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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 *vvhBA* 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).

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[1] The abbreviation used is: CRP, cAMP receptor protein.

TABLE I
Oligonucleotides used in this study

Oligonucleotide	Oligonucleotide sequence, 5′→3′ [a]	Location[b]	Use
VVH9901	AGAGTAGGGTAAGGCGTCCTACA	140 to 162	Primer extension
VVH001	CGCGGTACCTATATTAGATCACTTTTAAAAC	−110 to −99	Promoter deletion
VVH002	GTGGGTACCACAGTGAGCCAAAAAATACTTTTAT	−63 to −39	Promoter deletion, amplification of *vvhB*
VVH003	CTCGGTACCTTATTTATATGAAATATTTTCAGGA	−37 to −13	Promoter deletion
VVH004	CACGGATCCAAGAGAAAGGGTAAACAGAGTCA	635 to 657	Promoter deletion, amplification of *vvhB*
VVH005	CTTGGATCCAGATTGTTTGGTTCGTCCTT	33 to 52	Promoter deletion
VVH010	TAGATATGCACCAAAATCCTG	7 to 27	DNase I footprinting, gel-mobility shift assay
VVH011	CAACGCCCACATTAATCAAT	−142 to −161	DNase I footprinting, gel-mobility shift assay
HIS-CRP021	ATAGGATCCATGGTTCTAGGTAAACCTCAAA	Chromosomal DNA	Amplification of *crp*
HIS-CRP022	TGTGAATTCCTTAACGAGTACCGTAAACAC	Chromosomal DNA	Amplification of *crp*

[a] Regions of oligonucleotides not complementary to corresponding genes are *underlined*.
[b] Oligonucleotide position, where +1 is the transcription start site of *vvhBA* operon.

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.

Measurement of Cell Growth and Hemolysin Activities—Cultures of *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 A_{600} of the cultures. Cultures with an $A_{600} > 0.8$ 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.

RNA Purification and Northern Blot Analysis of the vvhBA Transcript—Total cellular RNAs from *V. vulnificus* strain ATCC29307 and an isogenic *crp* mutant, KC74, were isolated using a TRIzol reagent kit according to the manufacturer's instructions (Invitrogen, Gaithersburg, MD). For Northern blot analysis, RNA was separated by agarose gel electrophoresis, transferred to nylon membrane, and hybridized as previously described (14). A series of reactions was performed according to standard procedures (12) with 20 μg of total RNA for Northern dot blot analyses. Two DNA probes, VVHAP and VVHBP, were labeled with [α-^{32}P]dCTP using the Prime-a-gene labeling system (Promega, Madison, WI) and used for hybridizations, as previously described (Fig. 1) (14). The VVHAP probe was prepared by labeling the 1.0-kb *Sac*II-*Pst*I DNA fragment internal to *vvhA*. A 0.7-kb DNA fragment containing the coding region of *vvhB* was amplified by PCR using oligonucleotide primers, VVH002 and VVH004 (see Table I), and then labeled for VVHBP probe. The blots were visualized and quantified using a phosphorimaging analyzer (model BAS1500, Fuji Photo Film Co. Ltd, Tokyo, Japan) and the Image Gauge (version 3.12) program.

Primer Extension Analysis—Primer extension experiments were carried out with SuperScript II RNase H$^-$ reverse transcriptase (Invitrogen) according to Sambrook *et al.* (12). A 23-base oligonucleotide (VVH9901, see Table I) complementary to the open reading frame of *vvhB* was used as the primer. The primer was end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The cDNA products were purified and resolved on a sequencing gel alongside sequencing ladders generated with the same primer used for the primer extension. The nucleotide sequence of pHK0202 (see Table II) was determined using the dideoxy-chain termination method with Top^{TM} DNA polymerase (Bioneer, Seoul, Korea) following the manufacturer's protocols. The gels were dried, visualized, and then quantified as described above for Northern analysis.

