INFECTION AND IMMUNITY, Nov. 1997, p. 4634–4641 Vol. 65, No. 11
0019-9567/97/$04.00+0

Transcription of the *Corynebacterium diphtheriae hmuO* Gene Is Regulated by Iron and Heme

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Received 6 June 1997/Returned for modification 23 July 1997/Accepted 15 August 1997

The *hmuO* gene is required for the utilization of heme and hemoglobin as iron sources by *Corynebacterium diphtheriae*. The product of *hmuO* has homology to eukaryotic heme oxygenases which are involved in the degradation of heme and the release of iron. To investigate the mechanism of *hmuO* regulation, a promoterless *lacZ* gene present on the promoter-probe vector pCM502 was placed under transcriptional control of the *hmuO* promoter. In *C. diphtheriae* C7, optimal expression from the *hmuO* promoter was obtained only in the presence of heme or hemoglobin under low-iron conditions. Expression of *hmuO* in high-iron medium containing heme was repressed five- to sixfold from that seen under low-iron conditions in the presence of heme. Transcription from the *hmuO* promoter in the absence of heme or hemoglobin was fully repressed in high-iron medium and was expressed at very low levels in iron-depleted conditions. Expression studies with the *hmuO-lacZ* fusion construct in C7hm723, a *dtxR* mutant of C7, and in a *hmuO* mutant of *C. diphtheriae* HC1 provided further evidence that transcription of the *hmuO* promoter is repressed by DtxR and iron and activated by heme. In *Escherichia coli*, the *hmuO* promoter was expressed at very low levels under all conditions examined. Gel mobility shift assays and DNase I footprinting experiments indicated that DtxR binds in a metal-dependent manner to a sequence that overlaps the putative *hmuO* promoter. Total cellular RNA isolated from *C. diphtheriae* was used to identify the transcriptional start site for the *hmuO* gene. Northern blot analysis suggested that the *hmuO* mRNA was monocistronic and that transcription was heme inducible.

Corynebacterium diphtheriae is a gram-positive bacterium that is the causative agent of the severe respiratory disease diphtheria. Diphtheria toxin, which is synthesized and secreted by virulent toxigenic strains of *C. diphtheriae*, is the best characterized virulence determinant in this pathogen (34). The structural gene for diphtheria toxin (*tox*) is negatively regulated at the transcriptional level by iron and the diphtheria toxin repressor protein, DtxR (1, 40, 45). The DtxR protein is an iron-dependent DNA-binding protein that regulates various genes in *C. diphtheriae* (43, 51) and is functionally similar to the ferric uptake repressor protein (Fur) in gram-negative bacteria (14). DtxR homologs have been found in genera related to *C. diphtheriae*, including species of *Mycobacterium* (5, 44), *Streptomyces* (12), and *Brevibacterium* (32). Although DtxR and Fur are both iron-dependent global regulatory proteins, they share little if any amino acid sequence homology (1, 40).

The environmental niche within the host that is colonized by many pathogens is deficient in easily accessible iron (53). Much of the extracellular iron in eukaryotes is sequestered by the iron-binding proteins transferrin and lactoferrin, while intracellular iron is predominately bound to heme which is associated with protein (33, 53). To overcome this iron deficiency, bacterial pathogens have evolved a variety of mechanisms to extract iron from eukaryotic compounds. Numerous bacterial pathogens produce siderophores, low-molecular-weight iron-chelating molecules, that can remove iron from transferrin and lactoferrin (2, 10, 26, 54). Virulent species of the genera *Haemophilus* and *Neisseria* extract iron from transferrin and lactoferrin through a direct interaction between these host proteins and outer membrane receptors exposed on the surface of the bacteria (27, 33). Numerous gram-negative pathogens also possess transport mechanisms for obtaining iron from heme and heme-containing proteins (23). In these organisms, heme is thought to be transported through the outer membrane by a TonB-dependent outer membrane receptor and then subsequently transported into the cytosol by a periplasmic-binding-protein-dependent system (3, 17, 19, 29, 47, 48). Many of the genes involved in these various iron acquisition systems in gram-negative bacteria are regulated by iron and Fur (23). A notable exception to this mode of regulation occurs in pathogenic *Haemophilus* species where the synthesis of hemoglobin- and transferrin-binding proteins is heme repressible and unaffected by the iron concentration (7, 20, 31). Similarly, heme transport in *Porphyromonas gingivalis* is thought to be regulated by heme (11, 22). The molecular mechanism of heme regulation in these organisms has not been determined.

In contrast to gram-negative organisms, little is known about the molecular mechanisms by which gram-positive bacteria acquire iron from eukaryotic sources. Pathogenic species of the genera *Staphylococcus* and *Streptococcus* are known to utilize a variety of host compounds as iron sources (6, 9). While transferrin-binding proteins have been found in species of *Staphylococcus* (30) and heme-binding proteins have been identified in *S. pneumoniae* (50), the role of these proteins in the transport or utilization of iron has not been elucidated.

