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Requirements for Vibrio cholerae HapR Binding and Transcriptional Repression at the hapR Promoter Are Distinct from Those at the aphA Promoter

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Virulence gene expression in certain strains of Vibrio cholerae is regulated in response to cell density by a quorum-sensing cascade that influences the levels of the LuxR homolog HapR through small regulatory RNAs that control the stability of its message. At high cell density, HapR represses the expression of the gene encoding the virulence gene activator AphA by binding to a site between \(-85\) and \(-58\) in the aphA promoter. We show here that a second binding site for HapR lies within the hapR promoter from which it functions to repress its own transcription. This site, as determined by gel mobility shift assay and DNaseI footprinting, is located between \(+8\) and \(+36\) from the transcriptional start and is not strongly conserved with the site at the aphA promoter. At low cell density, when the expression of a transcriptional hapR-lacZ fusion was low, no autorepression was observed. However, at high cell density, when the expression of the hapR-lacZ fusion was approximately 15-fold higher, the presence of HapR reduced its expression. Introduction of a single base pair change within the binding site at \(+18\) prevented HapR binding in gel mobility shift assays. In the absence of HapR, this mutation did not significantly influence the expression of the hapR promoter, but in its presence, the expression of the promoter was increased at high cell density. These results indicate that HapR autorepresses from a single binding site in the hapR promoter and suggest a model for the temporal regulation of its expression as its intracellular levels increase.

Cholera is an often fatal epidemic diarrheal disease caused by oral ingestion of food or water contaminated with the bacterium Vibrio cholerae. The two primary virulence factors responsible for the disease are the toxin coregulated pilus (TCP), a critical colonization factor (24), and cholera toxin, which causes copious diarrhea that can quickly lead to severe dehydration and death. The expression of these genes from the Vibrio pathogenicity island (10) and the lysogenic cholera toxin phage (25), respectively, is dependent upon a transcriptional cascade involving multiple activator and repressor proteins (4) that is initiated at the tcpPH promoter by the regulators AphA and AphB (11, 23).

AphA is a member of a new transcriptional regulator family that shows homology to PadR, a repressor that controls the expression of genes involved in the detoxification of phenolic acids (1). Recent crystal structure determination of AphA revealed that it is a winged helix DNA binding protein with a unique antiparallel coiled coil domain that is involved in dimerization (5). AphA activates the transcription of the tcpPH promoter on the Vibrio pathogenicity island by an unusual mechanism that appears to require a direct interaction with the LysR-type regulator AphB, which binds at an adjacent and proximal site in the promoter (15). This interaction stabilizes the binding of AphB to its recognition site thereby facilitating transcriptional activation under the appropriate environmental conditions.

The expression of AphA in Vibrio cholerae is regulated by a quorum-sensing system that responds to cell density (19, 26). At low cell density, AphA levels are relatively high and virulence gene activation ensues. However, as the cell density increases, HapR, which is a LuxR homolog, binds to a specific site in the aphA promoter that represses its expression (13). This, in turn, reduces virulence gene expression. HapR was initially characterized as an activator of hemagglutinin (HA)/protease in V. cholerae (9) and has recently been shown to play a role in repressing biofilm formation (7, 27). Its expression in V. cholerae is dependent upon several quorum-sensing circuits that function in parallel (19, 26). System 1 is composed of the CqsS-dependent autoinducer CAI-1 and its sensor CqsR. System 2 is composed of the LuxS-dependent autoinducer AI-2 and its sensor LuxPQ. It appears that a third quorum-sensing system, not yet identified, also contributes to this process.

The quorum-sensing circuits function together to control the activity of the central response regulator LuxO (17, 19). According to the current model, at low cell density, when the concentrations of autoinducers are low, LuxO is phosphorylated by a relay from the sensor proteins. This activated form of LuxO, in association with \(\sigma^5\), activates the expression of several small regulatory RNAs (sRNAs) that in conjunction with the RNA binding protein Hfq destabilize the hapR message (16), thus permitting high-level expression of the virulence cascade. At high cell density, binding of autoinducers to their cognate sensors leads to dephosphorylation of LuxO. LuxO no longer activates the expression of the sRNAs. This increases hapR message stability and HapR, in turn, functions to downregulate expression of the virulence cascade by repressing expression from the aphA promoter.

