Promoter Analysis and Regulatory Characteristics of vvhBA Encoding Cytolytic Hemolysin of Vibrio vulnificus*

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Cytolytic hemolysin, a gene product of vvhA, is a putative virulence factor of the pathogenic bacterium Vibrio vulnificus. We have previously shown that hemolysin production is repressed by adding glucose to culture media and that production can be restored by adding cAMP. In this study, hemolysin activity and the level of vvh transcript were determined to reach a maximum in late exponential phase and were repressed when cells entered stationary phase. Northern blot and primer extension analyses revealed that vvhA is cotranscribed with a second gene, vvhB, located upstream of vvhA. Transcription of the vvhBA operon begins at a single site and is under the direction of a single promoter, P_vvh. A crp null mutation decreased hemolysin production and the level of vvhBA transcript by reducing the activity of P_vvh, indicating that the P_vvh activity is under the positive control of cAMP receptor protein (CRP). A direct interaction between CRP and the regulatory region of the vvhBA operon was demonstrated by gel-mobility shift assays. The CRP binding site, centered at 59.5 bp upstream of the transcription start site, was mapped by deletion analysis of the vvhBA promoter region and confirmed by DNase I protection assays. These results demonstrate that the vvhBA expression is activated by CRP in a growth-dependent manner and that CRP exerts its effects by directly binding to DNA upstream of P_vvh.

The pathogenic marine bacterium Vibrio vulnificus is the causative agent of food-borne diseases such as gastroenteritis in healthy persons and life-threatening septicemia in immunocompromised individuals (1–3). V. vulnificus infections are remarkable for their invasiveness, severe tissue damage, and rapidly fulminating course of disease. The characterization of somatic as well as secreted products of V. vulnificus has yielded a large list of putative virulence factors, whose known or putative functions are consistent with disease pathology (4).

Among the putative virulence factors is the cytolytic hemolysin encoded by the vvhA gene. Hemolysin can lyse red blood cells from a variety of animal species by forming small pores in the cytoplasmic membrane. Hemolysin also shows cytolytic activity against cultured cell lines (5, 6). Purified hemolysin, which has an estimated molecular mass of 51 kDa, is heat-labile and toxic for Chinese hamster ovary cells (7–9). When injected intravenously, the purified toxin is lethal in mice at levels of ~3 μg/kg body weight. It has been reported that hemolysin may bind to cholesterol and induce the release of K ions and to a lesser extent Na ions from liposome (10). Although there is a substantial body of literature concerning the biochemical and pathogenic properties of hemolysin, only a few studies have addressed the mechanisms whereby expression of the virulence factor is modulated (11).

A 3.4-kb DNA fragment of V. vulnificus strain EDL174, which encodes VvhA, has been cloned, and its nucleotide sequence has been reported (6). This DNA fragment contains two genes, vvhB and vvhA. The vvhA gene encodes hemolysin, but the function of the vvhB gene product is unknown. In a previous report (11), we showed that hemolysin production in V. vulnificus is repressed by adding glucose to culture media and that expression is derepressed by the addition of cAMP. These results suggested that hemolysin synthesis is regulated by cAMP-CRP (cAMP receptor protein)1 controlled catabolite repression. However, until now, no definitive analysis of the role of the CRP in vvhA expression has been reported. Neither the promoter(s) of the vvhA gene nor CRP binding sites upstream of vvhA has been previously identified. Furthermore, the question of whether CRP directly or indirectly affects hemolysin production has not been previously addressed.

This lack of the information on vvhA regulation makes it difficult to understand how hemolysin levels are modulated. Therefore, in an effort to elucidate the regulatory mechanisms of vvhA expression at a molecular level, we examined the influence of growth phase on hemolysin synthesis. We demonstrated that the vvhBA genes are cotranscribed, that transcription is growth phase dependent, and that vvhA transcription is initiated at a single site. The effects of a crp null mutation on hemolysin production, the cellular level of vvhBA transcript, and the activity of P_vvh were also examined. Finally, binding of CRP directly to the upstream portion of vvhBA was demonstrated, and the site for CRP binding was determined.

EXPERIMENTAL PROCEDURES

Strains, Plasmids, and Culture Media—The strains and plasmids used in this study are listed below (see Table II). Escherichia coli strains used for plasmid DNA replication or conjugational transfer of plasmids were grown in Luria-Bertani (LB) broth or on LB broth containing 1.5% (w/v) agar. Unless noted otherwise, V. vulnificus strains were grown in LB medium supplemented with 2.0% (w/v) NaCl (LBS). When appropriate, antibiotics were added to media at the following concentrations: ampicillin (100 μg/ml), kanamycin (50 μg/ml), and tetracycline (10 μg/ml).

