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Characterization of the Oxygen-Dependent Promoter of the *Vitreoscilla* Hemoglobin Gene in *Escherichia coli*

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The gene coding for the *Vitreoscilla* hemoglobin (VHb) molecule has been cloned and functionally expressed in *Escherichia coli*. By using a plasmid-encoded gene as well as single-copy integrants, the oxygen-dependent VHb gene (*VHb*) promoter was shown to be functional in *E. coli*. The promoter was maximally induced under microaerobic conditions (dissolved oxygen levels of less than 2% air saturation). Direct analysis of mRNA levels as well as the use of gene fusions with *lacZ* showed that oxygen-dependent regulation occurred at the level of transcription. Transcriptional activity decreased substantially under anaerobic conditions, suggesting the presence of a regulatory mechanism that is maximally induced under hypoxic but not completely anaerobic conditions in *E. coli*. Primer extension analysis was used to identify the existence of two overlapping promoters within a 150-base-pair region upstream of the structural *VHb* gene. The oxygen-dependent activity of both promoters was qualitatively similar, suggesting the existence of a common mechanism by which available oxygen concentrations influence expression from the two promoters. Analysis of promoter activity in *crp* and *cya* mutants showed that both cyclic AMP and catabolite activator protein were required for full activity of the promoter. The *VHb* promoter contained a region of significant homology to the catabolite activator protein-binding site near the *E. coli lac* promoter.

The response of bacteria at the level of gene regulation to profound environmental changes is a well-characterized phenomenon. In some of the better understood examples, such as changes in temperature, osmolarity, and nitrogen availability, this response involves sensing of the change by the cell and transmission of this information to a regulatory protein, which in turn affects the expression of a multigene system (37). Another important environmental parameter for both obligate and facultative aerobes is oxygen availability. In *Escherichia coli* and *Salmonella typhimurium*, several genes have been shown to be differentially regulated by varying dissolved oxygen (DO) levels and, in the extreme case, by switching from aerobic to anaerobic conditions or vice versa (8, 13, 18, 20, 21, 23, 30, 45). Multiple loci have been implicated in the regulation of these genes (1, 10, 18, 19, 21, 39, 45, 51). As would be expected, mutations in many of these regulatory loci are pleiotropic (1, 18, 21, 39). Likewise, the expression of some oxygen-dependent genes appears to be affected in more than one of these mutants (19, 21, 45), suggesting that these regulatory loci may function as components in one or more control cascades. Interestingly, many genes that are influenced by oxygen availability, such as those coding for some respiratory enzymes, are also influenced by other effectors. This has led to the proposal of the term "modulon" to describe a set of regulons that are subject to a common control mechanism (18). Oxygen-dependent control mechanisms revealed in some of the above studies include positive regulation by an activator protein (23), negative regulation by a repressor (19), and modulation of DNA superhelicity (10, 51).

Recently, we reported the discovery of an unusual oxygen-regulated genetic switch that appears to be controlled by a well-conserved mechanism in obligate as well as facultative gram-negative aerobic bacteria. The bacterium *Vitreoscilla* sp. is an obligate aerobe from the Beggiatoa family that synthesizes a hemoglobinlike molecule (VHb) in response to growth in oxygen-poor environments (4, 46). We have cloned and sequenced the gene (*VHb*) coding for this protein and demonstrated functional expression of the protein in *E. coli* from its native promoter (24). With this expression system on a multicopy vector, hemoglobin polypeptide levels as high as 15% of total cellular protein are readily obtainable in *E. coli* under low oxygen conditions (24; Khosla and Bailey, unpublished observations). Under well-sparged conditions, however, hemoglobin levels were observed to decline sharply, leading to the hypothesis that the oxygen-dependent regulatory element(s) that controls expression in *Vitreoscilla* sp. is also recognized in *E. coli* (25).

The purposes of the present study were to characterize the response of the promoter to changes in oxygen availability in the environment and to obtain initial insights about the mechanism(s) by which the promoter is controlled.

MATERIALS AND METHODS

Bacteria, bacteriophage, and plasmids. The bacteria, bacteriophage, and plasmids used in this work are listed in Table 1.

Media and growth conditions. A buffered complex medium containing (in grams per liter) Bacto-tryptone (Difco Laboratories), 10; yeast extract, 5; NaCl, 5; K_2HPO_4, 5; and KH_2PO_4, 1 (pH 7), was used to grow cells. Culture tube and shake-flask growth were conducted in a New Brunswick G24 shaker incubator operating at medium setting. Batch fermentations were performed in a New Brunswick Bioflo II 2.5-liter fermentor under pH and temperature control. All growth was at 37°C. Plasmid-containing cells were grown in the presence of ampicillin (100 mg/liter) except in the fermentor (to minimize foaming problems).

Construction of single-copy integrants of the *VHb* gene and its native promoter in *E. coli*. Plasmid pINT2 was constructed by subcloning the *Hin*dIII-*Bam*HI fragment containing the *VHb* gene into corresponding sites in pBR322. With the intention of constructing a transposable element, a

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TABLE 1. Bacteria, bacteriophage, and plasmids