In a previous report from this laboratory, *C. diphtheriae* was shown to utilize heme, hemoglobin, and transferrin as iron sources (39). Mutants in *C. diphtheriae* and in *Corynebacterium ulcerans* that were unable to utilize heme and hemoglobin as iron sources were generated by chemical mutagenesis. A gene designated *hmuO*, which was derived from a *C. diphtheriae* genomic library, was shown to complement several of these *Corynebacterium* mutants. The predicted product for *hmuO* (24,123 Da) has significant homology with eukaryotic heme oxygenases. Heme oxygenases have been well characterized in

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eukaryotes both biochemically and genetically (24), where they have an important role in heme metabolism. Heme oxygenases convert heme to biliverdin with the resulting production of CO and the release of iron from the porphyrin molecule. It was proposed that HmuO in *C. diphtheriae* is involved in the utilization of iron from heme through a heme oxygenase activity that results in the degradation of heme and the release of iron (39). While several heme transport systems have been found in gram-negative pathogens (23), no heme oxygenase homologs have yet been identified, and the mechanism by which iron is removed from heme in gram-negative organisms has not been determined.

In this study, the mechanism of *hmuO* regulation was examined by measuring the expression of the *hmuO* promoter in a *hmuO-lacZ* transcriptional fusion construct. DNA binding studies were done to locate DtxR binding sites at the *hmuO* promoter, and RNA analysis identified the transcriptional start site and indicated that the *hmuO* mRNA is monocistronic and activated by heme.

MATERIALS AND METHODS

Bacterial strains and media. *Escherichia coli* DH5α (Bethesda Research Laboratories, Gaithersburg, Md.) was used for routine maintenance and isolation of plasmid DNA. *C. diphtheriae* C7 (18), HC1 (4), and C7hm723 (21) were from the collection of R. K. Holmes. *C. diphtheriae* HC1-2 is a derivative of HC1 that is defective in the utilization of heme and hemoglobin as iron sources (39). *E. coli* was grown in Luria-Bertani (LB) medium (28), and *C. diphtheriae* strains were grown in heart infusion broth (Difco, Detroit, Mich.) containing 0.2% Tween 80 (HIBTW). Permanent stocks of all strains were maintained in 20% glycerol at −70°C. Antibiotics were added to LB medium for *E. coli* as follows: 100 μg of ampicillin/ml, 50 μg of kanamycin/ml, and 30 μg of chloramphenicol/ml. Two micrograms of chloramphenicol/ml was added to HIBTW for *C. diphtheriae*. LB or HIBTW medium was made low in iron by the addition of ethylenediamine-di(*o*-hydroxyphenylacetic acid) (EDDA) and was deferrated by the method of Rogers (35). EDDA was added at concentrations of 500, 50, and 25 μg/ml to cultures of *E. coli*, *C. diphtheriae* C7 and C7hm723, and *C. diphtheriae* HC1, respectively. Hemin (bovine) was added to medium at 100 μM, and hemoglobin (human) was added at 15 μM. Antibiotics, EDDA, Tween 80, hemin, and hemoglobin were from Sigma Chemical Co. (St. Louis, Mo.).

Plasmid construction. Construction of the promoter-probe vector pCM502 and the promoter fusion derivatives is detailed in Fig. 1. In step 1, plasmid pCM501 was produced by ligating a 3.1-kb *Sca*I-*Sal*I fragment from plasmid pQF50 (8), which carries a promoterless *lacZ* gene, to the *Bam*HI-*Sal*I sites in the *C. diphtheriae-E. coli* shuttle vector pCM2.6 (40). The *Bam*HI site in pCM2.6 was first made blunt by treatment with Klenow (37). In step 2, a 200-bp *Nde*I-*Bam*HI fragment from pQF50, which carries transcriptional termination sequences, was ligated to the *Sal*I site of pCM501 to generate pCM502. The *Nde*I, *Bam*HI, and *Sal*I sites were made blunt with Klenow prior to ligation. In step 3, the promoter-*lacZ* fusion plasmids pCZHMU and pCZORF were constructed by ligating the 350-bp insert from pAKS293, a subclone of plasmid pCD293 (39), into the multiple cloning site of pCM502 in both possible orientations (Fig. 1). Plasmid pCMtox was constructed by ligating a 200-bp fragment that carries the *C. diphtheriae tox* promoter into pCM502 (41). Expression of the *tox* promoter on this 200-bp fragment is regulated by iron and DtxR (41). Plasmid pCM502 and its derivatives, which contain the origin of replication from the *C. diphtheriae* plasmid pNG2 (46), replicate at low copy number in *C. diphtheriae* strains and at high copy number in *E. coli* (46). Plasmid pDSK29 carries the *dtxR* gene and was described previously (41). Plasmids were transformed into *C. diphtheriae* strains by electroporation (16) and were transformed into *E. coli* as described previously (13).

β-Galactosidase assays. *E. coli* and *C. diphtheriae* cultures were inoculated 1:100 from fresh 18-h cultures and grown for 16 to 18 h at 37°C with shaking. Supplements were added to the medium as indicated. LacZ activity was determined for *E. coli* according to Miller (28) and for *C. diphtheriae* as previously described (41).

DNA binding studies. DtxR was expressed from *E. coli* DH5α/pDtxR-7 with the T7 expression system and was purified by Ni^{2+} affinity chromatography as described previously (42). DNA fragments for binding experiments were derived from plasmid pAKS293. Gel mobility shift assays and DNase I protection experiments were done as described in a previous report (45). DNA fragments (0.1 to 0.5 nM) were labeled with ^{32}P at their 3′ ends by using the Klenow fragment (37), and DtxR was used at an approximately 500 μM concentration in all of the binding studies. Co^{2+} at a concentration of 150 μM was used in place of Fe^{2+} as the divalent metal corepressor for DtxR due to the greater redox stability of Co^{2+}. The Maxam and Gilbert G+A sequencing ladder was used as a size standard for the DNase I protection experiments (25).

