Study of *Vitreoscilla* globin (*vgb*) gene expression and promoter activity in *E. coli* through transcriptional fusion

Kanak L.Dikshit, Rajendra P.Dikshit and Dale A.Webster

Department of Biology, Illinois Institute of Technology, Chicago, IL 60616, USA

Received April 13, 1990; Revised and Accepted May 29, 1990

ABSTRACT

Bacterial hemoglobin (VtHb) is produced by the gram-negative bacterium, *Vitreoscilla*, in large quantity in response to hypoxic environmental conditions. The *vgb* gene coding for VtHb has been cloned in *E. coli* where it is expressed strongly by its natural promoter. The expression of the *vgb* gene in *Vitreoscilla* is transcriptionally regulated by oxygen. When *E. coli* cells were shifted from 20% to 5% oxygen, *vgb* specific transcript increased. In *E. coli* cells with plasmids carrying transcriptional fusions of the *vgb* gene promoter to either CAT (chloramphenicol acetyl transferase) or *xylE* (catechol-2,3-dioxygenase) genes, the promoter activity depended on the oxygen level. The concentration of CAT and *xylE* gene products in cells grown under 5% oxygen was 5–7 times that of aerobically (20% oxygen) grown cells. When the *vgb* gene promoter was deleted, VtHb was not produced under any conditions. When the promoter was replaced by the *E. coli* *tac* promoter, hypoxic oxygen did not affect the level of expression of *vgb*, but adding IPTG did increase the expression of this gene. These results indicate that the *vgb* gene promoter is transcriptionally regulated by oxygen even in *E. coli*, and that microaerobiosis is sufficient to induce *vgb* expression. The size of S1 nuclease-resistant hybrids, prepared using RNA transcripts protected with restriction enzyme fragments containing the promoter proximal region of *vgb*, was the same for both *Vitreoscilla* and *E. coli*, further evidence that the same promoter is used in both organisms. Transcriptional fusion of the *vgb* gene promoter to the *xylE* reporter gene on the broad host range plasmid, pKD-49, was used to demonstrate that the *vgb* promoter can be expressed in other gram-negative organisms, including *Pseudomonas*, *Azotobacter*, and *Rhizobium*.

INTRODUCTION

Vitreoscilla hemoglobin (VtHb), is the only hemoglobin of prokaryotic origin that is well characterized with regards to its structural and functional properties (1,2,3). It has been speculated, based on its oxygen binding kinetics (1), that the function of VtHb is to facilitate oxygen flux to vigorously respiring membranes of *Vitreoscilla*, which is an obligate aerobe usually found in hypoxic habitats. This assumption is also based on the fact that

the cellular level of heme in *Vitreoscilla* increases many fold when cells are exposed to hypoxic conditions (4,5).

The gene (*vgb*) responsible for VtHb production has been cloned in *E. coli* along with its natural promoter (3,6). A single copy of the *vgb* gene on the *Vitreoscilla* genome was identified after Southern blot analysis, and an approximately 500 base long RNA transcript specific for *vgb* was detected in *Vitreoscilla* after Northern hybridization (3). The nucleotide sequence of the coding region (6) of *vgb* matches the previously reported primary protein sequence of VtHb (2) and exhibits some sequence similarity with leghemoglobin genes (6). However, the non-coding upstream regulatory region is remarkably similar to *E. coli* promoters (7). The regulation of expression of *vgb* has been shown to be at the level of transcription (8,9). The cellular level of the enzymes, delta-aminolevulinic acid synthetase (ALAS) and methemoglobin (methHb) reductase, necessary for structural and functional integrity of VtHb, also increased in hypoxic conditions (8). Since the *vgb* gene is expressed at a high level in *E. coli* under its own promoter, we further characterized regulation of this gene in response to oxygen by monitoring its activity through CAT and *xylE* reporter genes after transcriptional fusion.

