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Dual Control of *Helicobacter pylori* Heat Shock Gene Transcription by HspR and HrcA

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The HspR repressor regulates transcription of the *groESL*, *hrcA-grpE-dnaK*, and *cbpA-hspR-orf* operons of *Helicobacter pylori*. Here we show that two of the HspR-regulated operons, namely, the *groESL* and *dnaK* operons, encoding the major cellular chaperone machineries are also regulated by the *H. pylori* homologue of the HrcA repressor. Similarly to the *hspR* mutation, deletion of the *hrcA* gene also leads to complete derepression of the P_{gro} and P_{hrc} promoters. The presence of both HspR and HrcA is therefore necessary for regulated transcription from these promoters. HrcA binds directly to P_{gro} and P_{hrc}, likely contacting two inverted repeats with similarity to the CIRCE motif, which are present on both promoters. HrcA regulation is, however, shown to depend on binding of the HspR protein, since deletion of the HspR-binding site of the P_{gro} promoter leads to loss of heat inducibility of this promoter. In contrast, transcription from the P_{cbp} promoter is regulated solely by HspR. HspR is also shown to form oligomers in vivo through a stretch of hydrophobic repeats between amino acid positions 66 and 97. The implications of these findings for the elucidation of the networks regulating heat shock gene expression in *H. pylori* are discussed.

Helicobacter pylori, a microaerophilic, spiral-shaped, gram-negative bacterium that colonizes the human gastric mucosa, is the principal causative agent of chronic active gastritis and gastric and duodenal ulcers and is a risk factor for the development of adenocarcinoma (23). Various bacterial factors contribute to the process of infection and colonization of the gastric epithelium, including urease, flagellins, the vacuolating toxin VacA, and the cytotoxin-associated protein CagA. The heat shock proteins of *H. pylori* have been shown to play an important role in the infectious process (15). The GroEL and GroES homologues of *H. pylori* are considered important modulators of the stability and activity of the urease enzyme, which protects the bacteria from the low pH of the stomach lumen (11, 12, 18), and both DnaK and GroEL are thought to contribute to the adherence of the bacteria to sulfated glycolipids on the surface of epithelial cells (17).

Expression of heat shock genes is generally tightly regulated, with a basal level ensuring cellular functions under normal growth conditions and a strong induction occurring after exposure to a variety of environmental stresses, including heat shock, osmotic or acidic shock, ethanol treatment, exposure to heavy metals, etc. Although this stress response is universally conserved throughout both the prokaryotic and eukaryotic worlds, the basic molecular mechanisms differ considerably between different species. Positive regulation is observed in *Escherichia coli* and most other gram-negative bacteria, where a specialized sigma factor (σ^{32}) induces the transcription of heat shock genes under stress conditions (7). In *Bacillus subtilis* and a variety of other gram-positive and gram-negative bacteria regulation is negative, involving a specialized transcriptional repressor (HrcA), which binds to an inverted repeat (CIRCE [for controlling inverted repeat of chaperone expression]) in the promoter regions of heat shock genes under nonstressed conditions but not under stressed conditions (30, 44). A variant of this mechanism is active in *Streptomyces* spp., in which HspR, a transcriptional repressor not related to HrcA, controls transcription of the *dnaK* operon by binding to three partially related inverted repeats (HAIR [for HspR-associated inverted repeats]) in the promoter region (6). The genome sequence of *H. pylori* revealed the absence of a homologue of the heat shock sigma factor σ^{32} and the presence of homologues of both the *B. subtilis* HrcA repressor and the *Streptomyces* HspR repressor (38). Previously, we have demonstrated that the *H. pylori* HspR protein negatively regulates transcription from the P_{gro} and P_{hrc} promoters, which are responsible for transcription of the *groESL* and *hrcA-grpE-dnaK* operons (35). HspR was found to autoregulate its own synthesis by repressing the P_{cbp} promoter responsible for transcription of the operon that contains the *hspR* gene itself, as well as the *cbpA* gene coding for a DnaJ homologue and a third gene of unknown function. Transcriptional repression is exerted by binding to large DNA regions of ca. 75 bp on the three chaperone gene promoters with the binding sites mapping to different positions with respect to the transcriptional start sites. Whereas binding occurs in a region overlapping the −35 and −10 promoter elements in the case of the P_{cbp} promoter, the HspR-binding sites of the P_{gro} and P_{hrc} promoters lie in positions centered around positions −72 and −117, respectively. This observation led us to hypothesize that HspR and another repressor, possibly binding further downstream with respect to the HspR binding site, might coregulate the *groESL* and *hrcA-grpE-dnaK* operons. In the present study, we demonstrate that