Strain, phage, or plasmid	Description	Origin or reference
E. coli		
MG1655	*E. coli* K-12	Cold Spring Harbor Laboratory
JM101	*supE thi* Δ(*lac-proAB*) (F′ *traD36 proAB lacI*^{q} *ZΔM15*)	52
HB101	F$^-$ *hsdS20 recA13 ara-1 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1 supE44* λ$^-$	35
GRO21	MG1655 *lac*::Tn*10*d (*VHb-kan*)	This work
GRO22	MG1655 *xyl*::Tn*10*d (*VHb-kan*)	This work
MC1061	*araD139* Δ(*ara-leu*)*7697* Δ*lacX74 galU galK* r$^-$ m$^+$ *strA*	7
MC4100	*araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB5301 ptsF25 deoC1*	6
GE1050	MC4100 Δ*crp cam*	G. E. Weinstock
GE1051	MC4100 Δ*cya-854 ilv*::Tn*10*	G. E. Weinstock
Bacteriophage P1 *vir*		44
Plasmids		
pRED4	pUC19 *VHb*	24
pOX1	pBR322 Δ*tet VHb*	This work
pINT2	pBR322 Δ*tet VHb*	This work
pINT4	pBR322 Δ*tet VHb kan*	This work
pMM12	pBR322 Δ*tet* Tn*10*d(*cam*) *tac*′-′IS*10*$_R$*tnp*	Cold Spring Harbor Laboratory
pINT6	pBR322 Δ*tet* Tn*10*d (*VHb-kan*) *tac*′-′IS*10*$_R$*tnp*	This work
pMS421	pSC101 Spc^r *lacI*^{q}	Cold Spring Harbor Laboratory
pRED503	pUC19 *VHb′*	This work
pRED509	pUC19 *VHb′*	This work
pYEJ001	pBR322 Δ*tet cam*	Pharmacia
pOX2	pBR322 Δ*tet VHb′-cam*	This work
pMLB1034	pBR322 Δ*tet ′lacZ lacY′*	3
pMLB1010	pBR322 Δ*tet* Φ(*trpA-lacZ*) (Hyb) *lac Y′*	3
pGE245	Φ(*recA-lacZ*)(Hyb) M13 IG region	48
pCO1	pBR322 Δ*tet ′lacZ lacY′* M13 IG region	This work
pCO2	pBR322 Δ*tet* Φ(*trpA-lacZ*) (Hyb) *lacY′* M13 IG region	This work
pOX11	pCO1 Φ(*VHb-lacZ*)(Hyb)	This work
pOX21	pCO2 Φ(*VHb-lacZ*)	This work

kanamycin resistance gene cartridge (Pharmacia) was inserted into the *Sal*I site downstream of the *VHb* gene on pINT2 to create pINT4. An *Eco*RI fragment containing both genes was inserted between the transposon Tn*10* inverted repeats on pMM12, replacing the original Cam^r gene. The resulting plasmid, pINT6, was transformed into MG1655 (pMS421). This strain was grown in the presence of 0.5 mM IPTG (isopropylthiogalactopyranoside) to induce transposition. Cells were plated on lactose-MacConkey and xylose-MacConkey plates to select for events that had inactivated the *lac* and *xyl* operons, respectively. The resulting mutations were transduced into MG1655 by using P1 phage. Two mutants, GRO21 and GRO22, were checked for the following phenotypes: Lac$^-$ Xyl$^+$ Kan^r Amp^s Spc^s, and Lac$^+$ Xyl$^-$ Kan^r Amp^s Spc^s, respectively (Amp and Spc are the plasmid markers). Although the assumption that the two strains contain single copies of the *VHb* gene was not confirmed by Southern hybridization, it is unlikely that both strains would contain more than one defective transposon, Tn*10*d(*VHb-kan*). Parallel investigations on two such independent strains eliminates the possibility of positional effects of chromosomal integration.

DNA manipulations. In vitro deletions were constructed by using the exonuclease Bal31 (35) as well as exonuclease III and S1 nuclease (17). For all other DNA manipulations, standard protocols were used (35).

DNA sequencing. The endpoints of the deletions constructed as described above were determined by dideoxy sequencing of pUC19-derived plasmids into which the deletions were cloned. For this purpose, the Sequenase kit (U.S. Biochemicals) and a modified protocol (28) were used. Both the universal and the reverse primers for pUC vectors were used (U.S. Biochemicals). The standard sequencing reactions used in primer extension assays were performed with a custom-made oligonucleotide primer (see Results section).

Preparation and analysis of RNA. Cells (1.5 ml) to be analyzed for mRNA content were centrifuged for 1 min in an Eppendorf centrifuge and suspended in 0.3 ml of 1.4% sodium dodecyl sulfate (SDS)–4 mM EDTA preheated to 65°C. Following further incubation at 65°C for 5 to 10 min, the samples were chilled and 0.15 ml of saturated NaCl was added. The cell debris was spun out, and nucleic acids were precipitated from the supernatant with ethanol and suspended in dH_2O containing Inhibit-ACE (5 Prime-3 Prime Inc.). The integrity of the RNA preparation was checked by observing intact rRNA bands in a 1% formaldehyde–agarose gel that was stained with ethidium bromide. All aqueous solutions involved were pretreated with diethyl pyrocarbonate.

Quantitation of hemoglobin mRNA content. Northern (RNA) blot analysis of hemoglobin mRNA was done by recommended protocols (Du Pont Co. NEN Products, catalog no. NEF-976, 1985). Plasmid pRED4, labeled by the random hexanucleotide priming technique (New England Nuclear), was used as the probe DNA. Hemoglobin mRNA content was quantitated by the method of Kornblum et al. (27). Counts thus obtained were normalized with plasmid DNA counts in order to obtain a measure of gene activity.

Primer extension analysis. Primer extension assays were done by the protocol of Kingston (26). Total cellular RNA, prepared by the method described above, was used for this purpose. In each case, the position of the transcription start point was determined by running sequencing reactions that used the same oligonucleotide as the sequencing primer.

Construction of *lacZ* operon and protein fusions. In order to construct *lacZ* fusions with the *VHb* promoter which would be useful for further genetic studies, derivatives of pMLB1010 and pMLB1034 which contained the IG region from phage M13 were created. pGE245 is an example of such a plasmid which contains a *recA-lacZ* gene fusion. The entire *recA* fragment was deleted from this vector by digestion with *Bam*HI followed by religation and identification of a white colony on indicator X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) plates. This resulted in plasmid pCO1, which contains the IG region and is suitable for making protein fusions with *lacZ*. An analogous vector, pCO2, for making operon fusions was constructed by replacing the *Bam*HI-*Sst*I fragment in pCO1, which contains most of the

truncated *lacZ* gene, with the corresponding fragment from pMLB1010, which includes a *trpA-lacZ* fusion. To construct *VHb-lacZ* fusions, two characterized deletions, pRED503 and pRED509 (see above as well as the Results section), were used. The protein fusion plasmid pOX11 was created by *Bam*HI digestion and mung bean nuclease treatment of pCO1, followed by ligation with pRED503 that had been digested with *Hin*dIII and *Xba*I and treated with mung bean nuclease. This resulted in an in-frame fusion which was identified by transforming MC1061 with the ligation mixture and picking blue colonies. The recombinant plasmid was checked by restriction mapping. To construct operon fusion plasmid pOX21, plasmid pCO2 was digested with *Sma*I and *Bam*HI, gel purified, and ligated with a mixture of two fragments produced by digestion of pRED509 with *Hin*dIII (end-filled with Klenow) and *Bam*HI. Resulting transformants in MC1061 were screened for the presence of a correctly oriented insert by restriction mapping.