The related marine organism Vibrio harveyi has a quorum-sensing circuit similar to that of V. cholerae in which LuxO...
controls the expression of bioluminescence in response to cell density (8, 16, 19). At high cell density, the \textit{V. harveyi} LuxR protein functions to activate the \textit{lux} promoter (20), and it also autorepresses its expression by binding to several sites in its own promoter (2). The parallels between HapR in \textit{V. cholerae} and LuxR in \textit{V. harveyi} prompted us to determine whether HapR also binds to its own promoter and similarly functions in autorepression. By constructing \textit{hapR-lacZ} transcriptional fusion strains we show here that HapR is capable of reducing its own expression at high cell density. Gel mobility shift assays and DNasel footprinting with purified HapR indicate that the protein recognizes a binding site downstream of its own promoter that extends from +8 to +36 relative to the start of transcription, consistent with a role in autorepression. To confirm that HapR binding to this site results in autorepression, a single A-to-G mutation was introduced into the site at +18. This mutation prevented HapR from binding in vitro in gel mobility shift assays and eliminated autorepression in vivo. Since the recognition sequence of the site at the \textit{hapR} promoter is only weakly conserved with that at the \textit{aphA} promoter and appears to have a lower binding affinity, this suggests these promoters are temporally regulated by HapR as its intracellular levels increase.

\section*{Materials and Methods}

\textbf{Bacterial strains and expression plasmids.} The \textit{V. cholerae} strains and expression plasmids used in this study are listed in Table 1. Strains were maintained at \textit{V. harveyi} density (8, 16, 19). At high cell density, the \textit{hapR} coding sequence expressed from a heterologous promoter in pMMB66EH.

\textbf{Construction of \textit{hapR} promoter mutations.} The base pair changes in the \textit{hapR} promoter were constructed by overlapping PCR using primers which contain the site for the type IIS restriction enzyme Earl. For each change, two 500-bp products were amplified from C6706 and then ligated into pKAS154 (15). The mutant products were amplified using primers HA24 (+18) (5'-GATCCGCTCT TCAGTTCTGTATGCTACTAAAGCC), HA23 (+21) (5'-GATCCGCT TTGATTTGCTATGCTACTAAAGCC), and HA25 (+18 and +21) (5'-GATCCGCTCTTGCTATGCTACTAAAGCCATG) together with primer HA16 (5'-GATCCGATTTGGTTAGATCTCCGT TACGG). The wild-type product was generated using primers HA22 (5'-GATCCGCTCTTGCTATGCTACTAAAGCC), and HA17 (5'-GATCCGCT TAGAGCTTTGGTTAGATCTCCGT TACGG). The RNA was subjected to 5 rapid amplification of cDNA ends (Invitrogen) as described previously (14), except that first-strand cDNA synthesis was carried out using the \textit{hapR}-specific primer HA11 (5'-CTTCTTGTTGAC C TAAACG) and the first and second nested primers were HA12 (5'-GGTGG GTTGTCATTTGTTAGATCTCCGT TACGG) and HA13 (5'-GATCCGCTCTTGCTATGCTACTAAAGCC). The resulting fragment was digested out of pKAS275 and ligated into pKAS180 (14). The resulting fusion in pKAS282 was then introduced in place of the \textit{lacZ} gene in KSK262, generating KSK2327.