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Reference or source
Strains		
Escherichia coli		
DH5α	*supE44* Δ*lacU169* (ϕ80 *lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	13
JH607	Lysogen of λ112O_sP_s-*cat* in strain AG1688	2, 43
Helicobacter pylori		
G27	Clinical isolate; wild type	42
G27(*hrcA*::*kan*)	G27 derivative; bp 156 to 375 of *hrcA* have been replaced by a *kan* cassette	This work
G27(*hspR*::*kan*)	G27 derivative; bp 66 to 334 of the *hspR* coding sequence have been replaced by a *kan* cassette	35
G27(*hrcA*::*kan; hspR*::*cat*)	G27(*hrcA*::*kan*) derivative; bp 66 to 334 of the *hspR* coding sequence have been replaced by a *cat* cassette	This work
G27 *hrcA-myc*(+)	G27(*hrcA*::*kan*) derivative; *hrcA-myc* complementing strain obtained by double homologous recombination of pVAC-*cat-hrcA-myc*(+)	This work
G27(P_{gro}::*cat*)	G27 derivative; bp −40 to −113 of the P_{gro} promoter have been replaced by a *cat* cassette	This work
Plasmids		
pGEM3	Cloning vector; Amp^r	Promega
pGEM-T	Cloning vector for PCR products; Amp^r	Promega
pBS	pBluescript KS II(+) cloning vector; Amp^r	Stratagene
pSL1190	Cloning vector; Amp^r	Pharmacia
pILL600	Plasmid carrying the *kan* gene from *Campylobacter coli*	21
pDT2548	Plasmid carrying the *cat* gene from *Campylobacter coli*	40
pGEM3(*hrcA*::*kan*)	pGEM3 derivative; a 1,400-bp BamHI fragment from pILL600 containing the *kan* gene is flanked by 451-bp EcoRI-BamHI and 365-bp BamHI-PstI PCR fragments (oligonucleotides hrc1 and hrc6 and oligonucleotides hrc3 and hrc4, respectively)	This work
pGEM3(*hspR*::*cat*)	Derivative of the plasmid used for knockout of the *hspR* gene (35); the *kan* cassette was replaced by an 800-bp HincII fragment of the *cat* gene from plasmid pDT2548	This work
pGEM3(P_{gro}::*cat*)	pGEM3 derivative; an 800-bp BamHI-XbaI fragment of the *cat* gene is flanked by a 626-bp EcoRI-BamHI and a 206-bp XbaI-PstI PCR fragment (oligonucleotides hsg1 and hsg2 and oligonucleotides hsg6 and hsg7, respectively)	This work
pVac::Km	pGEM3 derivative containing 564 bp of the 3′ end of the *cysS* gene upstream of *vacA* and a 753-bp fragment of the distal end of the *vacA* gene	9
pBS-*hrcA*	pBS KSII(+) derivative containing the *hrcA* gene with its promoter (oligonucleotides hrc1 and hrcHrev)	This work
pGEM3-FP1gro	pGEM3 derivative containing an ~665-bp EcoRI-BamHI PCR fragment (oligonucleotides gro1 and gro4) encompassing the P_{gro} promoter	This work
pGEM3-FP4hrc	pGEM3 derivative containing a 468-bp EcoRI-BamHI PCR fragment (oligonucleotides hrc1/hrc6) encompassing the P_{hrc} promoter	This work
pGEM3-cbp	pGEM3 derivative containing a 272-bp EcoRI-BamHI fragment encompassing the P_{cbp} promoter	35
pBS-*hrcA-myc*	pBS-*hrcA* derivative containing a 32-bp HindIII-XhoI insert, obtained by annealing of oligonucleotides mycA and mycB, and coding for a C-terminal human c-Myc fusion tag (EQKLISEEDL)	This work
pBS-*cat-hrcA-myc*	pBS-*hrcA-myc* derivative carrying a EcoRI-BamHI fragment of the *cat* gene from pDT2548	This work
pVAC-*cat-hrcA-myc*(+)	pVac::Km derivative linearized with BamHI and ligated to a 1,992-bp BglII-BamHI fragment obtained by partial digest of pBS-*cat-hrcA-myc*, carrying *cat-hrcA-myc* in the "+" orientation	This work
pGEM-1023	pGEM-T derivative carrying a 774-bp PCR fragment (oligonucleotides cbp4 and cbp5) that comprises the *hp1023-cbpA* intergenic region and the 5′ parts of the respective genes	This work
pGEM-dnaG	pGEM3 derivative containing an 858-bp EcoRI/BamHI PCR fragment (oligonucleotides gro1 and gro6) that comprises the *dnaG-groES* intergenic region and the 5′ parts of the respective genes	This work
pSL1190-112	pSL1190 derivative containing a 464-bp NcoI/BamHI PCR fragment (oligonucleotides hrc2 and hrc5) that comprises the *hrcA-hp112* intergenic region and the 5′ parts of the respective genes	This work
pBF21	pBR322 derivative expressing the bacteriophage λ *c*I repressor under the control of a tandemly repeated *lacUV5* promoter	37
pBF22	pBF21 derivative which an 866-bp HindIII-EcoRV fragment encoding the C-terminal oligomerization domain of *c*I has been deleted	37
pBFhrc	pBF21 derivative; the HindIII-BamHI fragment encoding the C-terminal oligomerization domain of *c*I has been replaced by an 877-bp fragment (oligonucleotides hrc-oli and hrcbam), which encodes the HrcA repressor protein (aa 1 to 276) of *H. pylori*	This work
pBFH1	pBF21 derivative; the HindIII-BamHI fragment has been replaced by a fragment (oligonucleotides hsp-oli and hspR-C) encoding aa 1 to 123 of HspR	This work
pBFH2	pBF21 derivative; the HindIII-BamHI fragment has been replaced by a fragment (oligonucleotides hsp-oli2 and hspR-C) encoding aa 44 to 123 of HspR	This work
pBFH3	pBF21 derivative; the HindIII-BamHI fragment has been replaced by a fragment (oligonucleotides hsp-oli and hsp-oli3) encoding aa 1 to 97 of HspR	This work
pBFH5	pBF21 derivative; the HindIII-BamHI fragment has been replaced by a fragment (oligonucleotides hsp-oli and hsp-oli6) encoding aa 1 to 43 of HspR	This work
pBFH6	pBF21 derivative; the HindIII-BamHI fragment has been replaced by a fragment (oligonucleotides hsp-oli4 and hspR-C) encoding aa 98 to 123 of HspR	This work
pBFH7	pBF21 derivative; the HindIII-BamHI fragment has been replaced by a fragment (oligonucleotides hsp-oli and hsp-oli7) encoding aa 1 to 65 of HspR	This work
pBFH8	pBF21 derivative; the HindIII-BamHI fragment has been replaced by a fragment (oligonucleotides hsp-oli5 and hspR-C) encoding aa 66 to 123 of HspR	This work

[a] Amp^r, ampicillin resistance.

TABLE 2. Oligonucleotides used in this study

Oligonucleotide	Sequence (5′ to 3′)[a]	Strand orientation	Site[b]	Position[c]
cbp4	attattgaaTTCATCTTGGCTGGCGTTTTCGCTC	−	EcoRI	1087698–1087665
cbp5	CGTTTAGGGAAGTAGgGATCCGATTCCCTTGC	+	BamHI	1086898–1086929
gro1	attattggatcCAGGGATGATGATGCCTGAACTGG	+	BamHI	9529–9563
gro6	TTTCAATGCCGTAATgAATTCTATTCGTGC	−	EcoRI	10388–10359
gro4	attattgaatTCTAAAACTATATTGAATCGGTGGG	−	EcoRI	10194–10170
hrc1	attattgaaTTCTTGGGTTAGGGGGATTTTAAGGG	−	EcoRI	119958–119924
hrc2	attattggatCCATTCTTGATGAAAGAACCCTCGC	+	BamHI	119640–119674
hrc3	attattggatCCCAGTTTGGAGCGTTTAGAAAGAG	−	BamHI	119291–119257
hrc4	TGTTTTGAAATTCTgCAGGGCGTGTTACTAGCATG	+	PstI	118903–118937
hrc5	ACCCTTTCTAAcCATGGATAATGAAATGC	−	NcoI	120126–120098
hrc6	ttttttggaTCCGCCAACTCTTTTAAGCGTTTAG	+	BamHI	119490–119523
hrcHrev	agtagaagcttCTGTTCCTCCTCAGAAATCGTTTG	+	HindIII	118824–118846
mycA	*agcttattagtgaggaagacctataatagatc*			
mycB	*tcgagatctattataggtcttcctcactaata*		BglII	
hsg1	CTTGCCTAGCACGCCgAATTCAATGAGCTTG	−	EcoRI	10438–10408
hsg2	atatatggatcCAAGGATTAGAGAAAGGGGCG	+	BamHI	9786–9817
hsg7	atatattctagAGGCTTGACTTATCCCTAAAAATGCGC	−	XbaI	9733–9696
hsg6	atatatctgcAGGCTTTTCCTTAGCGTTATCAGGG	+	PstI	9510–9544
hrc-oli	agctataagctttATGGTGATTGACGAGATTTTTCAAAT	−	HindIII	119666–119628
hrcbam	atatatggatccGGCTTAAATGATCGTGTTCTTGG	+	BamHI	118772–118806
hsp-oli	attattaagctttGTGTGCGATTATGATGAACCGC	+	HindIII	1088496–1088530
hsp-oli2	GACTGATGGGAAAATaaGCTTGTATTCCCAACG	+	HindIII	1088622–1088654
hsp-oli3	attattggatccTGTTTGTGCAGAGCGTCTTGCAAC	−	BamHI	1088813–1088778
hsp-oli4	attattaagcttaAAAAATACCAAAACCCCAACG	+	HindIII	1088793–1088826
hsp-oli5	attattgaagactaagcttgAATCTTGCGGGCGTGGATATTATC	+	BbsI,	1088687–1088730
hsp-oli6	attattggatcctaCATTTTCCCATCAGTCCTGCTAGG	−	BamHI	1088651–1088614
Hsp-oli7	atatatggatcctaCCCCATATCCCTTGTAAGGCG	−	BamHI	1088717–1088683
HspR-C	atatatggatccGGGTTTAAAAGCGAAGTCAGGCTC	−	BamHI	1088914–1088879
P112B	CCCTTTCTAAACATGGATAATG	−		120125–120104

[a] Capital letters indicate *H. pylori*-derived sequences, lowercase letters indicate sequences introduced for cloning purposes, underlined letters indicate restriction recognition sites, and italic letters refer to non-*H. pylori* sequences.
[b] Restriction recognition sites.
[c] Nucleotide positions refer to the genome sequence published by Tomb et al. (38).

both operons are corepressed by HspR and the *H. pylori* homologue of the HrcA repressor, encoded by the first gene of the *dnaK* operon. Transcription of the *cbpA-hspR-orf* operon is shown to be regulated exclusively by HspR. Evidence is furthermore provided that HspR is able to form oligomers in vivo and that binding of HspR to its target sites is required for HrcA-mediated regulation.