MATERIALS AND METHODS

Strains and Growth conditions

E. coli strains HB101 and JM83 and plasmid pUC8:16 (3) were used in this study. Growth conditions and maintenance of different oxygen levels have been described previously (3,8). When minimal medium was needed, M9CA (10) was used. Glycerol (0.2%) and potassium nitrate (0.5%) were added for anaerobic growth of *E. coli*. All restriction and DNA modifying enzymes were obtained from Bethesda Research Laboratories (Gaithersburg, MD) or New England Biolabs Inc. (Beverly, MA). Plasmid pDR 540 and promoter probe vector pKK232-8 (11) were obtained from Pharmacia, Piscataway, NJ, and pVDX18 (12) was a generous gift from Dr. Vojo Deretic.

Plasmid construction

The strategy for constructing reporter plasmids with CAT (chloramphenicol acetyl transferase) and *xylE* (catechol 2,3-dioxygenase) transcriptional fusion is shown in Fig. 2. Fusion with CAT gene was created by ligating the HindIII-MluI/Klenow fill-in fragment of plasmid pUC8:16 (Fig. 2B, this fragment is approximately 500 bp including about 180 bp upstream of the

structural gene) at the *Sma*I site of promoter probe vector pKK232-8 which contains the promoterless CAT gene preceding the multiple cloning site. The resulting plasmid pKD-10 (Fig. 2) contains the *vgb* promoter and two-thirds of the *vgb* coding region. Orientation of the insert with respect to the CAT gene was checked by *Afl*III digestion which has a unique site within the non-coding region of the *vgb*. Plasmid pKD-14, where only the *vgb* promoter region was directly coupled with CAT gene, was derived from pKD-10 by removing an *Afl*III-*Mlu*I restriction fragment and self ligation of the plasmid after filling the ends with the Klenow fragment of DNA polymerase. Both plasmid constructs provide chloramphenicol resistance to the host cells.

Plasmid pUC8:17 carrying the promoterless *vgb* gene was constructed by removing the *Hind*III-*Afl*III fragment from plasmid pUC8:16 (Fig. 2B) after treatment with these enzymes. The restriction fragments were separated on low melting agarose gel (1.1%) and the larger *Hind*III-*Afl*III fragment was isolated, purified through a Nick Pack column (BRL), and self-ligated with T4 DNA ligase after filling the ends with Klenow DNA polymerase.

Plasmids pDR 540:16 and pDR 540:17 which carry the *vgb* gene with its native promoter and the *E. coli* *tac* promoter, respectively, were constructed according to the scheme shown in Fig. 4, I. The *Hind*III-*Bam*HI fragment of plasmid pUC8:16, which contains the *vgb* gene along with its promoter, was ligated at the *Hind*III-*Bam*HI site of plasmid pDR 540 to produce pDR 540:16. In the control plasmid, pDR 540:17, the *vgb* promoter was replaced with the *E. coli* *tac* promoter by cloning the *Afl*III-*Sal*I fragment of pUC8:16 at the *Bam*HI site of pDR 540 after filling in the ends with Klenow DNA polymerase followed by blunt end ligation. Colonies expressing the *vgb* gene under control of the *tac* promoter were identified on plates supplemented with 5 mM IPTG on the basis of their color difference due to high expression of VtHb. The presence and orientation of the insert was checked by restriction enzyme analysis. *E. coli* JM 103 carrying the above plasmid constructs, i.e., pDR 540 (control), pDR 540:16, and pDR 540:17 were used to prepare RNA for comparative *vgb* transcript analysis after Northern blotting as described previously (8).

Another transcriptional fusion was created on the broad host range plasmid pVDX18 to assess *vgb* promoter activity in organisms other than *E. coli*. For that, the *Hind*III-*Afl*III fragment of pUC8:16 (*Afl*III end made blunt with the Klenow fragment of DNA polymerase) was ligated at the *Hind*III-*Eco*RI (*Eco*RI end made blunt) site of pVDX18, which keeps the *vgb* promoter in the right orientation with respect to the promoterless *xylE* gene. Clones that contained an insert which promoted transcription in the direction of the *xylE* gene were detected by the yellow color of the colonies which appeared after spraying with a solution of 100 mM catechol in 50 mM potassium phosphate buffer (pH 7.5).