Overexpression and Purification of V. vulnificus CRP—The coding region of *crp* was amplified using chromosomal DNA of *V. vulnificus* ATCC29307 as the template and oligonucleotide primers, HIS-CRP021 and HIS-CRP022[2] (see Table I). The 0.6-kb PCR product was digested with *Bam*HI and *Eco*RI and then ligated with a 6× histidine-tagging expression vector, pRSET A (Invitrogen, Carlsbad, CA), digested with the same enzymes. The resulting plasmid, pHK0201, encodes CRP with a His_6 tag at the amino terminus. His-tagged CRP protein was expressed in *E. coli* BL21, and the protein was purified by affinity chromatography according to the manufacturer's procedure (Qiagen, Valencia, CA). The final concentration of the purified protein was adjusted to 0.6 μg per microliter of elution buffer and kept frozen until use.

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 pHK0202 (Fig. 1) as the template. Primer VVH004 (see Table I) contained a *Bam*HI 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 lengths, respectively, of DNA upstream of *vvhA*. A *Kpn*I restriction site was added to these primers to facilitate cloning of the DNA fragments. The PCR products were digested with *Bam*HI and *Kpn*I 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.

Measurement of Cellular Luminescence—The *vvh-luxAB* reporters were mobilized into ATCC29307 and the *crp* mutant by conjugation. Cultures were grown to late exponential phase (A_{600} of 1.2), then 1-ml samples were taken from each culture, diluted 100-fold with phosphate-buffered saline (pH 7.4), and placed into cuvettes. 10 μl of a 0.3% (v/v) decanal stock solution was then added to each cuvette. The decanal stock solution was prepared by adding decanal to a 1:1 mixture of water and ethanol. Cellular luminescence was measured with a Lumat model 9501 luminometer (Berthold, Wildbad, Germany). Data are expressed as arbitrary relative light units.

Gel-mobility Shift Assay—Gel shift assays to measure binding of CRP to the regulatory region of *vvhBA* were performed as described by Sambrook *et al.* (12). The 188-bp upstream region of the *vvh* gene, extending from residues −167 to +27 with respect to the +1 transcription start site, was amplified by PCR using the ^{32}P-labeled VVH011 and unlabeled VVH010 as primers. The labeled 188-bp DNA (7 nM) fragment was incubated with varying concentrations of purified His-tagged CRP protein for 30 min at 37 °C in a 20-μl reaction mixture containing, 1× binding buffer (16), 200 μM cAMP, and 1 μg of poly(dI-dC) (Sigma, St. Louis, MO). Following the incubations, 3 μl of loading buffer (12) was added to each reaction, and the samples were separated by electrophoresis on a 6% nondenaturing polyacrylamide gel. For competition analyses, the same but unlabeled 188-bp DNA fragment was used as a competitor DNA. Various amounts of competitor DNA were added to the reaction mixture containing 7 nM of the labeled DNA prior to the addition of 300 nM CRP.

[2] S. H. Choi, manuscript in preparation.

TABLE II
Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Reference or source
Strains		
V. vulnificus		
ATCC29307	Clinical isolate, wild-type	Laboratory collection
KC74	ATCC29307, *crp*::*nptI*	(15)
E. coli		
DH5α	*supE44* Δ*lacU169*(ϕ*80 lacZ* ΔM15)*hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Laboratory collection
SM10 λ*pir*	*thi thr leu tonA lacY supE recA*::RP4–2-Tc::Mu λ*pir oriT* of RP4, Km^r; conjugational donor	(29)
BL21(DE3)	F^-, *ompT*, *hsdS*(r_B^-, m_B^-), *gal*(DE3)	Laboratory collection
Plasmids		
pRK415	IncP *arl;* broad host range vector; *oriT* of RP4; Tc^r	(17)
pHK0202	pUC19 with *vvhBA;* Ap^r	This study
pHK0011	pRK415 with promoterless *luxAB*; Tc^r	(15)
pHK0012	pHK0011 with 824-bp fragment of *vvhA* upstream region; Tc^r	This study
pHK0013	pHK0011 with 724-bp fragment of *vvhA* upstream region; Tc^r	This study
pHK0014	pHK0011 with 690-bp fragment of *vvhA* upstream region; Tc^r	This study
pHK0015	pHK0011 with 480-bp fragment of *vvhB* upstream region; Tc^r	This study
pHK0201	pRSET A with *crp*; Ap^r	This study
pKC0004	pRK415 with *crp*; Tc^r	(15)
pHK0004	pRK415 with his_6-*crp;* Tc^r	This study

[a] Ap^r, ampicillin-resistant; Km^r, kanamycin-resistant; Tc^r, tetracycline-resistant.