MATERIALS AND METHODS

Bacterial strains and media. *E. coli* strain DH5α was used for cloning and plasmid preparations, and strain JH607 was used for protein oligomerization assays. *H. pylori* G27 cells were recovered from frozen stocks on Columbia agar plates containing 5% horse blood, 0.2% cyclodextrin, and Dent's or Skirrow's antibiotic supplement under microaerophilic conditions (Oxoid) for 2 to 3 days. After passage on fresh plates, bacteria were cultured in a 5% CO_2 and 95% air atmosphere. Liquid cultures of *H. pylori* were grown in modified brucella broth containing Dent's or Skirrow's antibiotic supplement and 5% fetal calf serum. When required, kanamycin or chloramphenicol was added to final concentrations of 20 and 25 μg/ml, respectively. Heat shock was achieved by incubation of *H. pylori* cultures in a water bath at 42°C. *E. coli* strains were cultured in Luria-Bertani medium. Natural transformation of *H. pylori* G27 was carried out by adding 1 to 5 μg of plasmid DNA to a spot of fresh bacteria incubated for 5 h at 37°C. After overnight incubation at 37°C, bacteria were collected and streaked on selective agar plates. Single colonies were then selected for further analysis.

DNA techniques. DNA manipulations were carried out by general techniques as described by Sambrook et al. (29). Midi scale plasmid preparations were carried out with the Qiagen Midi column plasmid purification kit (Qiagen). DNA fragments or PCR amplification products for cloning purposes were purified from agarose gels with the QiaEX DNA purification kit (Qiagen). PCRs were performed in a Perkin-Elmer 2400 thermal cycler with Platinum-*Taq* DNA polymerase (Invitrogen). In each reaction, 100 ng of *H. pylori* G27 chromosomal DNA was mixed with 100 pmol of each specific primer in a 100-μl sample containing standard concentrations of deoxynucleotides and $MgCl_2$ (Roche). Reactions were performed by denaturing DNA at 94°C for 5 min, annealing at appropriate temperatures for 1 min, and extension at 72°C for 1 min. A total of 30 cycles was performed.

Construction of mutant strains. An isogenic *hrcA* mutant was obtained by transforming *H. pylori* strain G27 with plasmid pGEM3(*hrcA*::*kan*) (Table 1). Correct replacement of the wild-type sequence with the antibiotic resistance cassette was verified by PCR with primer pair hrc1-hrc4 (Table 2). For the construction of an *hrcA hspR* double mutant, the *hrcA* mutant strain was transformed with plasmid pGEM3(*hspR*::*cat*) (Table 1). Correct replacement of the wild-type *hspR* sequence with the antibiotic resistance cassette was verified by PCR with the oligonucleotide pair hsp1-hsp4 (35). Complementation of the *hrcA* mutant strain was achieved by transformation of G27 (*hrcA*::*kan*) with pVAC-*cat-hrcA-myc* (Table 1). Orientation of the *CAT-hrcA-myc* insert in the *vacA* locus (+/−) was verified by Southern blot analysis. Strain G27(P_{gro}::*cat*) lacking the HspR binding site in the P_{gro} promoter was constructed by transformation of *H. pylori* strain G27 with plasmid pGEM3(P_{gro}::*cat*) (Table 1). Replacement of the HspR-binding site with the chloramphenicol resistance gene was controlled by PCR with the oligonucleotide pair hsg1-hsg6.

RNA isolation and transcriptional analysis. Total *H. pylori* RNA was isolated as described previously (36). Transcription from the P_{gro} and P_{hrc} promoters was assayed by primer extension analysis with oligonucleotides groS and hrcA as detailed previously (35, 36). The P_{112} promoter was mapped by reverse transcription of RNA with oligonucleotide P112B (Table 2). To allow localization of the P_{112} primer extension product, the plasmid pSL1190-112 (Table 1) was sequenced in parallel with oligonucleotide P112B by using a T7 sequencing kit (Pharmacia). Transcription from the P_{cbp} promoter was assayed by S1 nuclease mapping analysis as described in Spohn and Scarlato (35). For mapping of the P_{1023} promoter, a 434-bp AvaII-EcoRI fragment obtained from the plasmid pGEM-1023 (Table 1) was 5′ end labeled at its AvaII site, hybridized to total *H. pylori* RNA at 45.5°C, and digested with S1 nuclease as described previously (35). For mapping of the P_{dnaG} promoter a 858-bp EcoRI-BamHI fragment obtained from plasmid pGEM-dnaG (Table 1) was 5′ end labeled at its EcoRI site, hybridized to *H. pylori* RNA at 43°C, and digested with S1 nuclease. For correct mapping of promoters, all labeled probes were sequenced in parallel to

the S1 nuclease reactions according to the method described by Maxam and Gilbert (24).

Immunoprecipitation of HrcA-DNA complexes (HrcA-IP). A total of 10 ml of *H. pylori* cultures [G27 and G27(*hrcA-myc*)] were grown to exponential phase (optical density at 600 nm [OD_{600}] 0.8 to 1.0) and cross-linked in 1% formaldehyde for 15 min at room temperature. To stop cross-linking, samples were equilibrated with 125 mM glycine and incubated further 10 min at room temperature. Bacteria were pelleted, washed twice in 10 ml of phosphate-buffered saline and once in 10 ml of 10 mM Tris-Cl (pH 8.0)–10 mM EDTA–150 mM NaCl–0.25% Triton X-100, and resuspended in 2 ml of TE (10 mM Tris [pH 8], 1 mM EDTA). The bacterial suspension was sonicated in a Branson Sonifier 250 (three 20-s pulses at 40 W, 80% duty cycle, empirically determined to generate 0.3- to 1.5-kb genomic DNA fragments) and centrifuged for 10 min at maximum speed in an Eppendorf centrifuge at 4°C to separate the soluble cell extract from the insoluble material. Prior to immunoprecipitation an aliquot of cell extract was reverted for 6 h at 65°C to check the correct size of fragmented DNA. Cell extracts (0.9 ml) were adjusted to 1× IP Buffer (150 mM NaCl, 50 mM Tris-Cl [pH 8.0], 1 mM EDTA, 0.25% sodium deoxycholate, 1% IGEPAL [Pharmacia]), precleared with 50 μl of ProtA-Sepharose (50% slurry equilibrated in IP Buffer) for 1 h at 4°C, and then incubated for 16 h at 4°C with a rabbit anti-human-c-Myc polyclonal peptide antibody (Sigma C3956 [1:33 dilution]). HrcA-DNA complexes were immunoprecipitated with 50 μl of ProtA-Sepharose slurry (equilibrated in 1× IP Buffer) for 4 h at 4°C in sterile minicolumns (Bio-Rad). Columns were drained by gravity flow, and the flowthrough was collected for further analysis. Bound protein-DNA complexes were washed five times in 1 ml of 1× IP Buffer, once in 1 ml of 250 mM LiCl–10 mM TrisCl (pH 8.0)–1 mM EDTA–0.5% sodium deoxycholate–0.5% IGEPAL, and twice in 1 ml of TE, all for 10 min at 4°C. Complexes were finally resuspended in 200 μl of TE–50 μg of RNase A/ml and incubated 30 min at 37°C. Then, 0.5 mg of proteinase K/ml and 0.5% sodium dodecyl sulfate were added, and the mixture was incubated overnight at 37°C. Cross-linking was reverted for 6 h at 65°C. DNA was extracted with phenol-chloroform, and the organic phase was back-extracted with TE; 2 μl of glycogen was added prior to precipitation in ethanol. Immunoprecipitated DNA fragments were finally resuspended in 50 to 100 μl of sterile water and denatured, and aliquots were blotted onto Hybond N+ membranes (Pharmacia) with a dot blot apparatus (Bio-Rad). Probes corresponding to the P_{hrc}, P_{gro}, and P_{cbp} promoters were obtained after EcoRI-BamHI restriction and gel excision of the plasmids pGEM3-FP4hrc, pGEM-FP1gro and pGEM3-cbp, respectively (Table 1) (35). Probes were labeled with an ECL nucleic acid labeling kit (Pharmacia) and hybridized to the HrcA-IP blots according to the manufacturer's instructions. Immunoprecipitations performed in parallel with the wild-type G27 strain, lacking the c-Myc fusion tag, served as a negative control.