S1 protection analysis of *vgb* transcripts

This was performed using a radioactive DNA probe containing the 5' promoter proximal region and part of the *vgb* coding region. To prepare the labelled probe, plasmid pUC8:16 was treated with *Mlu*I and the 5' end was labelled with polynucleotide kinase and [³²P]ATP, after removing the 5' terminal phosphate by treatment with calf intestine phosphatase, essentially as described by Maniatis et. al (10). The labelled fragments were separated on a 2% low melting agarose gel, and the *Hind*III-*Mlu*I fragment was isolated and purified through a Nick Pack column (BRL). Approximately 200 µg of RNA from each strain (i.e.,

Fig. 1. *vgb* gene expression and hemoglobin synthesis in response to oxygen levels. I. *E. coli* JM83 with plasmids pUC8 or pUC8:16 was grown in LB containing 100 µg/ml Ampicillin at 37°C under different oxygen levels. DO level was maintained by bubbling a mixture of air and nitrogen through the medium. Samples were withdrawn with a sterile syringe at different intervals and absorbance was measured at 600 nm. With pUC8 at 20% (●) and 5% (▲) oxygen levels and with pUC8:16 at 20% (○) and 5% (△) oxygen. II. CO-difference spectra of *E. coli* JM83 carrying plasmid pUC8:16 under different oxygen levels. Cells were suspended in 0.02 M phosphate buffer (7.2) at a final concentration of 20 mg/ml (wet wt) and CO-difference spectra were recorded after bubbling CO into the sample cuvette. A. Base line B. 20% oxygen and C. 5% oxygen. III. Relative level of *vgb* specific transcript in *E. coli* grown under 20% and 5% oxygen levels which were maintained throughout growth by bubbling continuously a mixture of air and nitrogen (8). Amount of total RNA blotted: A. 5 µg, B. 10 µg, C. 20 µg, D. 30 µg. The relative intensities of the blots measured with a densitometer for the 5% oxygen level were 1.00, 1.72, 2.34, and 3.09, respectively, and for the 20% oxygen were 0.12, 0.18, 0.33, and 0.25, respectively.

Vitreoscilla, and *E. coli* carrying plasmids pUC8:16 and pUC8:17) was mixed with the labelled DNA probe (20,000 cpm), denatured at 80°C for 25 min and allowed to anneal at 52°C for 6 h according to the procedures described in references 10 and 13. The DNA-RNA hybrids were then treated with 200 units of S1 nuclease (Pharmacia, NJ) and the protected fragments were analyzed on a 4% denaturing polyacrylamide urea (7 M) gel.

Isolation of RNA and dot blot analysis

Detailed procedures for RNA isolation and *vgb* specific transcript analysis have been described previously (8). Films were scanned with a Model EC910 Densitometer from E-C Apparatus Corp.

Enzymes assay

Promoter activity in cells harbouring the reporter plasmids was assayed by measuring the enzyme activity in cell free extracts.

Fig. 2. A. Detailed map of *vgb*-CAT and *vgb*-*xylE* fusion plasmids. Recombinant plasmids, pKD-14 and pKD-49 were created by fusing the HindIII-AflII fragment of pUC8:16 (shown in Fig. 2. B) at the SmaI site of pKK232-8 and the HindIII-EcoRI site of pVDX18, respectively. Details are given in Materials and Methods. Plasmid pKD-49 is a non-self transmissible RSF 1010 derivative and can be mobilized using helper plasmid pRK 2013 (32). B. Map of plasmid pUC8:16 showing cloned portion of the *Vitreoscilla* genome containing the *vgb* gene. Sites for restriction enzymes are denoted by A, AflII; B, BamHI; E, EcoRI; H, HindIII; K, KpnI; M, MluI; P, PstI; and S, SalI. Designations for genetic markers are ApR, ampicillin resistance; *xylE*, gene encoding catechol 2,3 dioxygenase; CAT, gene encoding chloramphenicol acetyl transferase; *vgb*, gene coding for globin part of *Vitreoscilla* hemoglobin; oriV, origin of replication; nic, relaxation nick site; mob, determinant essential for plasmid mobilization. The double arrows, $\longleftrightarrow$, indicate the portions of *vgb* that were cloned on the respective promoter probe vectors.