1 The abbreviation used is: CRP, cAMP receptor protein.
Oligonucleotide | Oligonucleotide sequence, 5′ — 3′ | Locationa | Use
--- | --- | --- | ---
VVH901 | AGAGTAGGGTTAAGGGCTTCTACA | 140 to 162 | Primer extension
VVH001 | CGCGGTACCTATATGATGATCTTTTAAAAAC | −110 to −99 | Primer deletion
VVH002 | DTTTGAGTACACAGGAGGAGCAGAAAATATGACTT | −82 to −39 | Primer deletion, amplification of vvhB
VVH003 | CTCTGAGACATTTATATGAAATATTTTCAGGA | 97 to 13 | Primer deletion
VVH004 | CAGGATGTCAGAGAGGGAATGCAAGCAATC | 635 to 657 | Primer deletion, amplification of vvhB
VVH005 | CTGTATGGAATCGTGGTTGTTGGTGTCCTT | 33 to 52 | Primer deletion
VVH010 | TATATGATGACAAATCTCGT | 7 to 27 | DNase I footprinting, gel-mobility shift assay
VVH011 | CAACGGCCATCATTAATCAT | −1 to −161 | DNase I footprinting, gel-mobility shift assay
c-HIS-CRP01 | ATAGGCTGCTTGATGTTGCTGAT | Chromosomal | Amplification of crp

c-HIS-CRP02 | TGGTAGTCTTTAACGAGTCCCTAAC | Chromosomal | Amplification of crp

a Regions of oligonucleotides not complementary to corresponding genes are underlined.

General Genetic Methods—Procedures for the isolation of plasmid DNA, genomic DNA, and transformation were carried out as described by Sambrook et al. (12). Restriction and DNA-modifying enzymes were used as recommended by the manufacturer (New England BioLabs, Beverly, MA). DNA fragments were purified from agarose gels using the Geneclean II kit (Bio 101, Inc., Vista, CA). DNA cloning and manipulation were conducted in E. coli DH5α, and restriction mapping was used to confirm that transformants contained the appropriate plasmids. PCR amplification of DNA was performed using a GeneAmp PCR system 2400 (PerkinElmer Life Sciences, Norwalk, CT) and following standard protocols.

RNA Purification and Northern Blot Analysis of the vvhBA Transcript—Total cellular RNAs from V. vulnificus strains were grown at 30 °C with aeration. 5-ml samples were removed at regular intervals for determination of cell densities, hemolysin activity, and cellular protein concentrations. Growth was monitored by measuring the A600 of the cultures. Cultures with an A600 > 0.8 were diluted prior to measurement. Hemolysin activities were determined as previously described (5, 11). A hemolytic unit was defined as the reciprocal of the maximal dilution showing 50% hemolysis of a suspension (1%, v/v) of human red blood cells. Protein concentrations were determined by the method of Bradford (13), with bovine serum albumin as the standard. Averages and standard errors of the mean (S.E.) were calculated from at least three independent determinations.

Construction of a Set of vvh-luxAB Transcriptional Fusions—A set of vvh-luxAB transcriptional fusion reporters were created by subcloning a series of DNA fragments that overlapped the vvhBA promoter region into pHK0011 (Fig. 6A). The later plasmid carries the promoterless luxAB luciferase genes (15). The subcloned fragments were amplified via PCR using pHK0020 (Fig. 1) as the template. Primer VVH004 (see Table I) contained a BamHI restriction site followed by bases corresponding to the 5′-end of the vvhA coding region. VVH004 was used in conjunction with one of the following primers to amplify DNA upstream of vvhA: VVH001 (for pHK0012), VVH002 (for pHK0013), or VVH003 (for pHK0014) (Table I). The primers were designed to amplify −824, −724, and −690 bp from DNA upstream of vvhA. A KpnI restriction site was added to these primers to facilitate cloning of the DNA fragments. The PCR products were digested with BamHI and KpnI and inserted into pHK0011 that had been digested with the same enzymes, to create the three vvh-luxAB reporter constructs. The reporter fusion of pHK0015, which carries 480 bp of vvhBA upstream DNA, was constructed using primers VVH005 and VVH001. All constructions were confirmed by DNA sequencing.