Oligomerization assay. To test the in vivo oligomerization capacity of HrcA and HspR, plasmids expressing different fusion proteins consisting of the N-terminal DNA-binding part of cI and various portions of HspR and HrcA were constructed (Table 1) and transformed into *E. coli* strain JH607. Overnight cultures grown in LB medium supplemented with 100 μg of ampicillin/ml were diluted 1:100 in LB medium containing 100 μg of ampicillin/ml, 70 μg of chloramphenicol/ml, and 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) and then grown at 37°C. The optical densities of the cultures were measured every hour.

RESULTS

HrcA and HspR act as corepressors of the *hrcA-grpE-dnaK* and *groESL* operons. The organization of the HspR-dependent chaperone encoding operons of *H. pylori* is shown in Fig. 1A. In order to investigate the role of HrcA in the transcriptional regulation of these operons, we constructed an isogenic *hrcA* mutant, as well as an *hspR hrcA* double mutant. The *hrcA* mutant was constructed by transforming *H. pylori* strain G27 with a suicide vector in which most of the coding sequence of the *hrcA* gene was replaced with a kanamycin resistance gene. From this strain, the *hspR hrcA* double mutant was then derived by replacing the entire *hspR* coding sequence with a chloramphenicol resistance gene (Fig. 1A). Correct replacement of the wild-type sequences with the respective antibiotic resistance cassette was verified by PCR with oligonucleotides complementary to regions flanking the insertion sites. Total RNA from the newly constructed mutants, as well as from the isogenic wild-type strain and the previously constructed *hspR* mutant (35), was isolated, and primer extension and S1 nuclease mapping were used to investigate transcription from the P_{gro}, P_{cbp}, and P_{hrc} chaperone promoters. As shown in the representative experiment of Fig. 2 (lanes 2, 6, and 10), the amount of each specific transcript is increased severalfold in the *hspR* background with respect to the isogenic wild-type strain (lanes 1, 5, and 9), thus confirming the role of HspR as a repressor of the chaperone gene promoters as previously reported (35). Furthermore, Fig. 2 shows that deletion of the

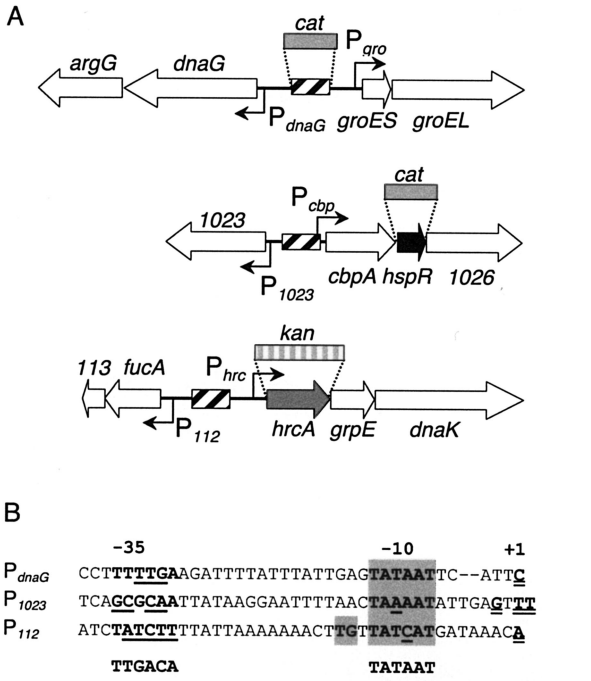

FIG. 1. (A) Structural organization of *H. pylori* chaperone genes and divergent genes and operons. Hatched boxes represent positions of HspR-binding sites. Open arrows indicate genes; solid and gray arrows indicate regulatory genes. All genes are marked according to their name or by the numbers of the genome sequence published by Tomb et al. (38); *groES* and *groEL* form a transcriptional unit (16) and code for the HspA (Hsp10) and HspB (Hsp60) proteins. *hrcA*, *grpE*, and *dnaK* are transcribed as part of a tricistronic transcript (16) and code for a homologue of the HrcA repressor of *B. subtilis* and the GrpE and DnaK (Hsp70) chaperones, respectively. *cbpA* codes for a homologue of cochaperone curved DNA-binding protein CbpA of *E. coli* (39), *hspR* codes for the negative regulator of the P_{gro}, P_{cbp}, and P_{hrc} promoters (35), and *hp1026* codes for a protein with homology to a putative helicase-like protein from *H. influenzae*. *dnaG* encodes DNA primase, *argG* encodes argininosuccinate synthase, *hp1023* encodes a putative outer membrane protein, *hp112* encodes a protein with 30% (50 of 164) amino acid identity to a putative fuculose-1-phosphate aldolase of *Aquifex aeolicus*, and *hp113* encodes a protein of unknown function. (B) Nucleotide sequences and transcription start sites of divergent promoters. Alignment of the promoter sequences with respect to their transcriptional start site (+1) is shown. Putative −10 hexamers are boxed. The $E\sigma^{70}$-recognized sequence is indicated (3); mismatches with respect to this sequence are underlined. The P_{112} promoter contains a TG motif at positions −14 and −15 typical of so-called extended −10 promoters (20). The P_{112} start site of transcription is located in the putative coding region of *hp112* as annotated by Tomb et al. (38). Possible alternative ATG or GTG start codons can be found at nucleotide positions +3, +69, and +138 with respect to the P_{112} start site of transcription; no obvious corresponding ribosome-binding sites can, however, be detected.