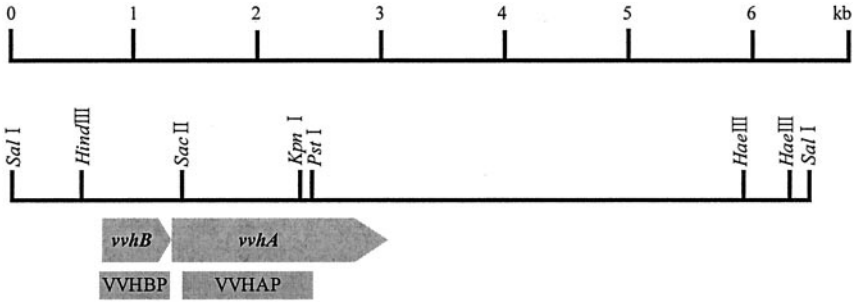

FIG. 1. **Schematic representation of the *V. vulnificus vvhBA* operon cloned on pHK0202.** The *arrows* represent the transcriptional directions and the coding regions of *vvhBA* genes. DNA probes, VVHAP and VVHBP, used for Northern blot and Northern dot blot analyses are depicted as *closed bars*.

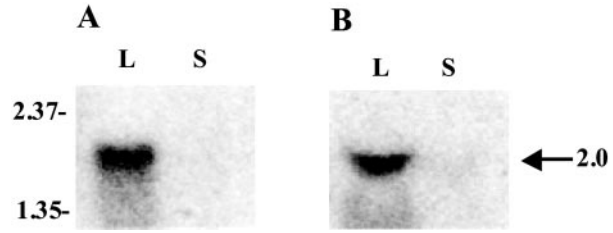

FIG. 2. **Northern blot analysis of the *vvhBA* transcript.** Total RNAs from strain ATCC29307 were separated and hybridized to ^{32}P-labeled DNA probes corresponding to the internal regions of *vvhA* (*A*) or *vvhB* (*B*). For both panels, total RNAs were prepared from cultures grown to exponential phase (A_{600} 1.2, *L*), and to stationary phase (A_{600} 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 cAMP, and various concentrations of CRP. The reaction mixtures were incubated at 25 °C for 20 min. 20 μl of 10 mM $MgCl_2$ and 5 mM $CaCl_2$ 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

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 pHK0202[2] (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. 2*A*). Based on the DNA sequence of *vvhBA*, 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. 2*B*). 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. 3*A*). 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. 3*B*). This result suggested that decreased hemolysin activity in the stationary phase correlated with a decrease in the level of *vvhBA* mRNA.

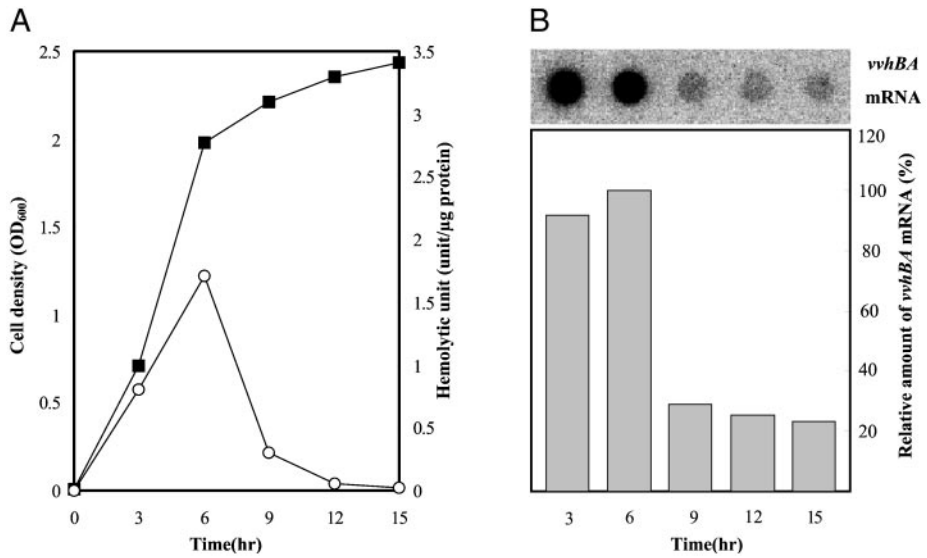

FIG. 3. **Growth kinetics and growth phase-dependent expression of *vvhBA*.** Samples of the cultures were removed at the time points as indicated. Growth of the wild-type strain is indicated by the *filled squares*. Samples were analyzed for hemolysin activity (*A*) and for *vvhA* mRNA (*B*). The relative amounts of the *vvhBA* transcript in each *dot* are expressed relative to the amount of the *vvhBA* transcript after 6 h of incubation. ■, cell density; ○, hemolysin activity.