OligonucleotideOligonucleotide sequence, 5′ — 3′UseVYH901AGAGTAGGGTTAAGGGCTTCTACA140 to 162Primer extensionVVH001CGCGGTACCTATATGATGATCTTTTAAAAAC−110 to −99Primer deletionVVH002DTTTTGAGTACACAGGAGGAGCAGAAAATATGACTT−82 to −39Primer deletion, amplification of vvhBVVH003CTCTGAGACATTTATATGAAATATTTTCAGGA97 to 13Primer deletionVVH004CAGGATGTCAGAGAGGGAATGCAAGCAATC635 to 657Primer deletion, amplification of vvhBVVH005CTGTATGGAATCGTGGTTGTTGGTGTCCTT33 to 52Primer deletionVVH010TATATGATGACAAATCTCGT7 to 27DNase I footprinting, gel-mobility shift assayVVH011CAACGGCCATCATTAATCAT−1 to −161DNase I footprinting, gel-mobility shift assay

c-HIS-CRP01ATAGGCTGCTTGATGTTGCTGATChromosomalAmplification of crp
c-HIS-CRP02TGGTAGTCTTTAACGAGTCCCTAACChromosomalAmplification of crp

a Regions of oligonucleotides not complementary to corresponding genes are underlined.

a Oligonucleotide position, where +1 is the transcription start site of vvhBA operon.
markers in kb. The arrow indicates the 2.0-kb mRNA would be transcribed from ATCC29307 cells and hybridized with the VVHAP DNA probe, formed. When total RNA was isolated from log phase ATCC29307 cells and hybridized with the VVHAP DNA probe, only a single ~2.0-kb transcript was detected (Fig. 2A). Based on the DNA sequence of vvhB, it was anticipated that a vvhA mRNA would be ~1.5 kb in length. Cotranscription of vvhA and vvhB was predicted to produce a 2.0-kb transcript. Therefore, it appeared that a single mRNA coded for both VvhB and VvhA. To test this possibility, Northern blot analysis was performed again using VVHBP as a DNA probe. VVHBP also hybridized to a 2.0-kb RNA (Fig. 2B). These combined results demonstrated that the vvhBA genes are transcribed as a transcriptional operon rather than as two independent genes.

Growth Phase-dependent Expression of vvhBA—To examine whether the production of hemolysin is influenced by growth phase, the hemolysin activities of ATCC29307 cultures were analyzed at various growth stages (Fig. 3A). Hemolysin activity appeared at the beginning of growth and reached a maximum in the late-exponential phase. Hemolysin activity then decreased in the stationary phase. Growth phase regulation of hemolysin production could be manifest at either the transcriptional or post-transcriptional levels. To distinguish between these two possibilities, levels of vvhBA mRNA were monitored during growth. The same amount of total RNA was isolated from ATCC29307 cells at different stages of growth. The results indicated that vvhBA mRNA levels decreased as the bacterial culture entered stationary phase (Fig. 3B). This result suggested that decreased hemolysin activity in the stationary phase correlated with a decrease in the level of vvhBA mRNA.

RESULTS

Northern Blot Analysis of the vvhBA Operon—A 6.5-kb DNA fragment from V. vulnificus ATCC29307, which carries the entire vvhBA operon, was cloned in pHK0202d (Table II). As shown in Fig. 1, an open reading frame for vvhB is present upstream of the vvhA coding region. The function of the vvhB gene product has not yet been determined. To determine whether vvhBA is expressed as a single transcript or as two independent transcripts, Northern blot analyses were performed. When total RNA was isolated from log phase ATCC29307 cells and hybridized with the VVHAP DNA probe, only a single ~2.0-kb transcript was detected (Fig. 2A). Based on the DNA sequence of vvhB, it was anticipated that a vvhA mRNA would be ~1.5 kb in length. Cotranscription of vvhA and vvhB was predicted to produce a 2.0-kb transcript. Therefore, it appeared that a single mRNA coded for both VvhB and VvhA. To test this possibility, Northern blot analysis was performed again using VVHBP as a DNA probe. VVHBP also hybridized to a 2.0-kb RNA (Fig. 2B). These combined results demonstrated that the vvhBA genes are transcribed as a transcriptional operon rather than as two independent genes.