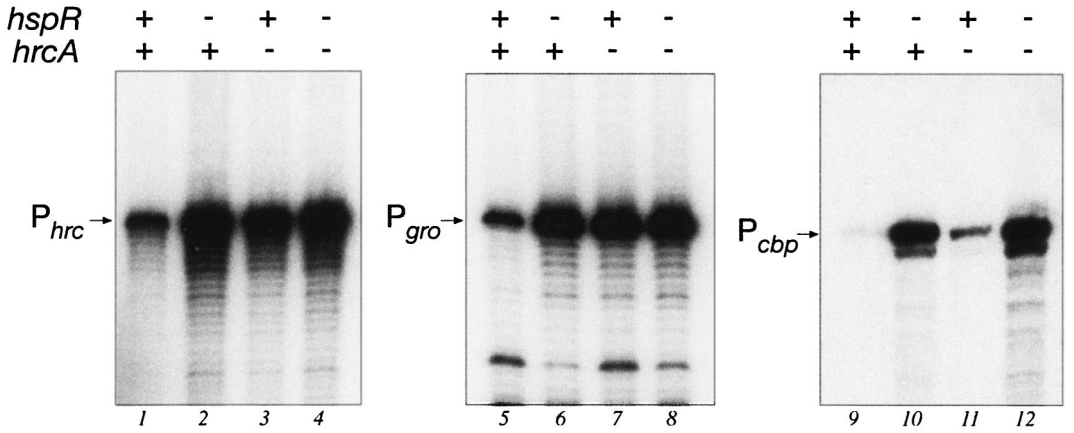

FIG. 2. Primer extension and S1 nuclease mapping analyses of heat shock promoters. Elongated primers or protected fragments are indicated by arrows and labeled "P." Lowercase letters refer to the first of the downstream genes. Total RNA was isolated from wild-type *H. pylori* G27, the *hspR* mutant, the *hrcA* mutant, or the *hspR hrcA* double mutant and annealed to specific oligonucleotides, which were elongated with reverse transcriptase or hybridized to specific DNA fragments and then digested with S1 nuclease. Each panel shows a representative experiment. Slight variations in band intensities (lanes 2 to 4 and lanes 9 and 11) were observed between different experiments.

hrcA gene leads to a strong increase in the transcripts associated with the P_{gro} and P_{hrc} promoters (lanes 3 and 7), while transcription of the P_{cbp} promoter (lane 11) remains essentially unaffected. In the double mutant, *hspR hrcA* (lanes 4, 8, and 12), all three specific transcripts are increased strongly with respect to the wild-type strain.

We conclude that the P_{gro} and P_{hrc} promoters responsible for expression of the major components of the *H. pylori* chaperone machineries are regulated by both the HspR and the HrcA repressors, whereas the P_{cbp} promoter regulating expression of the cochaperone CbpA and the HspR repressor itself is regulated only by HspR.

The structure of the chaperone encoding operons shows open reading frames that point in opposite directions (Fig. 1A). To investigate whether either HspR or HrcA influences expression of these divergent genes, we mapped the P_{dnaG}, P_{1023}, and P_{112} promoters (Fig. 1B) and assayed the effect of the different repressor mutations on their transcription, which revealed no significant differences (data not shown). This suggests that transcription from these promoters is not influenced by either HspR or HrcA.

In vivo binding of HrcA to P_{hrc} and P_{gro} but not to P_{cbp} promoters. Nucleotide sequence analysis of the P_{hrc} and P_{gro} promoters revealed the presence of inverted repeats with 61% (11 of 18) and 56% (10 of 18) identity, respectively, to the so-called CIRCE motif, known as the HrcA consensus binding site of *B. subtilis* (30, 44), suggesting that the HrcA protein of *H. pylori* may repress transcription by direct binding to these sequences centered to positions −42 (P_{hrc}) and +9 (P_{gro}) with respect to the transcriptional start sites (Fig. 3). To explore this hypothesis, we attempted first to overexpress and purify HrcA in *E. coli* to perform in vitro DNA-binding assays. However, we were unable to obtain significant amounts of functional protein, mainly due to its toxicity when expressed in *E. coli* (data not shown). To circumvent this limitation, we implemented an immunoprecipitation protocol for HrcA-DNA complexes in *H. pylori* (HrcA-IP), with anti-c-Myc antibodies to identify genomic targets bound by a HrcA-Myc fusion protein. This method is known as chromatin immunoprecipitation and has been successfully used in both eukaryotes and prokaryotes (22, 33).

To create a pseudo-wild-type *hrcA-myc* strain, a copy of the *hrcA* gene harboring the wild-type P_{hrc} promoter was fused at

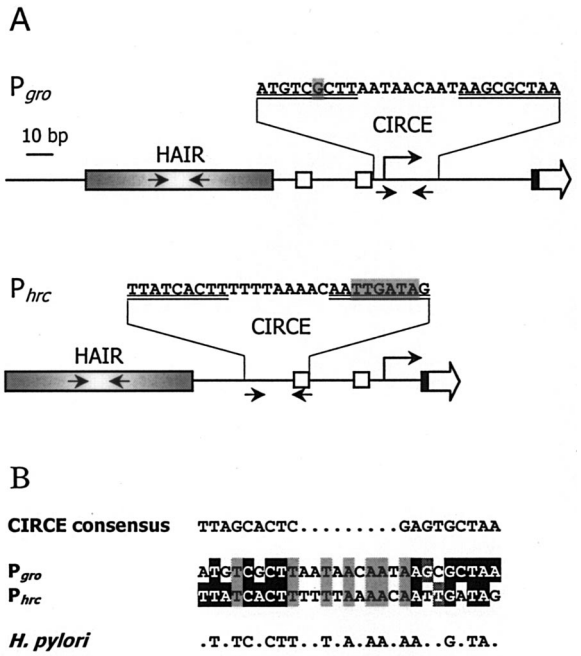

FIG. 3. Regulatory elements within the P_{gro} and P_{hrc} promoters. (A) Schematic representation of the key regulatory elements of the P_{gro} and P_{hrc} promoters (35). Bent arrows indicate direction of transcription; open boxes indicate the −10 and −35 elements of the promoter; the HspR-binding sites are indicated by gray boxes; the respective HAIR consensus sequences are marked by converging arrows. Inverted repeats and their sequences indicate possible CIRCE elements. (B) Alignment of putative HrcA-binding sequences on the P_{gro} and P_{hrc} promoters with the consensus CIRCE sequence. Nucleotides that are conserved with respect to the CIRCE sequence are shaded in black; those that are conserved between the two putative HrcA-binding sites are shaded in gray. A tentative consensus sequence for *H. pylori* HrcA binding is deduced.