\textbf{Identification of the \textit{hapR} transcriptional start site.} Total RNA was isolated from C6706 after growth for 7.5 h in LB medium at 37°C with TRIZOL reagent (Invitrogen). The RNA was subjected to 5 rapid amplification of cDNA ends (Invitrogen) as described previously (14), except that first-strand cDNA synthesis was carried out using the \textit{hapR}-specific primer HA11 (5'-CTTCTTGTTGAC C TAAACG) and the first and second nested primers were HA12 (5'-GGTGG GTTGTCATTTGTTAGATCTCCGT TACGG). The resulting fragment was digested out of pKAS275 and ligated into pKAS180 (14). The resulting fusion in pKAS282 was then introduced in place of the \textit{lacZ} gene in KSK262, generating KSK2327.
Binding reactions for HapR were carried out as previously described (13). The fragments were gel purified, treated with shrimp alkaline phosphatase, and kinased with upper-strand labeling, the inserts were excised with EcoRI and XbaI. For lower-strand labeling, the inserts were excised with BamHI (for the upper strand) and EcoRI (for the lower strand). Proteins were bound to the singly end-labeled DNA fragment as previously described (13). Binding reactions for HapR were carried out as previously described (13).

DINase footprinting. A 350-bp fragment was PCR amplified from C6706 with HAE3 (−198) (5′-GATCGGAAATGGATGATGCGTCCATAG) and HAE6 (158) (5′-GATCGGAATTCGTTGCACATTTTTCACCCAAC) and ligated into pBluescript (Stratagene), generating pWEL81. For upper-strand labeling, the inserts were excised with EcoRI and XbaI. For lower-strand labeling, the inserts were excised with BamHI and HindIII. The fragments were gel purified, treated with shrimp alkaline phosphatase, and kinased with [γ-32P]ATP (NEN; 3,000 Ci/mmol). Singly end-labeled fragments were obtained by digestion with BamHI (for the upper strand) and EcoRI (for the lower strand). Proteins were bound to the singly end-labeled DNA fragment as previously described (13).

RESULTS

HapR influences the transcription of the hapR promoter in V. cholerae. To determine whether the V. cholerae HapR protein functions similarly to LuxR as an autorepressor, a ΔhapR-lacZ transcriptional fusion strain, GK972, was constructed. In this strain, the hapR coding sequence was replaced with an E. coli lacZ gene containing its own ribosome binding site and ATG codon, thus rendering its expression independent of translational regulation through LuxO. A plasmid overexpressing HapR from a heterologous promoter, pKAS189, was then introduced into GK972. Although at low cell density (OD600 = 0.2) no significant difference in expression was observed compared to a vector control (Fig. 1), at high cell density (OD600 = 4.0) the expression of the fusion increased approximately 15-fold over that at low cell density, and under this condition the presence of HapR from pKAS189 resulted in a fourfold reduction in β-galactosidase activity (Fig. 1). To determine if a similar effect also occurs when hapR is in single copy, a hapR+ merodiploid strain was constructed by introducing the hapR-lacZ reporter into the lacZ locus by homologous recombination with regions of the flanking chrA and galR genes (14). The resulting strain, KSK2226, harbors the same hapR promoter-lacZ fusion as in GK972, but it is situated at the lacZ locus, leaving the hapR gene intact at its normal chromosomal location. KSK2226 was then compared with an isogenic strain containing a ΔhapR mutation. Again, at low cell density no significant difference between the strains was observed (Fig. 1), but at high cell density a twofold increase in expression was observed in the ΔhapR strain, consistent with a role for HapR in its negative autoregulation (Fig. 1).
Identification of the *hapR* transcriptional start site. The transcriptional start site for the *hapR* promoter was determined using 5′ rapid amplification of cDNA ends (6) with RNA isolated from wild-type C6706 grown in LB medium at 37°C to high cell density. A product corresponding to the size expected for the transcriptional start was identified, and sequencing of it revealed that the *hapR* promoter is a G 77 bp upstream of the ATG (Fig. 2). The −10 sequence, TACACT, shows two mismatches from the consensus TATAAT, and the −35 sequence, TTGACC, has only one mismatch from the consensus TTGACA.