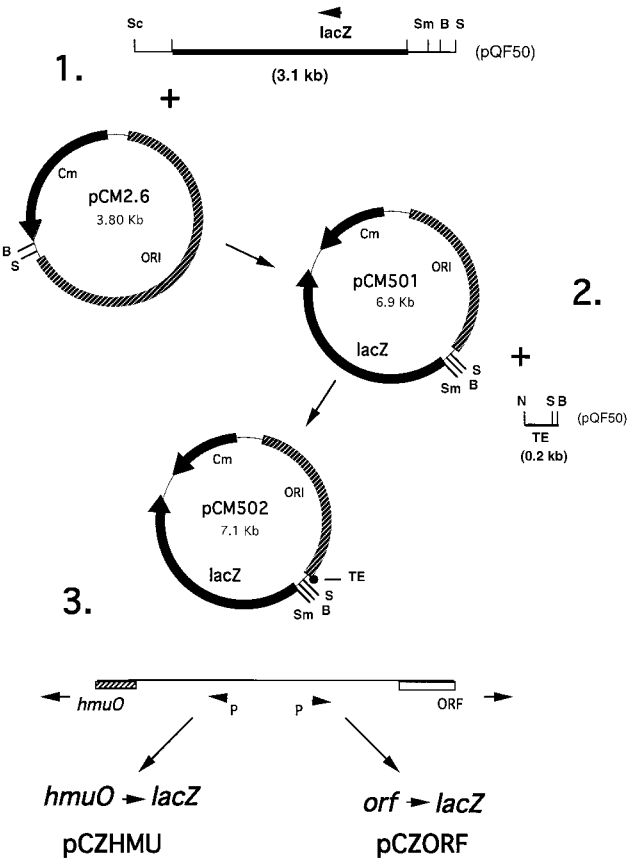

FIG. 1. Construction of the transcriptional fusion vector pCM502 and promoter-*lacZ* fusion plasmids pCZHMU and PCZORF. In step 3, pCZHMU and PCZORF were constructed by ligating a 350-bp fragment that contains the *hmuO* and *orf* intergenic region into the multiple cloning site of pCM502 in both possible orientations. Only the restriction sites used for plasmid construction are shown. B, *Bam*HI; N, *Nde*I; S, *Sal*I; Sc, *Sca*I; Sm, *Sma*I; Cm, chloramphenicol resistance determinant; ORI, pNG2 origin of plasmid DNA replication; TE, transcriptional termination sequences from plasmid pQF50; P, promoter regions. Plasmid maps are not to scale. See Materials and Methods for details on plasmid construction.

RNA analysis. *C. diphtheriae* strains were grown overnight in HIBTW medium and then diluted 1:10 into fresh HIBTW medium which contained various supplements as indicated. The bacteria were grown for an additional 6 to 8 h at 37°C with aeration, and then total cell RNA was isolated and purified as previously described (41). Primer extension experiments utilized a 30-bp single-stranded oligonucleotide primer designated HMPE (5′-GCGGTGGACTGCTTGAGTT CGACAGCGAGG-3′) that is complementary to the *hmuO* mRNA and is located approximately 75 bp downstream from the predicted −10 sequence of the *hmuO* promoter. The primer was labeled with ^{32}P at its 5′ terminus by using T4 DNA kinase (37), and primer extension experiments were done according to Sambrook et al. (37). Northern blot analysis was performed with *C. diphtheriae* RNA as described previously (37). Blots were probed with a 600-bp *Stu*I fragment derived from pCD293 (39). The *Stu*I fragment, which is located entirely within the *hmuO* coding region, was labeled with [^{32}P]dATP by using a random primer kit (Stratagene, LaJolla, Calif.). For primer extension studies and Northern blot analysis, 50 μg of RNA was used from *C. diphtheriae* C7 and HC1 and 10 μg of RNA was used from HC1-2. Higher concentrations of RNA were required for experiments using C7 and HC1, since these strains express *hmuO* at lower levels than HC1-2.

RESULTS

Transcriptional analysis of promoter-*lacZ* fusions. The transcriptional fusion vector pCM502, which contains a promoterless *lacZ* gene (Fig. 1), was constructed to measure promoter activity in the region upstream of the *hmuO* gene. DNA se-

TABLE 1. Expression of promoter-*lacZ* fusions in *E. coli* DH5α

Plasmid(s)	Iron[a]	LacZ amt (U)[b]
pCM502	+	<0.5
	−	<0.5
pCMtox, pDSK29 (*dtxR*)	+	3.3 ± 1.1
	−	105.0 ± 9.5
pCZHMU (P*hmuO*)	+	1.2 ± 0.1
	−	1.5 ± 0.4
pCZHMU, pDSK29	+	1.0 ± 0.4
	−	1.7 ± 0.3
pCZORF (P*orf*)	+	11.5 ± 1.2
	−	18.4 ± 4.1
pCZORF, pDSK29	+	1.2 ± 0.3
	−	4.8 ± 1.3

[a] +, high-iron medium (LB); −, low-iron medium, LB containing 500 μg of EDDA/ml.
[b] LacZ units (U) were determined according to Miller (28). Values are the means (± standard deviations) of three independent experiments.