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Strains and plasmids used in this study

| Strain or plasmid | Relevant characteristics | Reference or source |
|-------------------|-------------------------|---------------------|
| **Strains**       |                         |                     |
| V. vulnificus     | Clinical isolate, wild-type | Laboratory collection (15) |
| ATCC29307        |                         |                     |
| ATCC29307, crp::nptI |                     |                     |
| E. coli          |                         |                     |
| DH5α             | supE44 ΔlacU169::lacZ lacZ M215::R17 recA1 endA1 gyrA96 thi-1 relA1 | Laboratory collection (29) |
| SM10 apr         | thiA leuA tonA lacT supE recA4::RPA-2-Tc::Ma apr oriT of RP4, Km; conjugal donor |                     |
| BL21(DE3)        | F-, ompT, hsdS(rB, mB), gal(DE3) | Laboratory collection (29) |
| **Plasmids**     |                         |                     |
| pRK415           | IncP arl; broad host range vector; oriT of RP4, Tc' | (17) |
| pHK0001          | pUC19 with vvhBA; Ap'; | This study (15) |
| pHK0011          | pRK415 with promoterless luxAB; Tc' |                     |
| pHK0012          | pHK0011 with 824-bp fragment of vvhA upstream region; Tc' |                     |
| pHK0013          | pHK0011 with 724-bp fragment of vvhB upstream region; Tc' |                     |
| pHK0014          | pHK0011 with 690-bp fragment of vvhA upstream region; Tc' |                     |
| pHK0015          | pHK0011 with 840-bp fragment of vvhB upstream region; Tc' |                     |
| pHK0201          | pSET A with crp; Ap' | This study (15) |
| pKC0004          | pRK415 with crp; Tc' |                     |
| pHK0004          | pRK415 with his-crp; Tc' | This study (15) |

a Ap', ampicillin-resistant; Km', kanamycin-resistant; Tc', tetracycline-resistant.

FIG. 1. Schematic representation of the V. vulnificus vvhBA operon cloned on pRK0202. The arrows represent the transcriptional directions and the coding regions of vvhBA genes. DNA probes, VVHAP and VVHBP, used for Northern blot and Northern dot blot analyses are depicted as closed bars.

FIG. 2. Northern blot analysis of the vvhBA transcript. Total RNAs from strain ATCC29307 were separated and hybridized to 32P-labeled DNA probes corresponding to the internal regions of vvhA (A) or vvhB (B). For both panels, total RNAs were prepared from cultures grown to exponential phase (A,oo 1.2, L), and to stationary phase (A,oo 2.4, S). The numbers to the left of A correspond to molecular size markers in kb. The arrow on the right side indicates the 2.0-kb vvhBA transcript.

DNase I Protection Assay—DNase I protection assays were performed as described previously (16) with slight modifications. 300 ng of the same labeled 188-bp DNA, used for the mobility shift assay, was suspended in 20 μl of reaction solution containing 1× binding buffer (16), 1 μg of poly(dI-dC), 100 μM eAMP, and various concentrations of CRP. The reaction mixtures were incubated at 25 °C for 20 min. 20 μl of 10 mM MgCl₂ and 5 mM CaCl₂ mix were then added along with 1 μl of a DNase I solution (10 ng/μl, Sigma, St Louis, MO). Samples were then incubated for 1 min at 25 °C, the reactions were stopped by the addition of 80 μl of stop solution (12), and the DNA products were purified by ethanol precipitation. The purified DNA products were resolved on a sequencing gel alongside sequencing ladders of pHK0202 generated using VVH011 as primer. Gels were processed as described for the primer extension analyses.