Construction of a *VHb-cam* fusion. The vector pYEJ001 contains a promoterless *cam* gene fragment flanked by *Hin*dIII sites. Plasmid pRED509 was digested at the unique *Xba*I site downstream of the *VHb* promoter. The resulting ends were converted into *Hin*dIII cohesive ends with adaptor DNA (New England BioLabs). The vector was then ligated with the *cam* insert, resulting in a loss of both *Hin*dIII and *Xba*I sites. However, a new *Xmn*I site was introduced between the *VHb* promoter and the structural *cam* gene. The entire fusion was then isolated by digestion with *Hin*dIII and *Bam*HI and cloned into the corresponding sites of pBR322, giving rise to plasmid pOX2.

Hemoglobin activity determination. Hemoglobin activity was determined by the following method. Approximately 20 ml of cells was added to 10 ml of ice-cold 1% NaCl containing 400 μg of chloramphenicol per ml in order to inhibit further translation. Later, the cells were centrifuged, suspended in assay buffer (100 mM Tris chloride [pH 7.5], 50 mM NaCl), and sonicated. The soluble fraction was assayed for hemoglobin content by the CO difference spectrum method (47). Total soluble protein content was assayed by the Bradford method (BioRad Laboratories). Intracellular hemoglobin concentrations are reported as $\Delta A_{419-436}$/mg of total soluble protein.

Western immunoblotting of hemoglobin protein. Hemoglobin expressed in *E. coli* was purified from cell extracts (R. Hart and J. E. Bailey, manuscript in preparation). This preparation was used to generate rabbit anti-globin antiserum (Cocalico Biologicals). The globin content in samples of total cell protein was assayed by standard protocols (50).

Enzyme assays. β-Galactosidase assays were done by a slight modification of the Miller protocol (36). An appropriate volume of cells was diluted into 2 ml of Z-buffer plus β-mercaptol. Cells were lysed by adding 100 μl of chloroform and 50 μl of 0.1% SDS and vortexing well. The cell debris was removed by microfuging, and 1.5 ml of supernatant was equilibrated at 30°C prior to the assay. For the assay, 0.15 ml of 4-mg/ml ONPG (*o*-nitrophenyl-β-D-galactopyranoside; preequilibrated at 30°C) was added as the substrate, and the change in A_{420} was followed by a rate assay in a temperature-controlled spectrophotometer (Shimadzu UV 260). It has been observed that monitoring the time course of the change in absorbance improves the accuracy of the assay and also increases the dynamic range. Since the cell debris was spun down prior to the assay, measurement of A_{550} was unnecessary (36). Specific activity is expressed in Miller units (36).

Cells analyzed for chloramphenicol acetyltransferase (CAT) activity were disrupted by sonication. The soluble fraction was assayed with ^{14}C-labeled butyryl coenzyme A (New England Nuclear) according to recommended protocols (38). CAT activity is expressed in units of CAT per milligram of total soluble protein. Standard CAT (Sigma) was used to calibrate the assay.

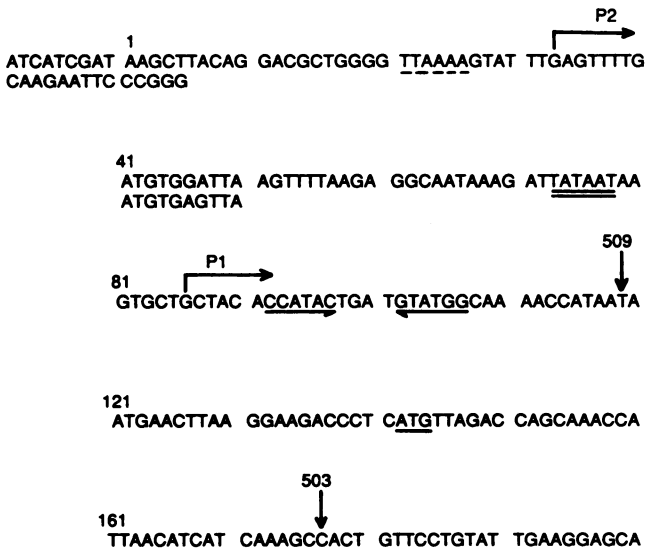

FIG. 1. Primary structure of the oxygen-regulated promoter element. The nucleotide sequence of the upstream region required for regulated expression of the *VHb* gene in *E. coli* is shown. The start codon of the structural gene is underlined. An inverted repeat element is shown with arrows. Bases marked 503 and 509 correspond to the 3′ end of the truncated promoter in plasmids pRED503 and pRED509, respectively. The nucleotide designated 1 corresponds to the 5′ end of the *VHb* gene-containing fragment isolated from a *Vitreoscilla* genomic library (24). The sequence upstream of this base represents adjacent pBR322 DNA on plasmid pOX1. The sequence of the junction between the 5′ end of the promoter fragment and vector DNA in plasmid pOX11 is shown immediately below this region. The origins of arrows designated P1 and P2 correspond to the transcriptional start sites of the two promoters. The putative Pribnow boxes of P1 and P2 are indicated with a double line and a dashed line, respectively. The sequence indicated below nucleotides 41 to 50 is a portion of the CAP-binding site upstream of the *lac* promoter, which was previously identified by footprinting studies (14). For details, see Results section.