TABLE 2. Expression of promoter-*lacZ* fusions in *C. diphtheriae* C7

Plasmid	Iron[a]	Heme[b]	LacZ amt (U)[c]
pCM502	+	−	<0.5
	−	−	<0.5
pCMtox	+	−	<0.5
	−	−	58.5 ± 5.2
	−	+	16.3 ± 2.7
pCZHMU (P*hmuO*)	+	−	<0.5
	−	−	0.5
	−	+	11.6 ± 1.5
	−	+[d]	12.8 ± 1.7
	+	+	2.2 ± 0.6
pCZORF (P*orf*)	+	−	0.9 ± 0.2
	−	−	1.9 ± 0.4
	−	+	0.7 ± 0.1

[a] +, high-iron medium (HIBTW); −, low-iron medium, HIBTW containing 50 μg of EDDA/ml.
[b] +, presence of heme; −, absence of heme.
[c] LacZ units (U) were determined according to Miller (28). Values are means (± standard deviation) of three independent experiments.
[d] Hemoglobin added instead of heme.

quence analysis of this upstream region revealed two putative overlapping promoters that are predicted to be divergently transcribed (39). To assess the activity of these putative promoters, a 350-bp fragment carrying this region was placed in both possible orientations in pCM502. Plasmid pCZHMU would detect transcriptional activity directed toward the *hmuO* gene, while pCZORF, which contains the fragment in the opposite orientation, would measure promoter activity directed toward an open reading frame (ORF) upstream of *hmuO* (Fig. 1). Plasmid pCMtox, which carries the *tox* promoter in pCM502, was used as a control for iron and DtxR regulation.

Transcriptional fusion studies in *E. coli*. LacZ expression was initially examined in *E. coli* DH5α under high- and low-iron conditions (Table 1). The pCM502 vector had no detectable activity in *E. coli*. The *tox* promoter on pCMtox was strongly regulated by the iron content of the medium in the presence of the cloned *dtxR* gene and was expressed at high constitutive levels in the absence of *dtxR* (not shown), which is consistent with earlier studies (41, 43). Transcriptional activity from the *hmuO* promoter (P*hmuO*) on pCZHMU in *E. coli* was very low and was not affected by either the iron concentration or the presence of the cloned *dtxR* gene (Table 1). Promoter activity on plasmid pCZORF (P*orf*) showed 11 to 18 U of LacZ activity in the absence of the cloned *dtxR* gene irrespective of the iron level and was repressed 10-fold in the presence of *dtxR* (pDSK29) in high-iron medium. A slight (fourfold) derepression of P*orf* was seen in low-iron medium in the presence of *dtxR*. These findings indicate that P*orf* is regulated by iron and *dtxR* in *E. coli*.

Analysis of promoter fusions in *C. diphtheriae* C7. Expression of the promoter-*lacZ* fusion constructs was next examined in *C. diphtheriae* strains grown in high- and low-iron medium in the presence or absence of heme. Transcription of the *tox* promoter (pCMtox) in wild-type *C. diphtheriae* C7 (*dtxR*$^+$) was strongly repressed in high-iron medium and derepressed in iron-depleted conditions (Table 2). In low-iron medium in the presence of heme, the *tox* promoter exhibited three- to fourfold repression from that observed in the absence of heme. The slight repression caused by heme is likely the result of heme functioning as an iron source for *C. diphtheriae*. Transcription from the *hmuO* promoter on pCZHMU was fully repressed in high-iron conditions, and only marginally detectable expression was observed in low-iron conditions. However, the presence of added heme or hemoglobin in the low-iron medium resulted in a 20- to 25-fold induction of the *hmuO* promoter. This enhanced expression in the presence of heme was repressed five- to sixfold in high-iron medium (Table 2). These results indicate that transcription of the *hmuO* gene in *C. diphtheriae* is activated in the presence of heme or hemoglobin and repressed in high-iron conditions.

Unlike in *E. coli*, only low levels of transcription were detected from P*orf*(pCZORF) in *C. diphtheriae* C7, and this expression was not appreciably affected by either iron or heme (Table 2).

Transcriptional fusion studies in *C. diphtheriae* C7hm723. To examine the effect of *dtxR* on the expression of the *hmuO* promoter, the transcriptional activity of pCZHMU was analyzed in *C. diphtheriae* C7hm723, a *dtxR* mutant of C7. As shown in Table 3, the *tox* promoter is strongly expressed at near-constitutive levels in C7hm723, which is consistent with previous findings (41, 49). The *hmuO* promoter exhibited between 7 and 13 U of LacZ activity in both high- and low-iron medium in C7hm723. Transcription from the *hmuO* promoter was enhanced three- to fivefold in the presence of heme, re-

TABLE 3. Expression of promoter-*lacZ* fusions in *C. diphtheriae* C7hm723 (*dtxR* mutant)

Plasmid	Iron[a]	Heme[b]	LacZ amt (U)[c]
pCMtox	+	−	47.8 ± 9.6
	−	−	62.4 ± 12.4
	−	+	45.0 ± 8.7
pCZHMU (P*hmuO*)	+	−	13.3 ± 2.4
	−	−	7.3 ± 0.4
	−	+	32.0 ± 5.5
	+	+	33.5 ± 3.7

[a] +, high-iron medium (HIBTW); −, low-iron medium, HIBTW containing 50 μg of EDDA/ml.
[b] +, presence of heme; −, absence of heme.
[c] LacZ units (U) were determined according to Miller (28). Values are means (± standard deviation) of three independent experiments.