HapR binds to a site in the *hapR* promoter downstream of the transcriptional start. Gel mobility shift assays were used to localize the HapR binding site in the *hapR* promoter. As shown in Fig. 3, a single shift was observed in the presence of increasing amounts of purified HapR protein on a DNA fragment encompassing the region from −61 to +96. However, no shift was observed on a fragment from +41 to +158, indicating that the HapR binding site is located between −61 and +41 in the promoter. No additional complexes with slower mobility were observed on a larger fragment extending upstream in the promoter to −116 (data not shown), indicating that HapR does not recognize any other sites in the promoter with a similar affinity.

To further localize the binding site between −61 and +41, DNaseI footprinting was utilized. On a 350-bp fragment extending from −198 to +158, a single site of strong protection was observed (Fig. 4). On the top strand, this site extended from +12 to +36, and on the bottom strand, it extended from +8 to +33 (Fig. 2). No other strong regions of protection were observed on this fragment, suggesting that a single site is necessary for HapR autorepression. Comparison of a 22-bp sequence of partial dyad symmetry within the footprint (Fig. 5A) to the site previously identified at the *aphA* promoter (13) reveals the lack of a strong HapR binding motif. Of the 10 bp which are conserved between the sites, six of these are symmetrical in both, indicating that there is a degree of functional similarity between them.

Identification of a point mutation in the HapR binding site that prevents binding. We have previously shown that a naturally occurring base pair change in classical biotype strains at position −77 from G to T in the HapR binding site at the *aphA* promoter.
promoter completely prevents HapR binding, indicating it is critical for its recognition (13). Since the analogous position in the HapR binding site at the hapR promoter (\(13\)) is not conserved (Fig. 5A), nor does it appear to be important for symmetry, identification of a base pair change that prevents the protein from binding to the promoter was not straightforward. Therefore, two base pairs were changed to G that appeared to be important for maintaining the dyad symmetry of the site (A at position \(13\) and A at position \(13\)). When these base pair changes were introduced into the \(13\) fragment which is capable of binding HapR (Fig. 3), the +18 change prevented binding whereas the +21 change did not (Fig. 5B). As expected from this, a fragment containing both mutations was also defective for binding (Fig. 5B). These results indicate that HapR binding at its own promoter can be disrupted by a single base pair change at position +18.

The G +18 mutation in the HapR binding site prevents autorepression. To determine if the +18 A-to-G change that interferes with HapR binding to its own promoter also prevents autorepression, its influence on hapR-lacZ fusions in \(\Delta\)hapR and hapR\(^{+}\) strains was determined. As shown in Fig. 6, the expression of the \(\Delta\)hapR-lacZ fusion containing the G +18 mutation (KSK2316) was only slightly higher than that of the wild-type fusion GK972 at both low and high cell density, indicating that the point mutation does not significantly influence the expression of the HapR promoter in the absence of HapR. However, in the presence of HapR, the expression of the fusion containing the G +18 mutation (KSK2337) showed approximately twofold derepression at high cell density such that it appeared similar to the wild-type fusion lacking HapR. These results indicate that HapR autorepresses by binding to the site identified in the hapR promoter that extends from +8 to +36. Consistent with this, the level of hapR expression as determined by real-time RT-PCR in C6706 containing the +18 A-to-G change at high cell density was approximately twofold higher than in the wild type (Fig. 7).

**DISCUSSION**

In strains of *V. cholerae* with a functional HapR protein, quorum sensing plays an important role in influencing various cellular processes. At low cell density, hapR message is destabilized by sRNAs in conjunction with Hfq such that its levels are insufficient to significantly repress phaA and the expression of virulence genes or the expression of polysaccharide genes required for biofilm formation. As the cell density increases, hapR message becomes stabilized, and this leads to an accumulation of HapR which downregulates virulence and polysaccharide gene expression and activates HA protease expression. The work presented here sheds additional light on the control of hapR expression in *V. cholerae* by showing that HapR is capable of repressing its own transcription at high cell density. At low cell density, the expression of a transcriptional hapR-lacZ fusion is fairly low and regulation is primarily achieved at the level of translation (16). However, as the cell density increases, the stability of the hapR message increases as well as
its transcription, and HapR serves to downregulate this transcription through autorepression.