RESULTS

Primary structure of the *VHb* upstream region. The nucleotide sequence of the promoter region of the hemoglobin gene is shown in Fig. 1. Deletion analysis of the region 3′ to the structural gene showed that the sequences downstream of the putative transcriptional terminator (24) were unessential for oxygen-dependent regulation (data not shown). This was corroborated by the results of the gene fusion experiments discussed below. Analysis of the sequence of the upstream region revealed a consensus Pribnow box and a ribosome-binding site (24), as well as a perfect inverted repeat element downstream of the Pribnow box (Fig. 1). Interestingly, despite the high strength of this promoter, it apparently lacked a consensus −35 region. However, approximately 20 base pairs (bp) upstream of the Pribnow box was a region of strong homology with the catabolite activator protein (CAP)-binding site near the *lac* promoter (14) (Fig. 1). This led to the hypothesis that a functional cAMP-CAP

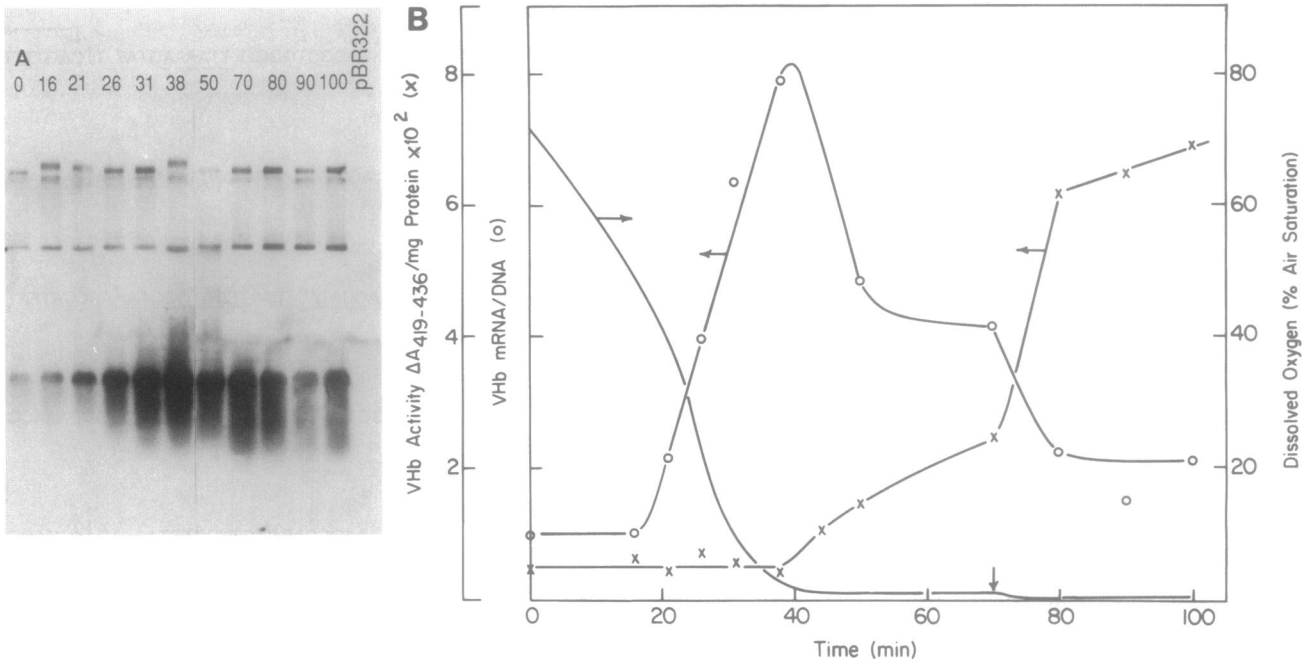

FIG. 2. Oxygen-dependent regulation of the *Vitreoscilla* hemoglobin gene in *E. coli*. HB101(pOX1), grown in a fermentor (see Materials and Methods), was subjected to a transient decrease in DO concentration (see Results), and hemoglobin mRNA and protein activity were monitored. (A) Northern blot analysis of hemoglobin mRNA content. The numbers above the lanes indicate the times (minutes) at which the samples were taken. The initial point corresponds to the sample taken before commencing the transient decrease in DO. RNA from HB101(pBR322) was included as a control in the blot. The higher-molecular-weight bands correspond to plasmid DNA. (B) Normalized intracellular RNA content (○) and protein activity (×) (see Materials and Methods) plotted against time. The DO trajectory is also shown (solid line). The vertical arrow on this curve corresponds to the point at which anaerobic conditions were introduced by sparging nitrogen into the fermentor. Since the experiment was not continued beyond 100 min, the significance of the decrease in the *VHb* mRNA level at 90 min (see panel A) is unclear.

complex is required to induce the promoter to its normal level of activity. This hypothesis was investigated.

Oxygen-dependent synthesis of hemoglobin mRNA and protein. Plasmid pOX1 was constructed by inserting the *Hin*dIII-*Sph*I fragment containing the hemoglobin gene from pRED4 into corresponding restriction sites in pBR322. In order to determine the response of the promoter to varying DO levels, strain HB101(pOX1) was grown to mid-log phase under well-aerated conditions (DO, greater than 60% air saturation) and then subjected to a transient (initially approximately linear) decrease in DO level. The duration of this transient decrease (ca. 40 min) was greater than the characteristic time (ca. 5 min) for response of mRNA levels in *E. coli*. During such a relatively slow change in DO, one will observe at each time a *VHb* mRNA level which corresponds approximately to the steady-state value for that instantaneous DO concentration. Thus, by using a slow ramp-shaped transient change instead of a step change, the DO level at which induction occurs can be observed. More importantly, one can determine approximately the DO value at which induction is maximum.

Regulation of the hemoglobin gene was transcriptional (Fig. 2). When present on a multicopy plasmid, the gene appeared to be induced when the DO fell below 40% air saturation; however, this value may be different when only a single gene copy is present. Nevertheless, maximal gene expression occurred at DO levels of about or below 2% air saturation (i.e., below the lower limit of sensitivity of the galvanic DO probe used to make these measurements). An interesting and potentially important observation is that the level of transcription decreased as the oxygen availability was reduced still further and dropped to almost the uninduced level when strict anaerobic conditions were obtained by sparging nitrogen into the fermentor. This result was checked by growing the cells in sealed bottles in deaerated medium that was preequilibrated with nitrogen. Cells thus grown were neither red (indicating the presence of functional hemoglobin) nor contained inclusion bodies. Reddish cells containing inclusion bodies are characteristic of cells grown under hypoxic but not quite anaerobic conditions, such as in a culture tube (25).

Figure 2 illustrates a lag in active hemoglobin synthesis relative to message synthesis. This could be due to the requirement of elevated heme biosynthesis, or it could indicate the presence of an additional regulatory mechanism(s) operating at the posttranscriptional level (see below).