TABLE 4. Expression of promoter-*lacZ* fusions in *C. diphtheriae* HC1 (*hmuO*$^+$) and HC1-2

Plasmid	Iron[a]	Heme[b]	LacZ amt (U)[c]
C. diphtheriae HC1			
pCMtox	+	−	11.4 ± 6.4
	++	−	0.9 ± 0.3
	−	−	139.6 ± 22.8
	−	+	56.5 ± 9.6
pCZHMU	+	−	11.6 ± 2.8
(P*hmuO*)	++	−	1.2 ± 0.5
	−	−	4.6 ± 2.4
	−	+	37.5 ± 9.2
C. diphtheriae HC1-2			
pCMtox	++	−	0.7 ± 0.2
	−	−	173.3 ± 10.5
	−	+	91.4 ± 16.7
pCZHMU	++	−	0.8 ± 0.2
(P*hmuO*)	−	−	9.1 ± 3.6
	−	+	135.3 ± 12.5

[a] +, high-iron medium (HIBTW); ++, HIBTW with 100 μM FeSO4; −, low-iron medium, HIBTW containing 25 μg of EDDA/ml.
[b] +, presence of heme; −, absence of heme.
[c] LacZ units (U) were determined according to Miller (28). Values are means (± standard deviations) of three independent experiments.

gardless of the iron concentration (Table 3). This finding suggests that DtxR is responsible for the iron-dependent repression of the *hmuO* promoter.

Transcriptional fusion studies of *C. diphtheriae* HC1 and HC1-2. *C. diphtheriae* HC1 is defective in its ability to transport iron and is thought to carry a mutation in a gene(s) required for uptake of the *C. diphtheriae* ferric-siderophore complex (4, 36). In a previous study, mutants of HC1 that were deficient in the ability to utilize heme and hemoglobin as iron sources were isolated (39). One of these mutant strains, HC1-2, was complemented by a plasmid carrying only *hmuO*, suggesting that HC1-2 is defective in HmuO activity. To determine if a defect in HmuO, a presumed heme-degrading enzyme, affected the expression of the *hmuO* promoter, plasmid pCZHMU was moved into HC1-2 and HC1. In high-iron medium (HIBTW with no added iron) expression from the *tox* promoter (pCMtox) produced approximately 11 U of LacZ activity (Table 4). When 100 μM $FeSO_4$ was added to this medium, expression was repressed over 10-fold, indicating that HC1 requires higher iron concentrations than the wild-type strain C7 to fully repress the *tox* promoter. This finding is consistent with a defective iron acquisition system and with earlier studies which indicated that HC1 produced diphtheria toxin under iron-replete conditions (4). In low-iron medium (HIBTW with 25 μg of EDDA/ml), expression of *tox* was strongly derepressed, and the addition of heme to this medium resulted in a slight (twofold) repression, similar to the effect observed in C7. Expression of the *hmuO* promoter in HC1 was similar to that of the *tox* promoter in high-iron medium. In low-iron medium, 4 to 5 U of LacZ activity were detected from the *hmuO* promoter, and the addition of heme resulted in a seven- to eightfold induction in promoter activity, which indicates that the *hmuO* promoter in the HC1 strain is activated by heme.

Expression of the *tox* promoter in HC1-2 was similar to that detected in the parent strain HC1 (Table 4). However, expression from the *hmuO* promoter in HC1-2, in low-iron medium in the presence of heme, was three- to fourfold higher than that found in HC1, indicating that the defect in HC1-2 results in an enhanced heme-dependent activation of the *hmuO* promoter.

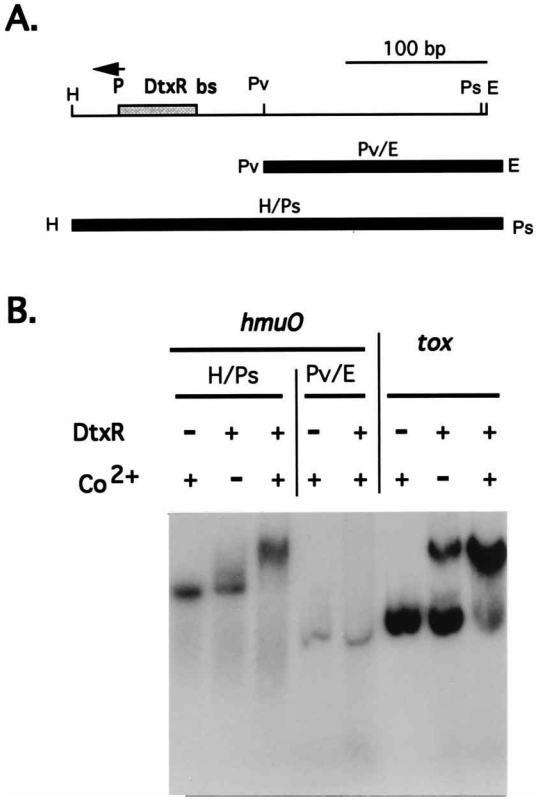

FIG. 2. (A) Partial restriction map of a 350-bp region upstream of the *hmuO* gene. This fragment was derived from plasmid pAKS293 and shows the approximate location of a putative promoter site (P) and the DtxR binding site (DtxR bs). Below the map are two fragments used in the gel shift assays: a 160-bp *Pvu*I-*Eco*RI fragment (Pv/E) and the full-length *Hin*dIII-*Pst*I fragment (H/Ps). E, *Eco*RI; H, *Hin*dIII; Ps, *Pst*I; Pv, *Pvu*I. The *Eco*RI, *Hin*dIII, and *Pst*I sites are present in the vector. (B) Gel mobility shift assays. DNA fragments were end labeled with [^{32}P]dATP and incubated in the presence (+) or absence (−) of DtxR and Co^{2+}. A 200-bp fragment carrying the *tox* promoter was used as a positive control. Experimental conditions are described in Materials and Methods.