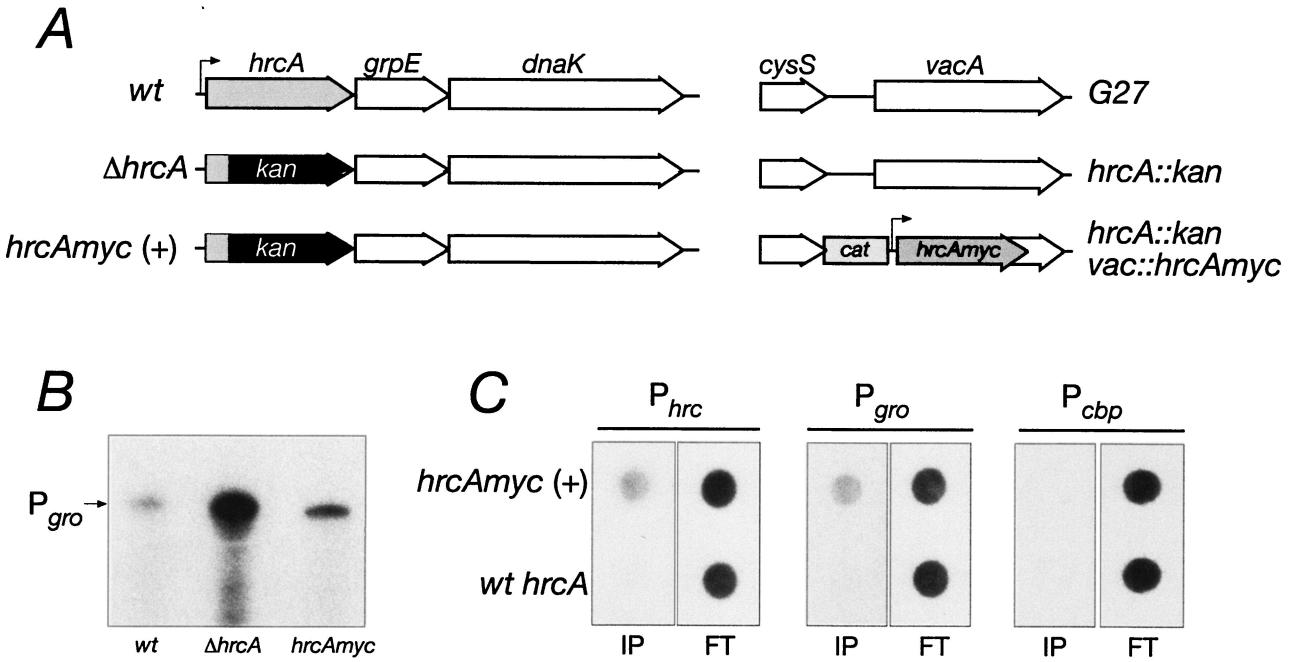

FIG. 4. Immunoprecipitation of HrcA genomic targets (HrcA-IP). (A) Construction of a complementing *hrcA-myc* strain from the isogenic G27 (*hrcA*::*kan*) knockout mutant. Arrows indicate orientation of the open reading frames. Kanamycin (*kan*) and chloramphenicol (*cat*) resistance cassettes are depicted, respectively, by black and gray boxes. (B) Primer extension analysis of the P_{gro} promoter with primer gro1 (Table 2) with equal amounts of RNA extracted from G27 wild-type (wt), *hrcA* mutant (Δ*hrcA*), and *hrcA-myc* complementing ($hrcA_{myc}$) strains, respectively. Restoration of P_{gro} repression indicates functional expression of the HrcA-Myc fusion protein. (C) Dot blot analysis of genomic fragments encompassing heat shock promoters (P_{hrc}, P_{gro}, and P_{cbp}) enriched after HrcA-IP. The G27 wild-type strain (wt *hrcA*), lacking the *hrcA-myc* fusion construct, was used as negative control. HrcA targets were enriched by immunoprecipitation with a c-Myc antibody from complementing strains harboring the *hrcA-myc* construct in the forward (+) orientation. Comparable amounts of unbound DNA (FT, flowthrough control) indicate that P_{hrc} and P_{gro} enrichment is not due to reduced amounts of target DNA in the wild-type *hrcA* control cell extract.

the 3′ end to a human c-*myc* tag (coding for the peptide EQKLISEEDL) and inserted with a *cat* cassette by homologous recombination in the *vacA* locus (*HP0887*) of the *hrcA* strain, thereby complementing the *hrcA* mutation (Fig. 4A; *hrcA*::*kan; vac*::*cat-hrcA*$_{myc}$). Clones with double antibiotic resistance were selected and tested for correct insertion of *hrcA-myc* by Southern blotting. Whole protein extracts were subjected to immunoblot analysis with antibody against c-Myc, which confirmed expression of the fusion protein (data not shown). In order to assess functional complementation by the *hrcA-myc* fusion gene, we carried out primer extension analysis on the *groESL* transcript (Fig. 4B). As expected, in a *hrcA* background the P_{gro} promoter is strongly derepressed, whereas its repression is restored in the *hrcA-myc* strain, indicating complementation of *hrcA* by the HrcA-Myc fusion protein. Thus, we proceeded to immunoprecipitate genomic DNA fragments bound by HrcA-Myc in vivo. Bacterial cultures were grown to mid-late exponential phase, cross-linked with formaldehyde, and sonicated. HrcA-DNA complexes were immunoprecipitated with a commercially available anti-c-Myc antiserum, and the DNA fragments, extracted and precipitated after reversal of the cross-linking by heat treatment, were blotted onto nylon membranes (Fig. 4C, IP). Aliquots of DNA extracted from the immunoprecipitation experiment and unbound by HrcA (flowthrough) were blotted in parallel (Fig. 4C, FT). Immunoprecipitations performed in parallel in the G27 wild-type strain, lacking the *hrcA-myc* gene, were used as negative control. HrcA-IP blots were subsequently hybridized with 468-, 665-, and 272-bp EcoRI-BamHI probes corresponding to the P_{hrc}, P_{gro}, and P_{cbp} promoters (Materials and Methods), respectively, with the results shown in Fig. 4C.

Genomic DNA fragments corresponding to P_{hrc} and P_{gro} are significantly more abundant after immunoprecipitation in the *hrcA-myc* strain compared to the wild-type control. Comparable amounts of unbound DNA (FT) indicate that the observed enrichment is not due to reduced quantities of target sequences in the wild-type control. It can therefore be concluded that HrcA binds in vivo genomic targets encompassing P_{hrc} and P_{gro}. In contrast, DNA fragments corresponding to the P_{cpb} promoter are not enriched in *hrcA-myc* strains. Together, these results strongly support HrcA-mediated transcriptional regulation of P_{hrc} and P_{gro} (but not P_{cbp}), exerted in vivo by direct binding of the repressor to these promoters.

HrcA regulation depends on HspR binding. In order to investigate whether HrcA regulation occurs independently of HspR or if it depends on binding of HspR to its target sites, we decided to specifically delete the HspR binding site of the HrcA/HspR-coregulated promoter P_{gro} and to assay the effect of this deletion on the heat shock response at this promoter. *H. pylori* strain G27 was therefore transformed with a suicide vector in which nucleotides between positions −40 and −113 of the P_{gro} promoter were replaced by a chloramphenicol resistance cassette (Fig. 1A). Correct replacement of the wild-type sequences with the antibiotic resistance gene was verified by PCR with oligonucleotides complementary to regions flanking the insertion sites. The resulting strain G27(P_{gro}::*cat*) was

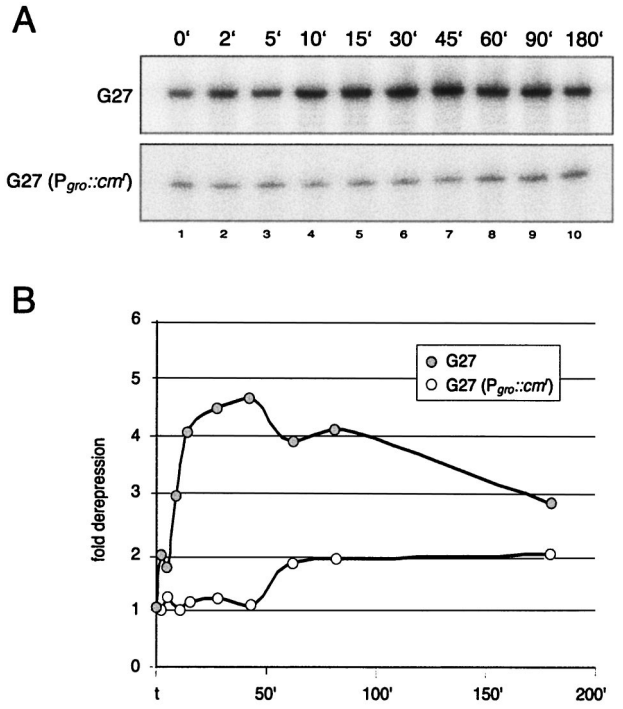

FIG. 5. Heat shock response of the P_{gro} promoter in *H. pylori* G27 and in G27(P_{gro}::*cat*). (A) Primer extension analyses of *H. pylori* RNA extracted from cells grown at 37°C (0 min, line 1) or upon temperature shift to 42°C (lines 2 to 10). A time interval at which RNA was extracted is indicated in minutes above each line. (B) Pattern of RNA accumulation at the P_{gro} promoter obtained by PhosphorImager quantifications of the radioactive bands shown in panel A.

devoid of the HspR-binding site between positions −46 and −118 but maintained the putative HrcA-binding site centered at position +9. Both G27(P_{gro}::*cat*) and the parental strain G27 were grown at 37°C, and total RNA was isolated before and at different time intervals after temperature upshift of the cultures to 42°C. P_{gro}-specific mRNA was detected as before by primer extension and quantified by exposure of acrylamide gels to a PhosphorImager. The results of this analysis are represented in Fig. 5. Figure 5A shows that the parental strain exhibits a typical heat shock response at the P_{gro} promoter with transcript amounts increasing rapidly after temperature upshift, starting already at about 2 min and reaching a maximum level at 15 min. The transcript quantification in Fig. 5B shows that the maximal induced level is ~5-fold higher than in nonstressed cells, thus confirming previous results obtained in our laboratory (36). In contrast, in G27(P_{gro}::*cat*) cells virtually no increase in transcription can be observed at the P_{gro} promoter after temperature upshift, indicating that transcriptional control is lost in this mutant. Unexpectedly, transcript amounts detected in G27(P_{gro}::*cat*) appear lower than those observed in heat-shocked wild-type cells or *hspR* and *hrcA* mutant cells. Although this phenomenon was not investigated, we speculate that replacement of the HAIR site with the chloramphenicol cassette could have altered the promoter context and its activity.