Two potential sources of artifacts may have contributed to the above results. First, the experiment was conducted with the gene present on a multicopy plasmid. This was necessary to obtain globin activity that could be quantitated with reasonable accuracy in a moderately sized sample. However, possible effects due to changes in copy number or plasmid DNA superhelicity cannot be ignored. Second, the relatively short time scale of the transient change in dissolved oxygen concentration may have precluded some physiologically relevant event from occurring in the cell that could affect promoter response (e.g., de novo synthesis of a cascade of regulatory proteins). Both these issues were qualitatively addressed by constructing two single-copy integrant *E. coli* strains containing the globin gene under the regulation of its native promoter. The construction of two

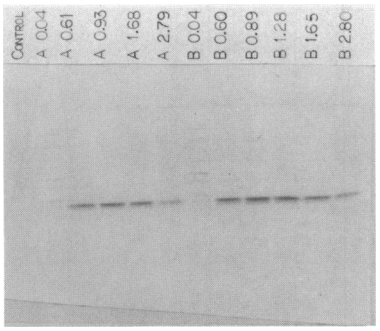

FIG. 3. Oxygen-dependent synthesis of globin in *E. coli* cells containing a single copy of the *VHb* gene. Western blot analysis of total cellular protein is shown. *E. coli* strains MG1655 (control), GRO21 (lanes A), and GRO22 (lanes B) were grown in 250-ml shake flasks containing 100 ml of medium. Samples were taken at different points along the growth curve. The number above each lane represents the OD_{600} of the sample. Each lane contains total cell protein that corresponds to 5 OD_{600} units per μl of cells. Thus, the relative intensities of the globin bands are indicative of the specific globin concentration in the samples. The faint bands that appear above the major band (VHb) are the result of cross-reaction of the antiserum with other antigens. This is probably due to the presence of trace quantities of contaminant proteins in the purified VHb sample that was used to raise the antiserum.

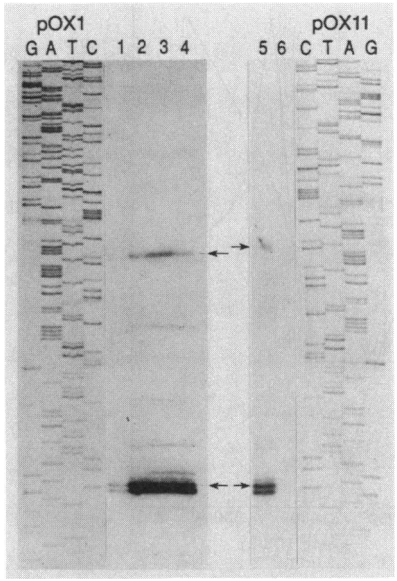

FIG. 4. Mapping the transcriptional start sites within the oxygen-dependent promoter element. The sequence ladders labeled pOX1 and pOX11 were generated by running the sequencing reaction products of the two plasmids with the same 30-mer oligonucleotide primer (see text). Lanes 1 to 4 contain the primer extension products from RNA templates in the 21-, 38-, 70-, and 100-min samples in Fig. 2A, respectively. Lanes 5 and 6 contain products from RNA templates in MC1061(pOX11) and MC1061(pCO1) (control), respectively. These total RNA samples were prepared from cells grown in culture tubes (5 ml) up to an OD_{600} of 0.8. The two main reaction products in the above samples are indicated by arrows.

such strains, GRO21 and GRO22, is described in the Materials and Methods section. Examination of two such independent strains helped eliminate the possibility of positional effects of chromosome integration of a foreign gene in *E. coli*. GRO21 and GRO22 were grown in shake flask cultures, wherein the available oxygen concentration would be expected to decline monotonically with time (as the cell density increases) throughout the growth phase of the experiment. Figure 3 shows the specific VHb concentration of cell samples as a function of increasing cell density (i.e., decreasing DO). VHb was virtually absent from early-exponential-phase cells that were well aerated. In both cases, intracellular VHb concentrations increased in response to oxygen limitation (as evidenced by a decrease in specific growth rate) and eventually decreased as the cell density increased beyond a certain point. Hence, even though the results in Fig. 3 do not provide any evidence for the level at which oxygen-dependent control occurs, they corroborate the conclusions drawn from the experiment with the *VHb* gene on a multicopy plasmid.

Mapping the transcriptional start site(s) of the promoter. Nucleotide sequencing of the promoter element revealed a putative Pribnow box that matched the consensus sequence (see above). However, the Northern blot in Fig. 2A suggested the possible existence of a second (weaker) promoter located somewhere upstream of the major promoter element. To identify the promoter element(s) that is used in vivo, the 5′ end(s) of *VHb* mRNA was mapped by primer extension analysis. For this purpose, a few representative samples of total mRNA from the experimental described in the legend to Fig. 2 and the previous section were used. These corresponded to different oxygen concentrations. A 30-mer oligonucleotide probe corresponding to bases 142 to 171 (Fig. 1) was hybridized to the RNA preparations. The same oligonucleotide was used as a primer in standard sequencing reactions with pOX1 as the template (Fig. 4). The products of the primer extension reaction are shown in Fig. 4 (lanes 1 to 4). The major product was a polynucleotide that terminated at position 86 (Fig. 1). A second, less intense band corresponding to position 33 (Fig. 1) was also observed. Some minor products were also observed which may be the result of premature termination of transcription by reverse transcriptase caused by the secondary structure of the mRNA in this region. Since DNA-RNA hybridization in primer extension analysis is carried out under conditions of excess oligonucleotide, the amount of extended product observed is indicative of the amount of *VHb* mRNA in the sample. Thus, both the promoters were induced similarly in response to oxygen limitation. This also agrees with the coarse resolution obtained from the Northern blot (Fig. 2A) and therefore suggests that oxygen-dependent expression from the two promoters is controlled by the same mechanism. The major (downstream) transcriptional start site was located at the expected position relative to the consensus Pribnow box; this promoter was designated P1 (Fig. 1), and its structure is discussed in an earlier section. Located 6 bp upstream of the weaker promoter (designated P2, Fig. 1) was the sequence TTAAAA, which may serve as a −10 region. However, since this hexamer was located only 20 bp downstream of the *Hin*dIII site, which corresponds to the 5′ end of the globin gene fragment isolated from a *Vitreoscilla* sp. genomic library (24) (numbered 1 in Fig. 1), the physiological significance of this promoter may be questionable.