The location of the HapR binding site centered at +22 in the hapR promoter is consistent with its role as a transcriptional repressor. In gel shift and DNaseI footprinting experiments, HapR did not appear to bind strongly to any other sites in the promoter. This is in contrast to the luxR promoter, where LuxR was found to bind independently to two sites that contribute to autorepression: one around +25 similar to that at the HapR promoter and the other upstream of the transcriptional start between \( +107 \) and \( +52 \) (2). The finding that a single base pair change from A to G at \( +18 \) in the hapR promoter prevents HapR binding and virtually eliminates autorepression is consistent with the notion that HapR binding to this single site is responsible for this activity. Since LuxR appears to interfere with RNA polymerase binding (2), HapR likely functions by a similar mechanism.

HapR has previously been shown to bind to a recognition site at the aphaA promoter from which it represses (13). The location of this site at \( -71 \) in the promoter raises the possibility that the protein employs different modes of action at the hapR and aphaA promoters to repress transcription. It was somewhat surprising that the recognition sequences for HapR at the aphaA and hapR promoters are only weakly conserved. The position in the aphaA promoter that is critical for HapR binding (G at \( +77 \)) is not conserved in the site at the hapR promoter nor does it contribute to its symmetry. Although the position in the hapR promoter that is critical for HapR binding and autorepression (A at \( +18 \)) is conserved in the aphaA promoter, it is not yet known whether this position is important for HapR binding to this site. Since it takes considerably less HapR protein to shift the site in the aphaA promoter (13), it is likely that the affinity for the site in the hapR promoter is lower, such that HapR is only able to repress at high protein levels. In contrast, experiments have indicated that HapR is capable of binding to the aphaA promoter and reducing its expression to some degree even at low cell density (13). Thus, the differences in the recognition sequences of HapR at the aphaA and hapR promoters likely contribute to their different binding affinities, and this is important for the temporal regulation of these promoters as the intracellular levels of HapR increase.

Since HapR autorepression downregulates the transcription of the hapR promoter at high cell density, this suggests that autoregulation may serve to prevent runaway expression under this condition. It was somewhat unexpected that at low cell density the expression of the hapR-lacZ fusion was not also reduced in the presence of overexpressed HapR. One possibility is that the activity measured at low cell density is a background level derived by read-through from upstream of the promoter and therefore is not subject to regulation by

FIG. 6. Influence of the +18 A-to-G change on \( \Delta \text{hapR} \) and \( \text{hapR}^{-} \lacZ \) fusions in \( V. \) cholerae. Strains were grown in LB medium at 37°C for 2 h (low cell density, OD\(_{600}\) = 0.2) or 8 h (high cell density, OD\(_{600}\) = 4.0). From left to right: GK972 (\( \Delta \text{hapR-lacZ} \)), KSK2316 (\( \Delta \text{hapR-lacZ} +18G \)), KSK2226 (\( \text{hapR}^{-} \lacZ \)), KSK2337 (\( \text{hapR}^{-} \lacZ +18G \)). wt, wild type.

FIG. 7. Relative levels of hapR expression determined by real-time PCR. Cultures were grown in LB medium at 37°C to an OD\(_{600}\) of 2.0. RNA was isolated, converted to cDNA, and analyzed by real-time PCR. C6706 (wild type [wt]), KSK2339 (+18G).
HapR. Alternatively, the levels of HapR from the overexpression plasmid after only 2 h of induction were insufficient to cause a reduction in the expression of the promoter.

Although the mechanisms involved in the transcriptional activation of the hapR and luxR promoters in V. cholerae and V. harveyi are not clearly understood, the cyclic AMP receptor protein appears to play a role in this process at both (3, 22). However, regulation by MetR appears to be different. In V. harveyi, MetR represses the luxR promoter (3), whereas a ΔmetR mutation in V. cholerae does not appear to influence the expression of hapR (data not shown). It is not surprising that at least some of the factors influencing bioluminescence in V. harveyi and virulence/biofilm formation in V. cholerae are different. Further investigation into the regulation of these promoters will shed additional light on the mechanisms involved in these processes.

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