We conclude that the HspR-binding site of the P_{gro} promoter is necessary for the regulated expression of *groESL* and full promoter activity and that expression of *hrcA* is not sufficient to mediate the transcriptional response to a sudden temperature upshift.

HspR oligomerizes in vivo. The activity of heat shock transcription factors is often regulated by their oligomeric state. This has been extensively studied for the eukaryotic heat shock transcription factor, for which thermal upshift induces formation of the transcription-competent trimer form (26); this has also been hypothesized for the HspR protein of *H. pylori* (10). In order to investigate whether HspR is able to oligomerize in vivo, we fused the *hspR* gene to the N-terminal DNA-binding part of the lambda *c*I repressor and tested the ability of the resulting fusion protein to confer a chloramphenicol-sensitive phenotype to *E. coli* strain JH607. As outlined in Fig. 6A, chloramphenicol sensitivity in this strain is achieved only when the P_s promoter controlling transcription of the *cat* gene is repressed by binding of an oligomeric fusion protein to the O_S operator overlapping the RNA polymerase binding site. Figure 6B shows that strain JH607 transformed with plasmid pBFH1, which expresses a fusion protein consisting of the N-terminal part of *c*I and the entire HspR protein, showed the same strong chloramphenicol sensitivity as the control strain harboring plasmid pBF21 expressing the entire *c*I protein, including the original C-terminal oligomerization domain. Similar chloramphenicol sensitivity was measured when cells harboring plasmids pBFH1 and pBF21 were grown at 42°C. Conversely, transformation of JH607 with plasmid pBF-hrc coding for a *c*I-HrcA fusion protein resulted in chloramphenicol resistance comparable to the level obtained by transformation with plasmid pBF22, suggesting that either HrcA is not able to form oligomers in vivo or expression of the plasmid pBF-hrc does not result in a stable fusion protein.

We therefore conclude that under the experimental conditions used here, HspR is able to form oligomers in vivo in cells adapted to grow at 37 and 42°C. Unfortunately, transient changes in oligomerization, as are typical of the heat shock response (28, 34, 36), cannot be measured with this assay.

The HspR oligomerization domain resides within the region from aa 66 to 97. In order to map the domain of HspR that accounts for the oligomerization capacity of the protein, we constructed a series of *c*I-HspR fusions carrying different N- or C-terminal deletions of the HspR coding sequence. As shown in Fig. 6B, plasmid pBFH3 expressing *c*I fused to amino acids (aa) 1 to 97 of HspR and plasmids pBFH2 and pBFH8 expressing *c*I fused to aa 44 to 123 and aa 66 to 123 of HspR, respectively, were able to confer chloramphenicol sensitivity to strain JH607. On the other hand, bacteria harboring plasmids pBFH7, pBFH5, or pBFH6 expressing fusion proteins containing aa 1 to 65, aa 1 to 43, or aa 98 to 123 of HspR, respectively, showed no chloramphenicol sensitivity. We thus conclude that the oligomerization domain of HspR is confined to the region between aa 65 and 97. Amino acid sequence analysis of this region shows the presence of an array of heptad repeats containing hydrophobic residues at positions a and d (Fig. 6C).

DISCUSSION

Bacteria have evolved different strategies to regulate the transcriptional response to a sudden exposure to environmental stresses. *E. coli* and most other gram-negative bacteria use