To address this issue, the primer extension assay was repeated with total mRNA samples from MC1061(pOX11) and the same oligonucleotide primer. Plasmid pOX11 contains a translational fusion between the *VHb* gene and *lacZ* (see Materials and Methods section and below). As shown in Fig. 1, the nucleotide sequence upstream of the TTAAAA site was significantly different in this plasmid. The results in Fig. 4 (lanes 5 and 6) indicate that not only was the activity

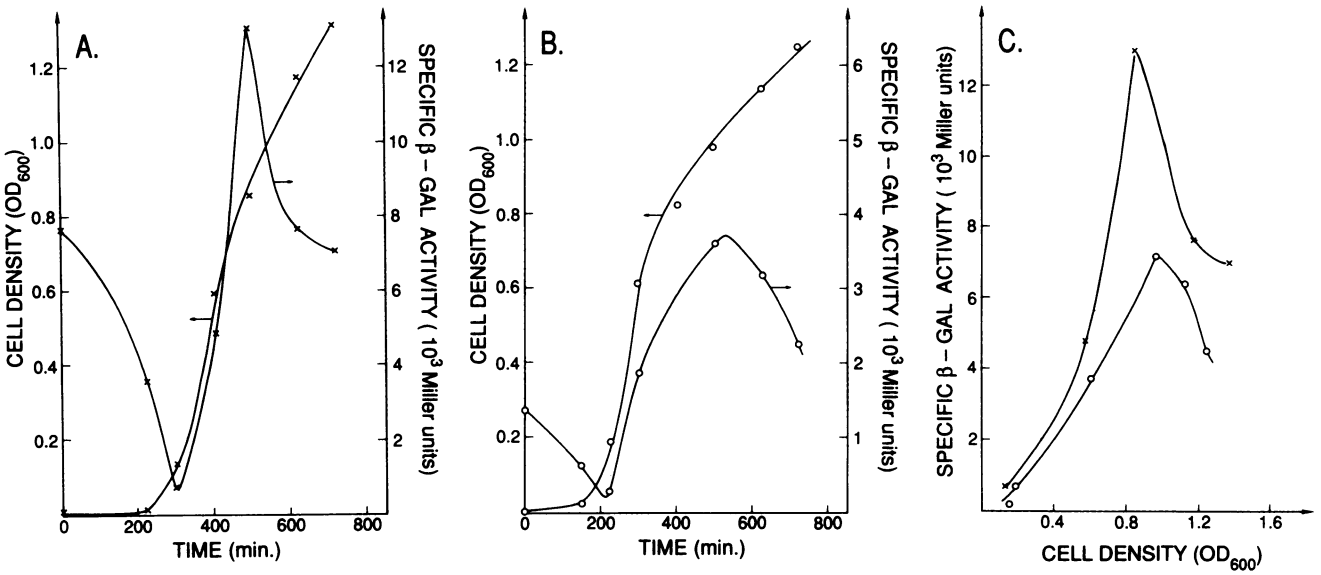

FIG. 5. Oxygen-dependent expression of β-galactosidase from *VHb-lacZ* fusions. Shake flasks (250 ml) containing 100 ml of liquid volume were inoculated with 0.1% overnight cultures, and the specific activity of β-galactosidase (β-GAL) was monitored through the growth phase. Cell density and specific activity are shown as a function of time for (A) MC1061(pOX11) (×) and (B) MC1061(pOX21) (○). The data from panels A and B are represented in panel C with the specific activity shown directly as a function of cell density. In this figure, the specific activity of MC1061(pOX21) is shown on double scale.

of P2 retained, but the strength of P2 relative to that of P1 remained similar. This suggests that the relevant domains involved in recognition of P2 by RNA polymerase are likely to be present on the *Vitreoscilla* genomic fragment. However, conclusive evidence with regard to this must await further genetic and biochemical experiments. Furthermore, the possibility that additional regulatory loci exist within the flanking sequence in the *Vitreoscilla* genome remains to be investigated.

Characterization of promoter function with *lacZ* fusions. In order to identify the regions in the upstream sequence that are important for oxygen-dependent regulatory activity and to study the promoter function via gene fusions, 5′ and 3′ deletion mapping experiments were conducted. The unique *Hin*dIII site at the 5′ end and a unique *Bsu*36I site approximately 150 bp into the structural gene were used to generate 5′ and 3′ deletions, respectively. Several 3′ deletions were isolated; however, the promoter region was extremely resistant to generating viable 5′ deletions by both of the in vitro techniques that were tried. The smallest deletion that was isolated mapped 2 bp upstream of the start codon. Whether this is an artifact of the deletion techniques used or is due to the potential lethality of an improperly regulated strong promoter is as yet unknown. The deletion endpoints of two 3′ deletions, pRED503 and pRED509, are shown in Fig. 1. These were used to create translational and transcriptional fusions, respectively, for further genetic analysis.

To facilitate further genetic analysis of the promoter function, a *lacZ* protein fusion, pOX11, and a *lac* operon fusion, pOX21, were constructed by using the promoter deletions in pRED503 and pRED509, respectively. The construction of these plasmids is described in the Materials and Methods section. The plasmids were transformed into MC1061. To test for oxygen dependence of *VHb-lacZ* expression in the two strains, the specific activity of β-galactosidase was monitored over the time course of shake flask cultivations (Fig. 5A and B). Since oxygen levels can be assumed to decrease monotonically over the course of growth, the specific activity can be alternatively plotted as a function of cell density for the two strains grown under identical conditions (Fig. 5C). This provides a basis for evaluating the oxygen dependence of the promoter in a qualitative sense without making DO measurements. As can be seen in Fig. 5c, both deletions retained the central features of oxygen-dependent expression: the promoter was induced in response to oxygen limitation, and there was an optimum oxygen concentration (somewhere in the microaerobic region) beyond which the β-galactosidase activity fell. Furthermore, since the response of both fusions to oxygen availability was similar, one can rule out the possibility of any oxygen-dependent control at the posttranscriptional level.