RESULTS

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Growth Phase-dependent Expression of vvhBA—To examine whether the production of hemolysin is influenced by growth phase, the hemolysin activities of ATCC29307 cultures were analyzed at various growth stages (Fig. 3A). Hemolysin activity appeared at the beginning of growth and reached a maximum in the late-exponential phase. Hemolysin activity then decreased in the stationary phase. Growth phase regulation of hemolysin production could be manifest at either the transcriptional or post-transcriptional levels. To distinguish between these two possibilities, levels of vvhBA mRNA were monitored during growth. The same amount of total RNA was isolated from ATCC29307 cells at different stages of growth. The results indicated that vvhBA mRNA levels decreased as the bacterial culture entered stationary phase (Fig. 3B). This result suggested that decreased hemolysin activity in the stationary phase correlated with a decrease in the level of vvhBA mRNA.
The decrease in \( \text{vvhBA} \) mRNA in stationary phase could be the result of a decrease in the rate of mRNA transcription initiation or decreased mRNA stability. Northern blot analysis demonstrated that the 2.0-kb \( \text{vvhBA} \) transcript, observed in log phase cultures, was not detected in the total RNAs isolated from the stationary phase cells (Fig. 2, A and B). This result agreed well with our previous observation that growth phase-dependent production of hemolysin is regulated at the level of transcription (Fig. 3 B). It was possible that \( \text{vvhBA} \) mRNA stability was decreased in stationary phase. The likelihood for this seemed low, however, because we could not detect any partial degradation products of \( \text{vvhBA} \) mRNA with either the VVHAP or VVHBP DNA probe (Fig. 2, A and B). These results indicated that a decrease in the level of transcription initiation at the \( \text{vvhBA} \) promoter is the sole, or at least the major, mechanism whereby hemolysin activity is down-regulated in the stationary phase.

**Effect of a Mutation in the crp Gene on Hemolysin Production**—A previous study suggested that cAMP-CRP catabolite repression may play a role in the regulation of hemolytic activity in \( \text{V. vulnificus} \) (11). To confirm and expand these earlier results, we examined hemolysin production by a \( \text{crp} \) mutant, KC74. The \( \text{crp} \) mutant was constructed by allelic exchange in ATCC29307 (15). Cultures of ATCC29307 and the \( \text{crp} \) mutant were grown to log phase, and the hemolysin activities of each culture were determined. Although hemolysin activity was present at about 2.0 units per \( \mu \)g of cellular protein in the wild-type strain, KC74 appeared to produce much less hemolysin. The residual level of hemolysin activity in KC74 corresponded to approximately one-thirtieth of that in the wild-type strain (Fig. 4 A). Down-regulation of hemolysin due to the disruption of \( \text{crp} \) suggested that CRP acts as a positive regulator of \( \text{vvhBA} \).

To rule out the possibility that the decreased hemolytic activity in the \( \text{crp} \) mutant was due to polar effects of the \( \text{crp} \) insertion on downstream genes, we determined if reintroduction of \( \text{crp} \) on a plasmid could complement the mutation. For this purpose, plasmid pKC0004 was constructed by subcloning \( \text{crp} \) into pRK415 (15, 17). The resulting plasmid, pKC0004, was transferred into KC74 by conjugation. The hemolytic activity expressed by KC74 (pKC0004) was, at least, equivalent to that of ATCC29307 (Fig. 4, A and B). Therefore, the decreased hemolytic activity of KC74 resulted from inactivation of \( \text{crp} \) rather than reduced expression of any genes downstream of \( \text{crp} \).

To characterize the role of CRP in more detail, the levels of \( \text{vvhBA} \) mRNA in the wild-type strain and KC74 were compared by Northern dot blot analysis. When VVHBP was used as the DNA probe, the \( \text{vvhBA} \) transcript was almost undetectable in KC74 (Fig. 4B). The \( \text{vvhBA} \) transcript was readily detectable in the wild-type strain and in KC74 (pKC0004). Therefore, levels of \( \text{vvhBA} \) mRNA correlated with hemolysin production for all three strains (Fig. 4). These results indicated that CRP exerted its effects on the production of hemolysin at the level of \( \text{vvhBA} \).
transcription. Overall, these results led us to conclude that the expression of vvhBA in V. vulnificus is under the positive control of CRP, at least in log-phase cultures.

**Identification of a Transcription Start Site of vvhBA**—The one or more transcription start sites of vvhBA were mapped by a primer extension analysis. For this purpose, RNAs were prepared from strain ATCC29307 and KC74 harvested log phase and stationary phase cells. A single reverse transcript was produced from primer extension of RNA isolated from log phase and stationary phase cultures (Fig. 5). Using several different sets of primers, we were unable to identify any other transcription start sites by primer extension (data not shown). This indicated that the same transcription start site was used for the transcription of vvhBA in both log and stationary phases. The 5′-end of the vvhBA transcript is located 115-bp upstream of the translational initiation codon of VvhB and is subsequently designated +1. The putative promoter upstream of the transcription start site was named P\textsubscript{vvhBA}. Based on the intensity of the reverse transcripts, P\textsubscript{vvhBA} activity was significantly decreased in stationary phase. This observation supported our hypothesis that the decrease in the level of hemolysin activity in stationary phase is mainly due to the reduced activity of the vvhBA promoter.