The promoter activity from P*orf* in C7hm723, HC1, and HC1-2 was similar to that detected in *C. diphtheriae* C7, in which only low-level constitutive expression was detected (data not shown).

Gel mobility shift assays. The transcriptional fusion studies described in Tables 1 to 4 indicated that the promoters located upstream of the *hmuO* gene may be regulated by the DtxR protein and iron. An earlier report (39) identified a sequence that overlapped the putative *hmuO* promoter that shared homology with the consensus DtxR binding site (43). To determine if DtxR can bind to the region upstream of the *hmuO* gene, gel mobility shift assays were done with the DtxR protein and DNA fragments carrying the *hmuO* upstream region. Fig. 2A shows a partial restriction map of the region upstream of the *hmuO* gene and the approximate location of the *hmuO* promoter/operator sequence. Below the map are shown two DNA fragments used in the mobility shift assays: the full-length 350-bp *Hin*dIII-*Pst*I fragment and a right-end 160-bp *Pvu*I-*Eco*RI fragment. The results shown in Fig. 2B indicate that only the full-length *Hin*dIII-*Pst*I fragment exhibited slower mobility when incubated in the presence of DtxR and Co^{2+}. This result suggests that a DtxR binding site is present on the

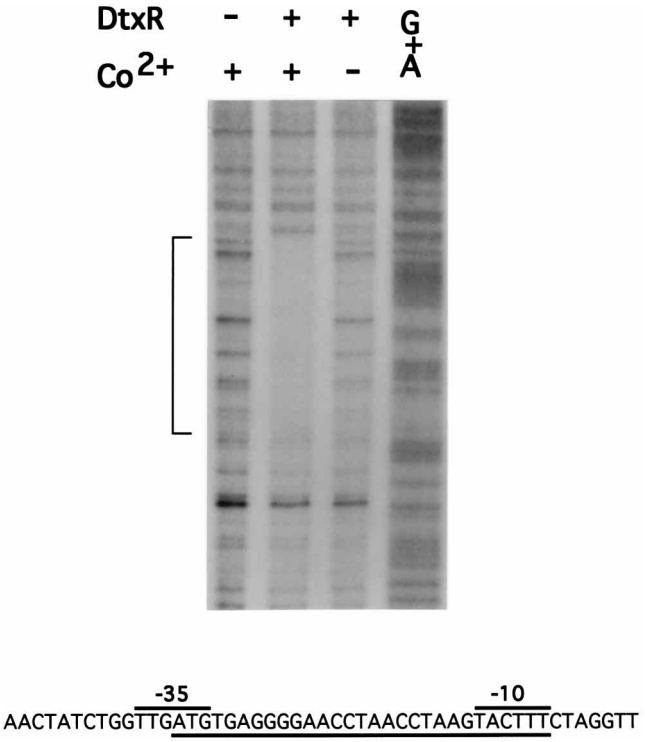

FIG. 3. DNase I protection experiments. The 350-bp *Hin*dIII-*Pst*I fragment used in the gel shift assays was end labeled at the *Hin*dIII site with [^{32}P]dATP and incubated in the presence (+) or absence (−) of DtxR and Co^{2+}. Experimental conditions are described in Materials and Methods. G+A, Maxam and Gilbert sequencing ladder (25). The DNA sequence of the *hmuO* promoter is shown below the gel, and the 29-bp region protected from DNase I digestion by DtxR is underlined. The bracket at the left of the gel also indicates the protected sequence.

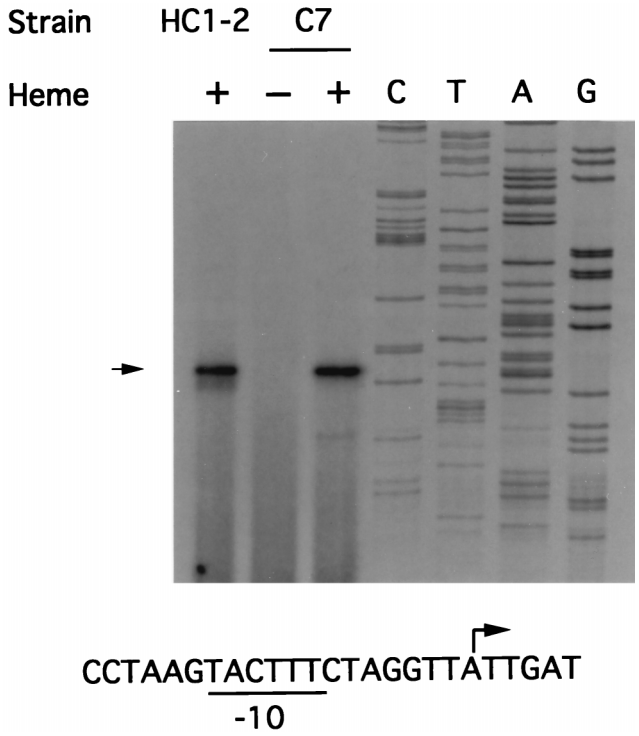

FIG. 4. Primer extension analysis. Total RNA was isolated from cultures of *C. diphtheriae* C7 and HC1-2 grown in low-iron medium in the presence (+) or absence (−) of heme. The arrow at left indicates the primer extension product. G, A, T, C, DNA sequencing reactions that were used as sizing standards (38). Shown below the gel is the sequence of the −10 region of the *hmuO* promoter, and the A residue marked with an arrow indicates the transcriptional start site.

left end of the 350-bp fragment which is in the same region that carries the putative *hmuO* promoter. A DNA fragment carrying the *tox* promoter/operator region was used as a control in these experiments (Fig. 2B).