Effect of catabolite repression on promoter activity. Sequence homology had implicated a possible involvement of CAP in the overall control of this promoter (see above). This is consistent with other observations that the presence of glucose reduced the amount of globin activity in *E. coli* cells. To address this question, plasmid pOX11 was transformed into MC4100, GE1050 (*crp*), and GE1051 (*cya*). Promoter activity was substantially reduced in strains that were unable to synthesize CAP or cAMP (Fig. 6). Furthermore, at comparable cell densities in a culture tube, specific β-galactosidase activity in GE1051 was 10-fold higher in the presence of cAMP. [At an OD_{600} of 0.8 to 0.85, the specific activity of β-galactosidase in GE1051(pOX11) was 500 Miller units in the presence of 4 mM cAMP and 50 Miller units in the absence of cAMP.] Together, these results suggest that the cAMP-CAP complex is involved in modulating the activity of this promoter either directly or indirectly.

Strategy for oxygen-dependent induction of gene expression. The *VHb* promoter offers a convenient (and economical) method for the heterologous expression of foreign genes in *E. coli*, particularly for larger scales of growth. High levels of expression of the *cam* gene product from plasmid pOX2

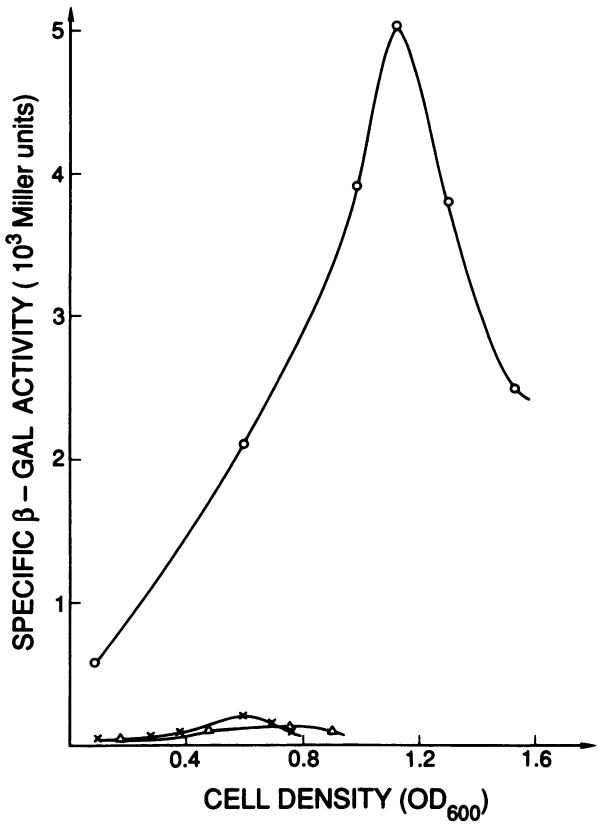

FIG. 6. Effect of cAMP-CAP on the activity of the *VHb* promoter. Shake flasks (250 ml) containing 100 ml of liquid volume were inoculated with 0.1% overnight cultures of MC4100(pOX11) (○), GE1050(pOX11) (△), and GE1051(pOX11) (×). The specific activity of β-galactosidase (β-GAL) was monitored along the growth phase. As in Fig. 5C, specific activity is directly plotted as a function of cell density.

(described in the Materials and Methods section) could be induced in a well-sparged fermentor by merely reducing the oxygen supply to the fermentor at the time of induction (Fig. 7). These results are in agreement with the above observations, which suggest that microaerobic conditions are required to achieve maximum activity at the promoter. In a log-phase culture, this state is achieved merely by allowing surface aeration.

DISCUSSION

Studies on regulatory responses to varying oxygen availability in diverse systems raise two key questions. First, what mechanisms are responsible for sensing changes in oxygen availability in the environment, and how is this information transduced to the regulatory elements that control gene expression? For example, the presence of abundant oxygen in the environment prevents the expression of nitrogen fixation genes in *Klebsiella pneumoniae* (a facultative aerobe) (2), *Rhizobium meliloti* (an obligate aerobe) (11), and *Bradyrhizobium japonicum* (an obligate aerobe) (12). However, the mechanism of oxygen influence is different in these bacteria. In *K. pneumoniae*, the oxygen status affects the supercoiling of the *nifLA* promoter (10) and also causes NifL to modulate the activity of the NifA activator protein (2). In *R. meliloti*, microaerobicity induces the expression of *nifA* (11), probably by influencing the activity of a transmembrane sensor-transcriptional activator couple (9). In *B. japonicum*, the presence of oxygen irreversibly inactivates the NifA activator protein, which contains a potential redox-sensitive metal-binding center (29). In the yeast *Saccharomyces cerevisiae*, at least two loci have been identified that are involved in the regulation of oxygen-dependent genes. Heme functions as an effector molecule in both these regulatory systems (33, 49). In the case of *HAP1*, which activates the expression of the *CYC1* and *CYC7* genes, heme mediates induction by counteracting the masking of the DNA-binding domain of the protein (40). Indirect evidence has also suggested that heme or a heme protein is involved in activation of erythropoetin in a hepatoma cell line in response to hypoxic conditions (15).

The second area of central questions concerns whether all these regulatory circuits respond in a monotonic fashion to increasing or decreasing DO levels. Do there exist mechanisms that, for example, are maximally induced under microaerobic conditions versus anaerobic or highly aerobic conditions? On biochemical grounds, the presence of different oxygen-sensitive sensors as discussed above might