In contrast, primer extension analysis, performed with RNA prepared from either log phase or stationary phase cells of KC74, failed to produce a visible product (Fig. 5). This result was not unexpected, because the vvhBA transcript was not detectable in Northern analysis of KC74 RNA (Fig. 4B). Although other explanations are possible, these data, combined with other results, indicate that vvhBA transcription is directed by the same promoter, P\textsubscript{vvhBA}, in log and stationary phase cells and that CRP affects the level of hemolysin production by activating P\textsubscript{vvhBA}.

**Deletion Analysis of the vvhBA Promoter Region**—To delineate the cis DNA sequences in the P\textsubscript{vvhBA} promoter region required for CRP activation, transcriptional fusions of the putative vvhBA regulatory region were made to the reporter gene, luxAB. The pHK-reporter fusions are shown in Fig. 6. The reporter constructs were transferred into the wild-type strain and KC74. Culture luminescence was used to quantify the capacity of each vvh upstream fragment to activate transcription of the vvhBA operon.

For the wild-type strain containing pHK0012, a plasmid that carries an intact regulatory region, luminescence activity was about 1.5 × 10\textsuperscript{6} relative light units (Fig. 6B). The light produced in the crp mutant carrying pHK0012 was significantly reduced, supporting the hypothesis that the expression of the P\textsubscript{vvhBA} is dependent on CRP. Luminescence was also reduced in the strains that carried pHK0013. Moreover, the levels of luminescence in ATCC29307 (pHK0013) and KC74 (pHK0013) did not significantly differ. Similar results were observed when the luminescence was compared between the wild-type and KC74 cells containing pHK0014 (Fig. 6B). These data indicated that the sequences necessary for activation of P\textsubscript{vvhBA} by CRP are absent from the vvh upstream regions carried on pHK0013 and pHK0014. Because the vvhBA upstream region on pHK0013 was deleted up to −63, it is reasonable to conclude that the cis-acting element important for activation of P\textsubscript{vvhBA} by CRP extends about 63-bp upstream of the vvhBA transcription start.

When transformed with pHK0015, the levels of luminescence in the wild-type and crp mutant were comparable to those observed in the cells containing pHK0012. The magnitude of the decrease in luminescence from pHK0015 in the crp mutant was similar to that observed in cells carrying pHK0012. This result suggests that the vvhB coding region does not harbor any cis-acting elements essential for vvhBA transcription. In addition to this, no significant difference was observed when the luminescence was compared between E. coli cells containing either pHK0012 or pHK0015 (data not shown). This observation indicates that VvhB does not affect the activity of P\textsubscript{vvhBA} at least in E. coli.

**CRP Interaction with the vvhBA Promoter in Vitro**—It was apparent that CRP affects the expression of vvhBA and that sequences located about 63-bp upstream of the vvhBA transcription start are required for CRP to activate P\textsubscript{vvhBA}. However, there were still several possible ways for CRP to affect the activity of P\textsubscript{vvhBA}. One was by binding directly to the 63-bp region upstream of the vvhBA to stimulate open complex formation of P\textsubscript{vvhBA}. A second possibility was that CRP either increased or decreased the cellular level of one or more unidentified trans-acting factors that may bind directly to the vvhBA regulatory region. To determine if CRP directly binds the vvhBA promoter, we measured binding of purified CRP to the 188-bp DNA fragment encompassing the residue −63. As shown in Fig. 7A, addition of CRP at a concentration of 100 nM resulted in a shift of the 188-bp DNA fragment to a single band with slower mobility. CRP binding was specific, because assays were performed in the presence of 1 μg of poly(dI-dC) as a nonspecific competitor. In a second gel-mobility shift assay, the same, but unlabeled, 188-bp DNA fragment was used as a self-competitor to confirm specific binding of CRP to the vvhBA promoter (Fig. 7B). The unlabeled 188-bp DNA competed for binding of CRP in a dose-dependent manner (Fig. 7B). It was apparent from these results that CRP binds specifically to the vvhBA regulatory region.