The cells were collected by centrifugation, washed in Tris-Cl buffer (pH 7.8) containing 30 μ M DTT, and sonicated for approximately two min (10 sec. pulses, with at least 15 sec incubation on ice between the pulses). Cell debris was removed after centrifugation and the supernatant was used for the enzyme assays. CAT activity was measured spectroscopically by the method of Shaw (14). Briefly, 40 μ l of crude lysate was added to 1.18 ml of CAT substrate (147S0008T) and equilibrated at 37°C for 30 min. The background rate of acetylation was determined by monitoring the change at 412 nm for 30 min. A 24 μ l aliquot of chloramphenicol (5 mM) was added and the reaction monitored for 3 min. Specific activity of enzyme was calculated as $A_{412}/0.0136/\text{mg protein}$. The activity of CDO (catechol 2,3-dioxygenase), the *xylE* gene product, was measured essentially as described by Konyecsni and Deretic (12). Protein concentrations were determined by the method of Bradford (15). Detailed methods for determination of heme and hemoglobin have been described previously (3).

RESULTS

Effect of oxygen on *vgb* gene expression and hemoglobin production in *E. coli*

Oxygen regulates the expression of the *vgb* gene and hemoglobin production in *Vitreoscilla*. To evaluate the role of oxygen and the mechanism by which it regulates VtHb production, we studied

expression of the *vgb* gene in *E. coli* in response to different oxygen levels. *E. coli* JM83 with recombinant plasmid pUC8:16 was grown at 20% and 5% oxygen and the amount of *vgb* specific RNA transcript was measured. Both VtHb mRNA and the protein itself were significantly more abundant in cells grown at 5% oxygen. Only a small amount of *vgb* specific transcript was detectable in cells grown at atmospheric oxygen (Fig. 1 II, III). The growth responses of cells containing plasmid pUC8 with and without the *vgb* gene under the above conditions were compared: the growth rate of cells with plasmid pUC8 alone was lower than with cells containing pUC8:16 with *vgb* present (Fig 1, I). These results confirm that the synthesis of hemoglobin in *E. coli* is regulated in response to oxygen as it is in *Vitreoscilla* (8) and that its presence is beneficial for these cells when oxygen is limited.

Coupling of *vgb* promoter with CAT and *xylE* reporter genes

Fig. 2 shows the construction of different transcriptional fusions which were created by putting promoterless CAT and *xylE* genes, which are present on promoter probe vectors pKK232-8 and pVDX18, respectively, under the control of the *vgb* promoter. Two constructs, pKD-10 and pKD-14 (Fig. 2 A, B) show different response in terms of chloramphenicol resistance, pKD-14 being more resistant (up to 300 μ g/ml chloramphenicol) than pKD-10 (100 μ g/ml); the latter contains two-thirds of the *vgb* gene in addition to the *vgb* promoter and the RBS (ribosome binding site). This may interfere because of transcriptional

polarity with efficient translation of the CAT gene from the second RBS site present on plasmid pKK232-8 (11) since pKD-14, which has a different attachment of the *vgb* promoter region, strongly expressed CAT. This plasmid is constructed from the HindIII-AflII fragment of pUC8:16, in which the AflII site is within the RBS site of *vgb*. When this fragment was cloned on pVDX18 in the proper orientation, the *xylE* gene was strongly expressed. The *xylE* fusion was specially created to assess the *vgb* promoter activity in organisms other than *E. coli* because the presence of this reporter gene is easily detectable by the yellow color of colonies on spraying plates with a solution of catechol.

Expression of CAT and *xylE* genes in response to growth phase and oxygen level

The observed increase in *vgb* specific transcript in both *Vitreoscilla* (8) and *E. coli* (Fig. 1, III) harboring pUC8:16 when oxygen was limited suggested that the transcriptional activity of the *vgb* promoter may be regulated by oxygen. To test this hypothesis, plasmids pKD-14 and pKD-49 having transcriptional fusion with CAT and *xylE* genes, respectively, were used to monitor and compare *vgb* promoter activity under various conditions. The specific activities of the protein products of these two genes increased sharply after mid-exponential phase in aerobically growing cells, reaching maximum values at a cell density of $A_{600} = 1.2$, and remaining fairly stable thereafter (Fig. 3). The specific activities were 5–8 times higher in cells grown under limited oxygen (5%). Our results also indicate that *vgb* promoter activity was induced earlier in the exponential phase of cells grown under low oxygen compared to cells grown at atmospheric oxygen (Fig. 3).