DNase I footprinting. To identify the DtxR binding site upstream of the *hmuO* gene, DNase I footprinting was done with the 350-bp fragment described for the mobility shift assays. The results of this experiment are shown in Fig. 3 and reveal that a 29-bp region is fully protected from DNase I digestion in the presence of DtxR and Co^{2+}. This protected sequence contains the entire −10 region and a portion of the −35 region of the putative *hmuO* promoter (Fig. 3).

Primer extension analysis. Total cell RNA isolated from *C. diphtheriae* strains was used to map the transcriptional start site for the *hmuO* gene. RNA was isolated from strains grown in low-iron medium in the presence or absence of heme. In *C. diphtheriae* C7, a single strong primer extension product was seen only in RNA preparations that were isolated from strains grown in the presence of heme (Fig. 4). An RNA product with an identical size to that observed with C7 was also detected in strains HC1-2 (Fig. 4) and HC1 (data not shown). The migration of this DNA fragment in relation to the DNA sequence standard indicates that the start site of transcription is an A residue located 8 bp downstream from the predicted −10 sequence of the *hmuO* promoter (Fig. 4).

Northern blot analysis. RNA preparations that were used for the primer extension studies were also analyzed in Northern blot experiments. RNA was transferred to nitrocellulose and probed with a 600-bp *Stu*I DNA fragment that is located entirely within the *hmuO* coding region (39). The probe hybridized to a single mRNA fragment that migrated with a predicted size of approximately 800 nucleotides (nt) (Fig. 5). This finding suggests that the *hmuO* mRNA is monocistronic, since the minimal mRNA size that would be predicted to carry the complete *hmuO* coding region is 694 nt and no open

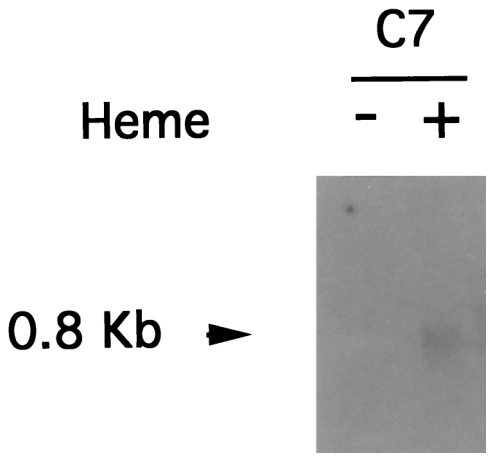

FIG. 5. Northern blot analysis. Total RNA was isolated from *C. diphtheriae* C7 grown in iron-depleted cultures in the presence (+) or absence (−) of added heme. The arrow indicates the 0.8-kb RNA product. RNA sizing standards (Bethesda Research Laboratories) were excised from the gel along with duplicate samples and were stained and photographed separately from the RNA that was used for hybridization (37).

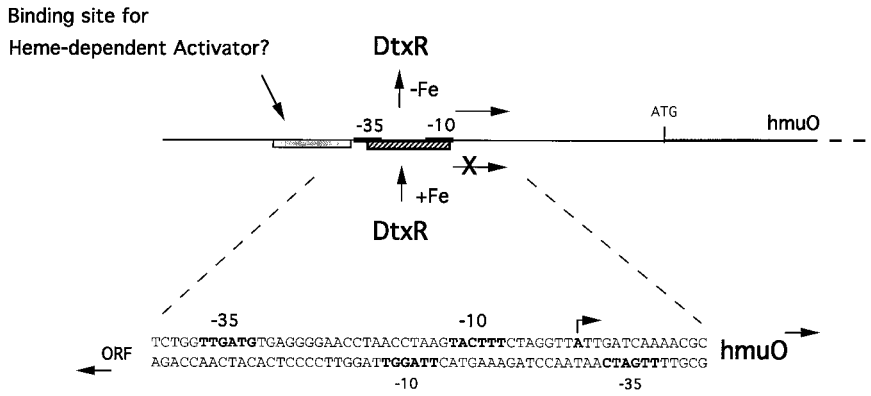

FIG. 6. Regulation of the *hmuO* promoter is under both positive and negative control. Transcription of the *hmuO* promoter is repressed by DtxR and iron in high-iron environments. Binding to the *hmuO* promoter by DtxR, which likely interferes with the ability of RNA polymerase to interact with the −10 and −35 sequences, is thought to directly block transcription. Positive control of the *hmuO* promoter is dependent on the presence of heme or a heme source such as hemoglobin. The *hmuO* promoter, which contains an unusually large 20-bp gap between the −10 and −35 sequences, is poorly expressed in the absence of heme, and like other promoters with abnormal sequences or structure, it may require an activator protein for optimal expression. An ORF located upstream of the *hmuO* gene is presumably transcribed from a weakly expressed promoter (P*orf*) that overlaps the *hmuO* −10 sequence.

reading frames are found immediately downstream of *hmuO* (39a). The 800-nt band was only detected in the presence of heme, which provides additional evidence that *hmuO* transcription is activated by heme.