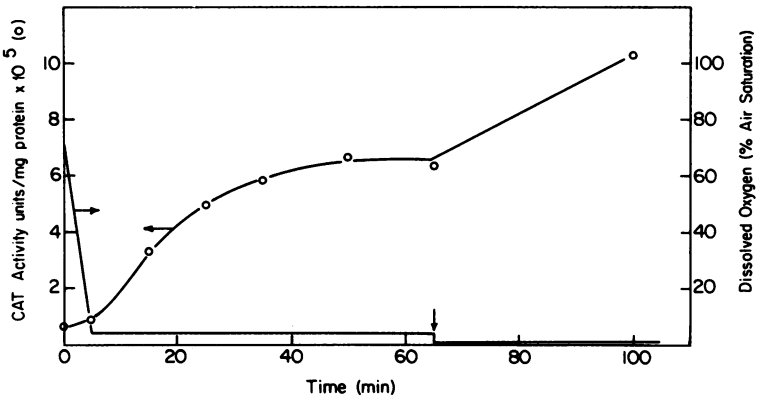

FIG. 7. Oxygen-dependent expression of CAT. HB101(pOX2) was grown in a fermentor under conditions described in the Materials and Methods section. At time zero, the air flow rate was decreased in order to reduce the DO level to the range of 2 to 5% air saturation very rapidly. This condition was maintained until steady state was reached with respect to CAT activity. Thereafter, the sparging system was completely turned off (indicated by the vertical arrow), allowing oxygen uptake to occur only at the liquid surface. Another assay was conducted after allowing sufficient time for attaining a new steady state with greater CAT activity.

argue for the existence of a variety of induction patterns. For example, optimal induction of gene expression under microaerobic conditions has been demonstrated in the case of NifA-mediated activation of the nitrogenase promoter in *R. meliloti* (11).

The main conclusion that can be drawn from this study is that the regulation of the *Vitreoscilla* hemoglobin promoter in *E. coli* is transcriptional. Gene expression attains a maximum under microaerobic conditions (DO less than 2% air saturation) and is substantially reduced under strict anaerobic conditions. This suggests that facultative aerobes like *E. coli* may possess multiple regulatory mechanisms which respond to changes in oxygen availability and that different mechanisms may have different maxima. For example, enzymes such as superoxide dismutase are required under high oxygen concentrations (22), whereas several proteins are expressed as part of the *fnr*-activated regulon under strictly anaerobic conditions (8, 23). In addition, there also exist mechanisms that activate proteins such as cytochrome *d* in response to decreasing oxygen concentrations (13). Whether these regulatory circuits share any common intracellular sensor or transducer molecules remains unknown.

Several studies of gene activation (and chemotaxis) in procaryotes in response to environmental changes have led to the emergence of a common theme (9, 42). Typically, a transmembrane protein detects an environmental change and transduces the signal to modulate the activity of a transcriptional activator. In the case of gene activation in response to decreasing oxygen availability, the inability of the cell membrane to function as an effective barrier against oxygen diffusion obviates the need for the sensing protein or metabolite to be membrane anchored. It might be noteworthy, however, that the receptor for aerotaxis in *Salmonella typhimurium* is cytochrome *o* (31). It is not known whether the same protein is involved, directly or indirectly, in gene regulation. More recently, two loci have been identified that mediate repression of enzymes of aerobic pathways (e.g., certain tricarboxylic acid cycle and aerobic respiratory enzymes) in *E. coli* (18, 19). It has been suggested that the products of these two genes form a sensor-regulator couple. Should this be the case, it is conceivable that one or both of these products are also involved in controlling genes that are expressed in the opposite way.

The functional regulatory activity of the *VHb* promoter in *E. coli* suggests that this mechanism of regulation is fairly well conserved among both obligate and facultative aerobes. In another study, constitutive expression of the *B. japonicum nifA* regulatory gene in *E. coli* activated a *nifA*-dependent *B. japonicum* promoter under microaerobic conditions (12). Among other possibilities, this suggested that both *B. japonicum* and *E. coli* possess a well-conserved mechanism for sensing oxygen changes in the environment and in turn altering the activity of regulatory proteins. The results of this study imply that both the sensing and regulatory mechanisms involved in this control circuit are conserved in a variety of gram-negative bacteria. Hence, genetic dissection of this promoter in *E. coli* might facilitate a similar investigation in other bacteria in which a homologous mechanism exists, particularly those for which genetic techniques are not as well developed (e.g., bacteria of the Beggiatoa family).

The presence of two promoters upstream of the *VHb* gene has interesting implications. It suggests that multiple factors may be involved in controlling the overall level of globin expression. For example, P1, which is the stronger promoter, may be influenced by catabolite repression. Thus, in the presence of a preferred carbon source, oxygen-limiting conditions could lead to globin expression primarily from P2. It is also possible that P2 is activated under conditions which lead to the presence of a different form of RNA polymerase holoenzyme. Isolation of *cis* and *trans* mutations that affect promoter function in *E. coli* may be helpful in addressing such questions. Since P1 and P2 respond to oxygen changes similarly, they are likely to be under the control of a common mechanism. Given the arrangement of the two promoters, a negative regulatory mechanism is a likely candidate.

The observation of a lag in the synthesis of active VHb protein suggests that the availability of free heme may be a limiting factor. Feedback regulation of heme biosynthesis by an intermediate metabolite has been suggested recently (32). In this case, the lag could imply the presence of a regulatory mechanism preventing translation in the absence of heme or improper folding of the nascent polypeptide, leading to eventual degradation. It could also indicate the existence of a metastable folded state of the protein that is capable of incorporating heme, when available, to form active VHb.

The regulatory role of the cAMP-CAP complex has been well studied in the context of catabolism of preferred carbon sources (34). Addition of cAMP to cells growing in the presence of glucose has also been shown to elevate the synthesis of some tricarboxylic acid enzymes (18) and cytochromes (5), as well as increase the efficiency of oxidative phosphorylation (16). Furthermore, heme biosynthesis is also under the control of this mechanism (41). Hence, although it is shown in this work that both CAP and cAMP are required for the activity of the *VHb* promoter, it is not clear whether this is a direct or indirect effect. The presence of a region homologous to the CAP-binding site of the *lac* promoter located around the −35 region of the major promoter argues for direct interaction between CAP and the promoter. However, alternative possibilities cannot be ruled out. For example, heme may be a component of an activator molecule in the overall regulatory circuit. If this is the case, heme levels decreased by the absence of active CAP could account for lower activity of the *VHb* promoter. However, should *E. coli* CAP interact directly with the *VHb* promoter, investigation of an analogous activity in *Vitreoscilla* sp. may be of interest.

The isolated promoter can be used to express high levels of a cloned gene product in an oxygen-dependent manner. Since high-cell-density batch cultures naturally progress toward an oxygen-limited regimen of growth, this promoter provides an attractive alternative for heterologous expression of genes in *E. coli* without the requirement for a gratuitous inducer or temperature shifts which may have deleterious side effects (43).

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