Fig. 3. Expression of *vgb*-CAT and *vgb*-*xylE* gene products in relation to growth phase and oxygen levels. *E. coli* JM83 with fusion plasmids pKD-14 or pKD-49 was grown in LB medium at 37°C under 20% and 5% oxygen. The oxygen levels were maintained by bubbling a mixture of air and nitrogen. Samples were removed from the culture periodically and specific enzyme activities measured. Specific activity of CAT under 20% (Δ) and 5% (▲) oxygen. CDO activity under 20% (○) and 5% (●) oxygen.

In cells grown under atmospheric oxygen the *vgb* promoter activity was not induced until stationary phase. To test whether microaerobiosis alone is sufficient to induce the *vgb* promoter, aerobically growing *E. coli* cells containing fusion plasmids were shifted to a different level of oxygen during early exponential phase (Table 1). The results indicated that *vgb* promoter activity is not dependent upon the growth phase and can be induced by oxygen limitation. Promoter activity was maximal at an oxygen level of 2%, 10–15 times higher than at an atmospheric level (Table 1).

Cells grown in limited oxygen have an increased level of *vgb* specific message (Fig. 1, III), higher hemoglobin content, and higher activity of reporter gene products (Table 1). These results indicate that oxygen has a repressive effect on the transcriptional activity of the *vgb* promoter which is relieved when oxygen falls below a certain level. To determine whether lowering oxygen below 2% would further increase expression of the *vgb* promoter, we grew *E. coli* JM83 harboring plasmids pUC8:16 and pKD-14 in the presence of different carbon sources in growth flasks flushed with nitrogen for 30 min and sealed (Table 2). Promoter activity under these conditions was higher when cells were grown in minimal media (containing 0.04% glucose and 0.2% casamino acids) supplemented with a carbon source, except additional glucose, than in the minimal medium alone and was maximum in the presence of glycerol. Glucose increased the growth rate but reduced the level of hemoglobin as well as reporter gene products. The *vgb* promoter activity was maximum in the presence of glycerol which does support the anaerobic growth of *E. coli* (Table 2). The repression of *vgb* expression by glucose in *E. coli* suggests the involvement of cyclic AMP (16,17).

Deletion and replacement of the *vgb* promoter and effects on *vgb* specific transcript

To ascertain whether the HindIII-AflII fragment of pUC8:16 contains the *vgb* promoter and whether the CAT and *xylE* fusions were utilizing this promoter and not some upstream vector sequences, we constructed plasmid pUC8:17 with the HindIII-AflII portion deleted. Cells transformed with pUC8:17 did not produce hemoglobin under any conditions tested, including oxygen limitation, indicating that this HindIII-AflII region is required for *vgb* expression. Additionally, when this fragment was inserted at the SmaI site of plasmid pKK232-8, CAT was expressed only when it was in the proper orientation and not in the reverse orientation, evidence that the CAT gene utilizes the *vgb* promoter for its expression.

Two plasmid constructs (Fig. 4, I) carrying the *vgb* gene with

Table 1. Expression of reporter proteins under control of the *vgb* promoter in response to oxygen shifts.

Oxygen level	CAT activity U/mg protein	CDO activity U/mg protein
20% (a)	16	14
20% (b)	44	68
5%	99	157
2%	198	240
Nitrogen (c)	191	241

E. coli JM83 harboring fusion plasmids were grown in LB medium at 37°C with vigorous shaking (250 rpm) for 3 h and shifted immediately to the specified oxygen level and maintained at that level as previously described (8). After 1 h incubation the amounts of reporter proteins were measured. a. 20% oxygen level was maintained by continuously bubbling air. b. Oxygen level was not maintained (standing culture). c. Culture was bubbled with nitrogen and sealed.

Table 2. Effect of carbon sources on expression of hemoglobin and CAT.