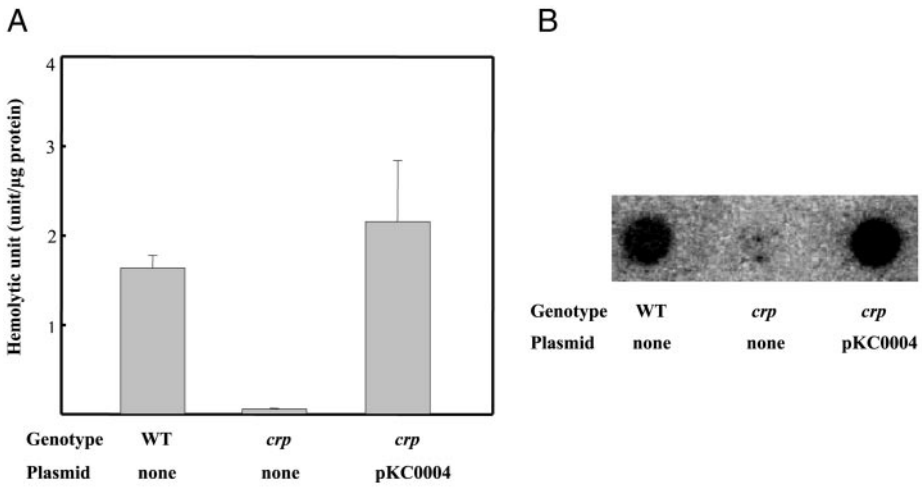

FIG. 4. **CRP dependence of hemolysin production by *V. vulnificus*.** Hemolysin activities (*A*) and relative amounts of the *vvhBA* transcript (*B*) were determined for the wild-type strain and the isogenic *crp* mutant, KC74, as indicated. Complementation of the *crp* mutation by functional *crp* (pKC0004) is also presented. For both panels, samples removed at an A_{600} of 1.2 were analyzed for hemolysin activity and *vvhBA* transcript. *Error bars* represent the S.E. *WT*, wild-type.

The decrease in *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 *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 *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 *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 *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 *V. vulnificus* (11). To confirm and expand these earlier results, we examined hemolysin production by a *crp* mutant, KC74. The *crp* mutant was constructed by allelic exchange in ATCC29307 (15). Cultures of ATCC29307 and the *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 μg 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 *crp* suggested that CRP acts as a positive regulator of *vvhBA*.

To rule out the possibility that the decreased hemolytic activity in the *crp* mutant was due to polar effects of the *crp* insertion on downstream genes, we determined if reintroduction of *crp* on a plasmid could complement the mutation. For this purpose, plasmid pKC0004 was constructed by subcloning *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 *crp* rather than reduced expression of any genes downstream of *crp*.

To characterize the role of CRP in more detail, the levels of *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 *vvhBA* transcript was almost undetectable in KC74 (Fig. 4*B*). The *vvhBA* transcript was readily detectable in the wild-type strain and in KC74 (pKC0004). Therefore, levels of *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 *vvhBA*