DISCUSSION

The findings from this study indicate that the *hmuO* gene is the newest member of the DtxR regulon and appears to exhibit regulatory features not found in the other DtxR-regulated genes in *C. diphtheriae*. The *hmuO* promoter was expressed poorly in *E. coli*, while a divergently transcribed promoter (P*orf*), which directs the transcription of an ORF upstream of *hmuO*, showed moderate expression and was regulated by iron and DtxR in *E. coli*. All DtxR-regulated genes examined prior to this study have shown good expression in *E. coli* and were regulated by iron and the cloned *dtxR* gene (43). A possible reason for the low level of expression of the *hmuO* promoter in *E. coli* may be due to its poor homology to *E. coli* sigma 70 promoters. The *hmuO* promoter has a relatively good match with the consensus −10 and −35 sequences of sigma 70 promoters, maintaining 4 of 6 residues in the −35 sequence (*hmuO*, **TTG**Atg; sigma 70, **TTG**ACA and 3 of 6 residues in the −10 sequence (*hmuO*, **TA**ctt**T**; sigma 70, **TA**TAA**T**). (The most highly conserved residues in the consensus sigma 70 promoter are indicated in bold.) However, there exists a 20-bp gap between the −10 and −35 sequences in the *hmuO* promoter (Fig. 6), which is unusual for sigma 70 promoters and is frequently associated with poor expression in *E. coli* (15). Other reasons for the low expression of the *hmuO* gene in *E. coli* include the lack of intracellular heme (assuming heme is required for optimal expression in *E. coli* as it is in *C. diphtheriae*) and the possible requirement for an activator protein for the expression of *hmuO*. The P*orf* promoter has about 50% homology to the −10 and −35 sigma 70 consensus sequences and contains a more typical 17-bp gap between these regions (Fig. 6), which may account for the higher levels of expression seen with this promoter in *E. coli*.

In *C. diphtheriae* C7, the *hmuO* promoter was not expressed efficiently in either high- or low-iron medium, and significant transcription from *hmuO* was only detected when a heme source was added to the medium. Activation by heme could be partially repressed in iron-replete conditions. Iron repression in the presence of heme was abolished in the *dtxR* mutant C7hm723, suggesting that iron repression is due to the DtxR protein and that heme activation must be caused by a factor other than DtxR. In HC1-2, transcription of the *hmuO* gene in the presence of heme was enhanced three- to fourfold relative to the transcription of *hmuO* in its parent strain HC1 (Table 4). This increased expression from the *hmuO* promoter in HC1-2 may be a result of higher levels of intracellular heme. Since HC1-2 is presumed to carry a defective HmuO protein, an enzyme involved in the degradation of intracellular heme, the absence of a functional HmuO protein may result in increased intracellular heme levels, which cause the increased activation of the *hmuO* promoter.

Only low levels of expression from P*orf* were detected in the *C. diphtheriae* strains under the various conditions examined, suggesting that either environmental factors other than heme or iron are required for optimal expression from this promoter or P*orf* is expressed at a low constitutive level. The ORF upstream of *hmuO*, which is predicted to encode a protein of 8,624 Da, has no significant amino acid sequence homology to proteins in the GenBank or SwissProt databases, and no function for this gene has been determined (39, 39a).

In Fig. 6, a model is presented describing a possible mechanism by which the *hmuO* promoter is regulated in *C. diphtheriae*. The transcriptional fusion studies suggest that in high-iron conditions, DtxR and iron repress expression of the *hmuO* promoter. DNA binding studies confirmed that DtxR binds in vitro to sequences which overlap the *hmuO* promoter, which provides further evidence that transcription of *hmuO* is regulated by DtxR and iron. In low-iron conditions, optimal expression of the *hmuO* promoter in the wild-type C7 strain requires heme, and while the mechanism of this heme induction is not known, it may require a *trans*-acting activator protein that acts either directly through heme, with heme serving as a coactivator, or indirectly, whereby the presence of heme results in the activation of regulatory factors which subsequently induce transcription of the *hmuO* promoter. Additional support for the presence of an activator includes the abnormal structure of the *hmuO* promoter (an unusually large 20-bp gap between the −10 and −35 sequences) and the very poor expression of this promoter in *C. diphtheriae* in the absence of heme. Interestingly, the inducible forms of heme oxygenase that are present in eukaryotes are also activated at the transcriptional level by heme (55).

Since it is known that high concentrations of heme result in the oxidation of macromolecules (52), it is possible that the heme activation seen at the *hmuO* promoter may be due to an oxidative stress response. However, hydrogen peroxide (a known inducer of proteins involved in the oxidative stress response in bacteria) at concentrations as high as 5 mM had no effect on the expression of the *hmuO* promoter in low-iron conditions (data not shown).

In this report, transcription of the *hmuO* gene was shown to be activated by heme and hemoglobin and repressed by DtxR and iron. These findings confirm that *hmuO* is a member of the DtxR regulon and that *hmuO* is the first described example of an iron-repressible gene in bacteria in which expression is also activated by heme.

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Editor: V. A. Fischetti