**Identification of CRP Binding Site Using in Vitro DNase I Protection Analysis**—To determine the precise location of the
CRP binding site in the vvhBA regulatory region, a DNase I footprinting experiment was performed using the same 188-bp DNA fragment used for the gel-shift assays. DNase I footprinting revealed a clear protection pattern in the upstream region of vvhBA between −50 and −66 (Fig. 8A). Several nucleotides showed enhanced cleavage, which is frequently observed in DNase I protection analysis of CRP binding sites (18). The protected region overlapped with a consensus sequence for CRP binding, extending from −52 to −67 (Fig. 8B). This CRP binding site is centered −59.5 bp upstream from the transcriptional start site of vvhBA. This position for CRP binding indicates that the P_{vvh} is a class I CRP-dependent promoter. For class I CRP-dependent promoters, CRP binding sites are centered near integral turns of the helix (i.e. n × 10.5 bp) from the transcription start site (19). These observations confirmed that CRP activates P_{vvh} directly, by binding to the upstream region of vvhBA.

Because purified His-tagged CRP protein was used for the gel-mobility shift and DNase I protection assays, it was possible, although unlikely, that the protection resulted from the effects of the His on the recombinant protein. Therefore, it was determined whether His-tagged CRP could complement the crp mutation of KC74. For this purpose, a plasmid, pHK0004, was constructed by subcloning the DNA fragment-encoding His-tagged CRP into pRK415. The levels of hemolytic activity and vvhBA transcription in KC74 containing pHK0004 were restored to levels comparable to those of the wild-type strain (data not shown). Therefore, protection of the vvhBA upstream region by the His-tagged CRP protein resulted from the binding of functional CRP rather than any effects of His_{6} on CRP binding site or CRP itself.

In summary, we have found that the V. vulnificus vvhBA genes are transcribed as a single transcriptional unit, under the control of a single promoter P_{vvh}, and that transcription is growth phase-dependent. Also, the activity of the P_{vvh} is under the positive control of CRP, and CRP exerts its effects by binding directly to a CRP binding site in the vvhBA upstream region.

**DISCUSSION**

A variety of endotoxins and exotoxins, including polysaccharide capsules (20), an elastolytic protease (21), and a phospholipase A2 (22), have been implicated as putative virulence factors for *V. vulnificus*. Another putative virulence factor is a cytolytic hemolysin (5, 6). Several different lines of evidence have led to the hypothesis that hemolysin is an important, if not essential, virulence factor for *V. vulnificus*. The role of hemolysin as a potential virulence factor has been established primarily by using the purified protein in animal models (7). However, when the pathogenesis of an isogenic mutant deficient in hemolysin production was compared with a wild-type strain, it appeared that hemolysin is less important than would have been predicted from examining the effects of the purified proteins on animals (23). It is noteworthy, however, that vvhBA is expressed at low levels under certain conditions, such as in stationary phase cultures (this study) or in the presence of glucose (11). Although other explanations are possible, the lack of significant difference in virulence between the vvhA mutant and the wild-type parent could be due to down-regulation of hemolysin production under particular conditions. This possi-

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**FIG. 6. Deletion analysis of the vvhBA promoter region.** A, construction of vvh-lux fusion pHK-plasmids. PCR fragments carrying the regulatory region of vvhBA and truncated derivatives thereof were subcloned into pHK0011 (15) to create each pHK-reporter construct. Filled blocks, the vvh coding regions; open blocks, luxAB DNA; solid lines, the upstream region of vvhBA. The wild-type vvhBA regulatory region is shown on top with the proposed −10 region, −35 region, and CRP binding site (CB). The −10 and the −35 regions were proposed on the basis of the transcription start site (P) as determined by the primer extension analysis. B, cellular luminescence values were determined for wild-type (filled bars) and the crp mutant (open bars) containing each pHK-reporter, as indicated. Cultures in log phase of growth were used to measure cellular luminescence values. Error bars represent the S.E.

**FIG. 7. Specific binding of V. vulnificus CRP protein to the vvhBA promoter.** The 188-bp DNA fragment of the vvhBA upstream region was radioactively labeled and then used as a probe DNA. A, increasing amounts of CRP (0, 100, 200, and 300 nM in lanes 1–4, respectively) were added to the radiolabeled probe (7 nM). B, for competition analysis, the same, but unlabeled, 188-bp DNA fragment was used as competitor DNA. Various amounts of competitor DNA were added to a reaction mixture containing 7 nM labeled DNA, prior to the addition of CRP. Lane 1, probe DNA alone; lanes 2–5, probe DNA incubated with 300 nM of CRP and 0, 350, 700, or 1400 nM of competitor DNA, respectively.
bility strongly underscores the need to verify expression levels of putative virulence factors during infection to understand the roles of the factors in pathogenesis.