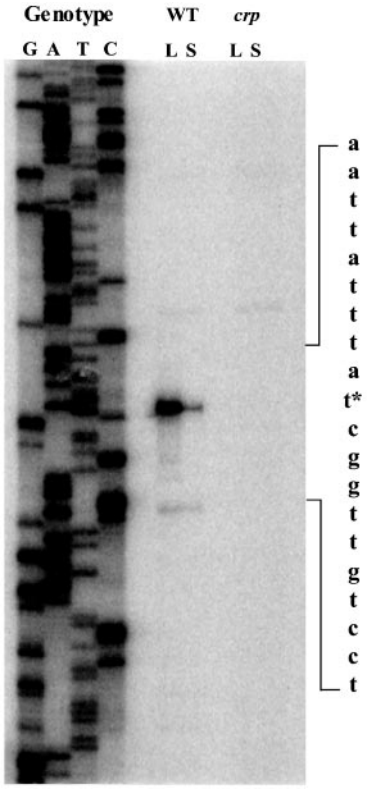

FIG. 5. **Identification of a transcription start site of the *vvhBA* operon.** The transcription start sites were determined by the primer extension of the RNA derived from wild-type and *crp* mutant (KC74) strains as indicated. Total RNAs were isolated from log phase (*L*, A_{600} 1.2) and stationary phase (*S*, A_{600} 2.4) cultures of each strain. *Lanes G*, *A*, *T*, and *C* represent the nucleotide sequencing ladders of pHK0202. The *asterisk* indicates the site of the transcription start. *WT*, wild-type.

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_{vvh}. Based on the intensity of the reverse transcripts, P_{vvh} 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. 4*B*). Although other explanations are possible, these data, combined with other results, indicate that *vvhBA* transcription is directed by the same promoter, P_{vvh}, in log and stationary phase cells and that CRP affects the level of hemolysin production by activating P_{vvh}.

Deletion Analysis of the vvhBA Promoter Region—To delineate the *cis* DNA sequences in the P_{vvh} 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 1.5×10^6 relative light units (Fig. 6*B*). The light produced in the *crp* mutant carrying pHK0012 was significantly reduced, supporting the hypothesis that the expression of the P_{vvh} 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. 6*B*). These data indicated that the sequences necessary for activation of P_{vvh} 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_{vvh} 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_{vvh} 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_{vvh}. However, there were still several possible ways for CRP to affect the activity of P_{vvh}. One was by binding directly to the 63-bp region upstream of the *vvhBA* to stimulate open complex formation of P_{vvh}. 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. 7*A*, 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. 7*B*). The unlabeled 188-bp DNA competed for binding of CRP in a dose-dependent manner (Fig. 7*B*). 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

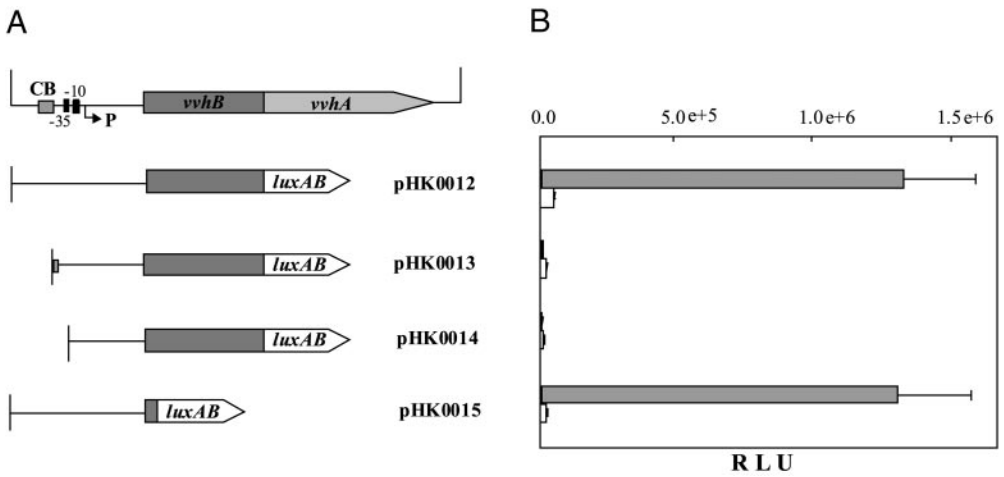

FIG. 6. **Deletion analysis of the *vvhBA* promoter region.** *A*, construction of *vvh-lux* fusion pHK-plasmids. PCR fragments carrying the regulatory region of *vvhBA* and truncated derivatives thereof were subcloned into pHK0011 (15) to create each pHK-reporter construct. *Filled blocks*, the *vvh* 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.

