination of ureB transcription
by 5’ureB-sRNA in an in vitro transcription system, we believe that transcription termination is a more efficient mechanism in vivo, compared to co-degradation, in tight control of UreAB expression. With the transcription termination mechanism, a limited amount of 5’ureB-sRNA is sufficient to regulate the relatively high level of ureAB transcript.
ACKNOWLEDGEMENTS

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complete genome sequence of the gastric pathogen *Helicobacter pylori*. Nature 388:539-547.


FIGURE LEGENDS

Figure 1. The pH responsive expression profile of an antisense sRNA, 5’ureB-sRNA, in *H. pylori* by Northern blot analysis. The total RNAs from *H. pylori* 43504 wild-type (indicated by “WT”) and transformant with pTM-P<sub>cagA</sub>-5’ureB-sRNA(+) that expressed high level of 5’ureB-sRNA (indicated by “sRNA+”) were harvested after treatment at pH 7.4 (lanes 1) and pH 4.5 (lanes 2) for 30 min. RNA samples (5 μg) were separated in 6% polyacrylamide-urea gels and then transferred to Zeta-Probe GT Membrane. The sRNA was detected with an oligonucleotide sense probe (5’-ureB 2-1S) corresponding to the 5’ ureB with overnight exposure (panels A), and the ureAB transcripts were detected with a strand-specific oligonucleotide antisense probe (5’-ureB 2-1AS) with 30-min exposure (panels C). The gels (stained with ethidium bromide) are shown as loading controls (right panels). The relative transcript levels normalized to the corresponding intensity of 23S and 16S rRNA for ~290 nt 5’ureB-sRNA (B) and intact 2.7 kb and truncated 1.4 kb ureAB transcripts (D) from *H. pylori* 43504 wild-type and sRNA-overexpression strains at different pH conditions are shown in the Bar-graphs.

Figure 2. 3’-end and 5’-end S1 mapping of the ureB 5’-region. (A) Physical map of the ureAB gene cluster with an antisense sRNA complementary to the 5’ third of ureB. The corresponding transcripts for ureAB gene (intact 2.7 kb and truncated 1.4 kb) are shown on the top. The 3’-end labeled and 5’-end labeled probes that are used for S1 mapping are shown on the bottom at the corresponding position. The stars indicate the position of the radioactive label. Please note that the probes are single-stranded cDNA fragments complementary to the ureB coding sequence and therefore orientated in an opposite direction from the ureB gene. (B) Scheme of the expected banding patterns in the S1 mapping experiments resulting from degradation of the intact mRNA.
of ureAB or transcription termination at a site downstream of sRNA coding region. In the case of co-degradation, both 3’- and 5’-end labeled probes should detect low-molecular-weight bands corresponding to protection of the labeled probe by transcripts representing both the 3’ and 5’ ends of the degrading RNA, in addition to a band corresponding to the full-length transcript resulting from protection by the non-degraded mRNAs. In the case of termination, the 5’-end-labeled probe should detect only the full-length mRNA species that have not been terminated, while the 3’-end probe should detect a band corresponding to un-terminated full-length mRNA and smaller distinct bands representing the 3’ end of the transcript up to the termination site. (C) Experimental results of the 5’- and 3’-end S1 mapping using a 32P-labeled probe and total RNA from wild-type H. pylori (WT). A control experiment (Probe) using the same probes only but without S1 nuclease treatment is also shown.

Figure 3. In vitro transcription of 5’ ureB as function of increasing amounts of purified 5’ ureB-sRNA. (A) An 813 bp DNA fragment corresponding to 5’ ureB (nucleotides 46-859 of ureB coding sequence) fused downstream of cagA promoter (P_cagA) was used as template with E. coli RNA polymerase, [α-32P]UTP, NTPs, without addition (lane 3), and with addition of increasing amount of 5’ureB-sRNA from 100 to 400 fmoles (lanes 4-10). The reaction mixtures were incubated with and without in vitro synthesized 5’ureB-sRNA for 30 min at 37°C. (B) A similar in vitro transcription assay with a 515-bp DNA fragment corresponding to 3’ ureB (nucleotides 1123-1608 of ureB coding sequence) fused downstream of P_cagA was used as a control. Lanes 1 marked as “M” is a marker (X174 DNA/Hinf III). Lanes 2 marked as “C” sever as a control with 1 µg rat liver mRNA but without 5’ureB-sRNA.
Figure 4. (A) The nucleotide sequence of the region encompassing the 3’ end of ureA and 5’ end of ureB is shown. The complete antisense sRNA, 5’ureB-sRNA (arrowed green line beneath the sequence), including 5’ end (+1) and the putative -10 promoter sequence (red arrow) for 5’ureB-sRNA are indicated. The nucleotides of loops of the stem-loop structures are highlighted (yellow for ureB mRNA and blue for 5’ureB-sRNA). (B) The putative stem-loop structures were generated using the RNAfold web server (http://www.tbi.univie.ac.at/). The complementary stem-loop structures (I-I*, II-II* and III-III*) are shown. Position 1 in the sense RNA structure corresponds to the nucleotide 151 of the ureB coding sequence (numbered on the right side of the sequence in (A). A YUNR U-turn motif is indicated in the stem-loop structures II and II*.

Figure 5. The mutation on YUNR U-turn motif in stem-loop structure II reduces the efficiency of transcriptional termination mediated by 5’ureB-sRNA. (A) The stem-loop structure II with YUNR U-turn motif in 5’ureB mRNA and base exchanges to create the mutated 5’ureB mRNA (Mut-YUNR) are shown. (B) An 813-bp 5’ ureB DNA fragment carrying mutation (Mut-YUNR) fused downstream of P_cagA was used as template for in vitro transcription with E. coli RNA polymerase, [α-32P]UTP, NTPs, without addition (lane 2), and with addition of increasing amount of 5’ureB-sRNA from 200 to 550 fmoles (lanes 3-10). The reaction mixtures were incubated with and without in vitro synthesized 5’ureB-sRNA for 30 min at 37° C. Lane 1 marked as “M” is a marker (X174 DNA/Hinf III).
A

\[ \text{stem-loop II in 5'} \text{ ureB mRNA} \]

Mut-YUNR

B

\[ 5' \text{ ureB-sRNA added (fmoles)} \]

~800 nt (Full)

~400 nt (Terminated)
Table 1. Oligonucleotide primers/probes used in this study

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a Sequences in uppercase letters are derived from the genome sequences of *H. pylori* 26695 {Tomb, 1997 #46}. Sequences introduced for cloning or mutation purposes are given in lowercase letters, and restriction recognition sites are underlined.

b Restriction recognition sites.

c Nucleotide positions refer to the genome sequence of *H. pylori* 26695 {Tomb, 1997 #46}.