In the present study, it was found that CRP activates the expression of the V. vulnificus hemolysin gene by binding directly to the vvhBA promoter, P_vvhBA. CRP (in conjunction with cAMP) plays a central role in carbon catabolite repression, by which a rapidly metabolizable carbon source, present in the growth medium, represses the synthesis of many enzymes required to metabolize other carbon sources. This global regulatory system has been well described especially for enteric bacteria (24). Besides regulating the synthesis of these catabolic enzymes, a direct role for CRP in regulating the expression of numerous virulence factors has been established for a number of pathogenic Enterobacteriaceae. A crp mutation leads to a reduced production of hemolysin, a potential virulence factor of avian-pathogenic E. coli (25). Our recent work has revealed that expression of V. vulnificus vvhBA operon is under the positive control of cAMP-CRP (15). In contrast, CRP protein negatively regulates the expression of cholera toxin and toxin-coregulated pilus genes in V. cholerae, a species closely related to V. vulnificus (26). In Salmonella typhimurium there is evidence that CRP plays a crucial role in the regulation of virulence gene expression and pathogenesis (27, 28).

From the standpoint of bacterial pathogenesis, the finding that numerous virulence factors are regulated by cAMP-CRP is, perhaps, not surprising. When bacteria invade the human body, many environmental changes, such as differences in type and concentrations of nutrients, would be encountered. Sensing concentrations of cAMP could be used by bacteria to recognize and respond to new environments that are encountered within an infected host. Responses to environmental signals often involve coordinated alterations in the expression of sets of genes and operons. Frequently, the products of environmentally regulated genes are virulence factors (29). Like many other symbiotic and pathogenic microorganisms, V. vulnificus exists in two distinct habitats: seawater and the human body. Previously, we reported that hemolytic activity can be increased, in a dose-dependent manner, by addition of cAMP (11). This observation, combined with the results of this study, suggests that the intracellular level of cAMP is a limiting factor for hemolysin synthesis. Therefore, cAMP is a major factor dictating hemolysin production by V. vulnificus.

Recently, we have identified the toxR gene of V. vulnificus. This gene is a homolog of V. cholerae toxR known to regulate V. cholerae virulence genes encoding cholera toxin, toxin-coregulated pilus, and other virulence factors. A toxR null mutation decreased hemolysin production in V. vulnificus by 50% (30). This suggests that ToxR can up-regulate production of the V. vulnificus hemolysin. However, when transcription of vvhBA was compared between a wild-type strain and an isogenic toxR mutant, no significant differences in vvhBA mRNA levels were detected (data not shown). Consistent with this result, no ToxR binding sites are present in the vvhBA regulatory region. ToxR is known to bind to tandem TTTGAT repeats found in the upstream region of the V. cholerae ctx (31, 32) and to inverted repeats found upstream of toxT (33, 34). These observations suggest that ToxR regulates hemolysin production at a post-transcriptional level, although it has not yet been established if ToxR directly or indirectly affects vvhBA expression. Combined with the positive regulation of the vvhBA promoter by CRP, post-transcriptional regulation may permit tighter regulation of hemolysin production in response to environmental and growth phase regulatory signals.

The exact type of sigma factor associated with RNA polymerase for the transcription of P_vvhBA has not yet been determined. We sequenced several hundred base pairs of DNA upstream of vvhBA and compared this sequence with several putative promoter sequences (Fig. 8). Based on homology to the consensus E. coli σ^70 promoter, a putative promoter sequence consisting of −10 and −35 segments separated by 17 nucleotides, located 6-bp upstream from the transcription start site, was identified. The putative −35 region (TTTATA) has 67% homology to the E. coli −35 consensus sequence (TTGACA) for promoters recognized by RNA polymerase with RpoD (σ^70). The sequence of the −10 region (TATTAA) also revealed substantial homology to the −10 consensus sequence (TATAAT). The homology of P_vvhBA with the E. coli consensus sequences suggests that P_vvhBA is most probably recognized by the V. vulnificus homolog of the σ^70 RNA polymerase holoenzyme. RpoD or σ^70 is the major housekeeping sigma factor in E. coli. Consistent with this assertion, neither the cellular level of vvhBA transcript nor hemolysin
activity was affected by a mutation in rpoS, which encodes an alternative sigma factor, RpoS (σ38) (data not shown). However, additional work is needed to determine if the P_vvh is transcribed by the RNA polymerase using RpoD as a sigma factor.

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