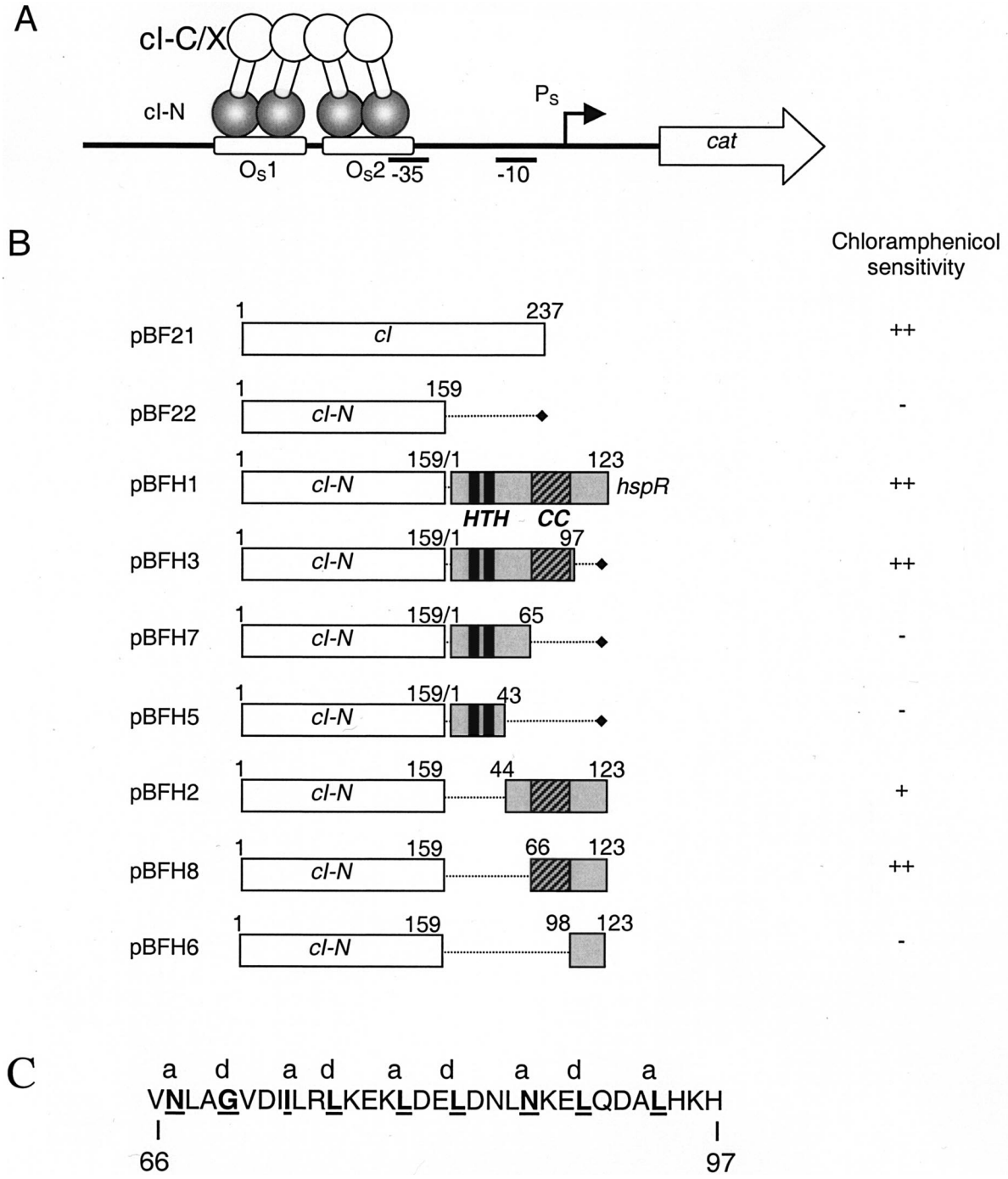

FIG. 6. In vivo oligomerization of HspR. (A) Principle of the assay. Fusion proteins between the N-terminal DNA-binding domain of lambda repressor *c*I and the protein of interest are expressed in *E. coli* strain JH607 and assayed for their ability to repress the P_S promoter, which in this strain controls expression of the *cat* gene coding for chloramphenicol acetyltransferase. Repression is exerted via binding to a particular regulatory site, which consists of one strong operator (O_S 1) in a promoter distal position and one weak operator (O_S 2) overlapping the −35 sequence of the P_S promoter. Whereas O_S 1 is able to bind any dimeric *c*I fusion protein with normal affinity, O_S 2 can bind *c*I fusion proteins only when their local concentration is increased as a consequence of a protein-protein interaction occurring with fusion protein bound to O_S 1. In this case the P_S promoter and therefore the *cat* gene becomes tightly repressed, leading to chloramphenicol sensitivity of bacterial cultures. The intracellular oligomerization capability of proteins can thus be measured as growth retardation of bacterial cultures in chloramphenicol-containing media. (B) Fusion proteins assayed for their oligomerization capability. The numbers indicate amino acid positions where the respective proteins are fused to each other. The helix-turn-helix motif of HspR putatively involved in DNA binding is indicated by black bars and labeled "HTH." A region of HspR containing hydrophobic heptad repeats putatively involved in the formation of intermolecular coiled coils is indicated by dashed areas and labeled "CC." A "++" indicates that strain JH607 transformed with the indicated plasmid shows the same growth retardation in chloramphenicol-containing media as the same strain transformed with the control plasmid pBF21. A "+" indicates that transformation of JH607 with the indicated plasmid leads to at least 70% of the growth retardation observed after transformation with pBF21. (C) Oligomerization domain of HspR. Amino acid residues at positions a and d of the heptad repeats are in boldface and underlined.

specialized sigma factors, which become activated after stress exposure and direct the RNA polymerase to their target promoters, whereas a subgroup of gram-negative and all gram-positive bacteria use specialized repressors that become inactivated under stress conditions, leading to derepression of target promoters. We have previously demonstrated that the major chaperone-encoding operons of *H. pylori* are negatively regulated by HspR, the homologue of the repressor of the *dnaK* operon of *Streptomyces* spp. (35). In the present study we showed that two of the HspR-regulated operons, *groESL* and *hrcA-grpE-dnaK*, are also regulated by HrcA, the homologue of the repressor of the *groESL* operon of *B. subtilis*. Deletion of the *hrcA* gene from the chromosome of *H. pylori* leads to derepression of the P_{gro} and P_{hrc} promoters, and the level of derepression corresponds to the one observed in *hspR* or *hspR*/*hrcA* deletion mutants (Fig. 2). The presence of both regulators is therefore necessary for maintaining P_{gro} and P_{hrc} in the repressed state. HspR binds to large operators located far upstream from these promoters (35), whereas the HrcA operators are likely to be localized in close proximity to the respective transcription start sites. The selective immunoprecipitation of HrcA targets P_{hrc} and P_{gro}, encompassing the promoter regions, including the CIRCE-related inverted repeats centered around positions −42 and +9 with respect to the respective transcription start sites (Fig. 3), suggests that HrcA binds the putative CIRCE sequences, thereby repressing transcription by direct interference with RNA polymerase binding. In silico inspection of the amino acid sequence revealed a putative transmembrane domain spanning amino acids 140 to 156 (http://psort.nibb.ac.jp/form.html and http://psort.nibb.ac.jp/form.html). This suggests a membrane localization of HrcA, similarly to the membrane-bound activator ToxR of *Vibrio cholerae*, which regulates virulence gene expression by sensing environmental stimuli by its periplasmic domain and transmitting them directly into a transcriptional response mediated by its cytoplasmic DNA-binding domain (19, 32). Accordingly, it can be speculated that stress signals sensed by the putative periplasmic domain of HrcA might induce a conformational change, which causes the loss of DNA-binding activity in the cytoplasmic part, thereby leading to transcriptional derepression. HrcA-mediated regulation depends, however, clearly on the presence of HspR, as demonstrated by deletion of the *hspR* gene and of the HspR-binding site, which both lead to promoter deregulation (Fig. 2, lanes 2 and 6, and Fig. 5). It should therefore be assumed that HrcA is not able to bind its target sequences in the absence of a functional HspR repressor. The reason for this dependence might be found in protein-protein interactions between the two repressors occurring either in solution or upon DNA binding, which may be a prerequisite for the formation of a stable repression-competent complex. Alternatively or in addition, HspR-induced topological changes of the DNA surrounding the HspR binding site might be necessary for HrcA to bind to its target site, since affinity for CIRCE elements may be impaired by the low conservation with consensus. Evidence for such topological changes comes from footprinting data, which showed the induction of various DNase I-hypersensitive sites upon HspR binding, indicating the introduction of torsional stress into the DNA in the proximity of the heat shock promoters (35) and from the characterization of the HrcA-mediated repression in *Chlamydia trachomatis* (41). Members of the MerR family of transcriptional regulators, to which HspR belongs, are known to regulate transcription via bending and twisting mechanisms (1, 14), and it is therefore tempting to speculate that HspR operates with similar mechanisms.

Oligomerization of HspR likely plays an important role in heat shock regulation, as suggested by the large operators of the protein (35). The oligomerization domain revealed heptad repeats of hydrophobic residues, present also in the eukaryotic heat shock transcription factor HSF, where they have been found to mediate trimerization through the formation of coiled coils between monomers (28, 34). This phenomenon was shown to depend on temperature shift, and thus it will be of interest to explore whether HspR regulation is similarly controlled through a transient temperature-dependent oligomerization state of the protein, or if it uniquely relies on chaperone-mediated folding mechanisms.

Expression of HspR, which is controlled by the P_{cbp} promoter, is exclusively regulated by HspR itself and is not influenced by HrcA. Repression of this promoter is exerted via direct interference of HspR with RNA polymerase binding, and this repression is not affected in an *hrcA* knockout strain (Fig. 2, lane 11). On the other hand, both HspR and HrcA are required for the regulation of HrcA expression, which depends on the P_{hrc} promoter (Fig. 2, lanes 2 to 3). This suggests a cooperative and as-yet-unknown interplay between the two repressors.

Several species, including *Streptomyces* spp. and *B. subtilis*, contain more than one heat shock repressor. The target genes of these repressors are, however, distinct (27, 31). Interestingly, a dual heat shock regulation mode by CtsR, a stress response regulator, and HrcA was recently reported in *Staphylococcus aureus*, in which the two repressors synergistically control the transcription of *dnaK* and *groESL* operons (8). HspR and HrcA similarly coregulate heat shock operons in *H. pylori*. The existence of chaperone-mediated feedback regulation of HspR by DnaK (4, 5) in *Streptomyces coelicolor* and of HrcA by GroEL in *B. subtilis* (25) combines additional posttranscriptional regulatory capabilities to such systems. It will be interesting to dissect similar circuits in *H. pylori*.

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