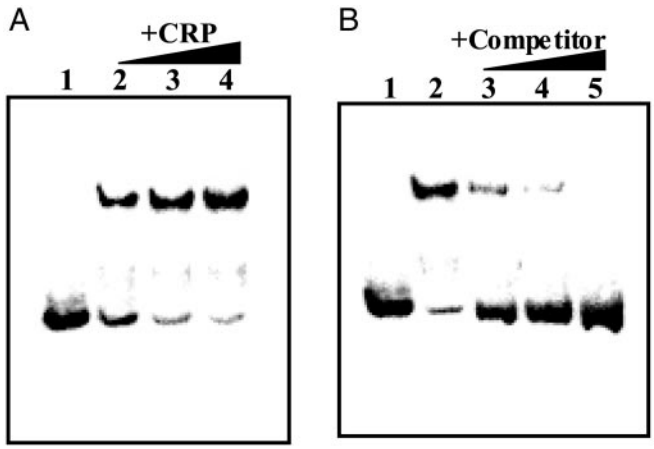

FIG. 7. **Specific binding of *V. vulnificus* CRP protein to the *vvhBA* promoter.** The 188-bp DNA fragment of the *vvhBA* upstream region was radioactively labeled and then used as a probe DNA. *A*, increasing amounts of CRP (0, 100, 200, and 300 nM in *lanes 1–4*, respectively) were added to the radiolabeled 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.

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. 8*A*). 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. 8*B*). 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.* $n \times 10.5$ 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* transcript 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-

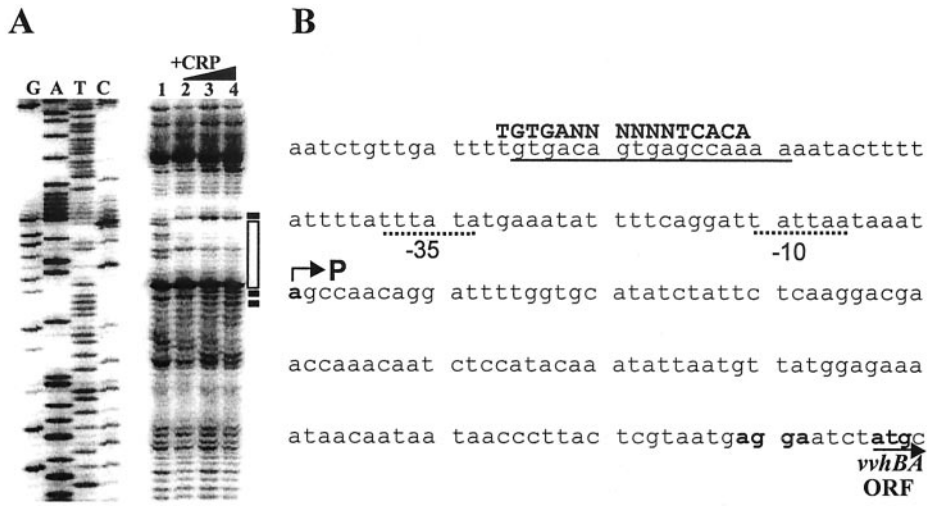

FIG. 8. **CRP binding site for the *vvhBA* promoter.** *A*, DNase I protection analysis of CRP binding to the wild-type *vvh* regulatory region. *Lane 1*, no CRP added; *lanes 2–4*, CRP at 100, 200, and 300 nM, respectively. *Lanes G*, *A*, *T*, and *C* represent nucleotide sequencing ladders of pHK0202. Nucleotides showing enhanced cleavage in the presence of CRP are indicated by the *thick lines*, and the region protected by CRP is indicated by the *open box* (not all hypersensitive and protected bands are indicated). *B*, sequence analysis of the *vvhBA* upstream region. The transcription start site is indicated by the *bent arrow* (*P*). The region protected from DNase I by CRP and the putative promoter region (−10 and −35) are *underlined* with *continuous* and *broken lines*, respectively. Conserved nucleotide sequences for CRP binding (24) are indicated by *capital letters above* the *V. vulnificus* DNA sequence. The ATG translation initiation codon and putative ribosome binding site (AGGA) are indicated in *boldface*.

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_{vvh}. 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 vvpE*-encoding elastase 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 (30), 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-transcription 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_{vvh} 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_{vvh} with the *E. coli* consensus sequences suggests that P_{vvh} 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 (σ^{38}) (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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