Carbon sources	Growth atmosphere	Total Hb nmol/g wet wt	CAT activity U/mg protein
Control	air	183	81
Succinate	N ₂	215	101
Lactate	N ₂	201	122
Glucose	N ₂	63	38
Glycerol	N ₂	297	202

E. coli JM83 cultures with plasmids pUC8:16 or pKD-14 were grown overnight in 100 ml minimal media (9) supplemented with nitrate and different carbon sources (0.2%) under the indicated growth atmosphere at 37°C. Oxygen was flushed by bubbling nitrogen into each 250 ml flask for 30 min through a sterilized 0.45 μ Millipore filter and the flasks sealed with a rubber stopper plus parafilm.

either its native promoter (pDR 540:16) or the *E. coli* tac promoter (pDR 540:17) were used to study the effect of oxygen on the expression of this gene. The level of expression was determined by using the *vgb* gene as a probe (the nick-translated HindIII-MluI fragment, Fig. 2 B) to measure the amount of *vgb* transcript after Northern blotting. At atmospheric oxygen (20%) *E. coli* JM103 carrying plasmid pDR 540:16 exhibited a relatively low level of *vgb* specific RNA in comparison to cells carrying plasmid pDR 540:17 (Fig. 4, II A and C). However, when exposed to hypoxia (2% oxygen) a large increase in *vgb* transcript was observed in cells carrying the former but not the latter plasmid (Fig. 4, II B and D). This result shows that expression of *vgb* is induced in response to oxygen limitation only when it is under the control of its natural promoter. When the *vgb* gene

Fig. 4. I. Construction of plasmids from pDR 540 containing the *vgb* gene under control of its native promoter and the *E. coli* tac promoter. A detailed description is given in Materials and Methods. Restriction enzyme sites are A, AflII; B, BamHI; E, EcoRI; H, HindIII; and S, SalI. S/B designates the junction of the SalI and BamHI sites after blunt end ligation. Genetic markers are designated Amp^R, ampicillin resistance; gal K, gene coding for galactokinase; *vgb*, gene coding for globin portion of *Vitreoscilla* hemoglobin; , promoter region of *vgb*; , *vgb* coding region; , tac promoter (ptac). II. Northern blot analysis of *vgb* transcript controlled by different promoters in response to oxygen. Total RNA was isolated as described previously (8) from *E. coli* JM103 carrying plasmids pDR 540, pDR 540:16, and pDR 540:17. 50 μ g of RNA from each sample was fractionated on a 1.2% agarose gel containing 2.2 M formaldehyde (9), immobilized on nitrocellulose paper, and hybridized with excess ³²P labelled HindIII-MluI fragment (Fig. 2, B), containing part of the *vgb* gene. The RNA was prepared from cells containing plasmids grown under 20% or 2% oxygen as follows (the number in parentheses represents the relative intensity of that blot):

A. pDR 540:16, 20% oxygen (1.00).
B. pDR 540:16, 2% oxygen (6.79).
C. pDR 540:17, 20% oxygen (3.46).

D. pDR 540:17, 2% oxygen (3.08).
E. pDR 540:17, 20% oxygen + 5 mM IPTG (3.88).
F. pDR 540 (control).

was cloned under control of the tac promoter it did not respond to hypoxia, but addition of IPTG, an inducer of the tac promoter, increased the level of *vgb* transcript (Fig. 4, II E). No *vgb* specific mRNA was detected in cells containing control plasmid pDR 540 (Fig. 4, II F).

S1 protection analysis of *vgb* transcripts

To determine whether transcription of *vgb* is initiated in both *Vitreoscilla* and *E. coli* using the same promoter we performed S1 mapping with *in vivo* RNA transcripts which protected restriction enzyme fragments containing the promoter-proximal region of the *vgb* gene. RNA was prepared from *Vitreoscilla* and *E. coli* JM83 carrying recombinant plasmid pUC8:16 during the stationary phase of growth and hybridized with a radioactive probe labelled at the 5' end of the MluI site within the *vgb* gene (Fig. 2 B). The size of the S1 nuclease resistant hybrids, analyzed on a denaturing polyacrylamide gel (Fig. 5) was the same for both *Vitreoscilla* and *E. coli* which shows that the same promoter is utilized in both organisms. An additional faint band observed in the case of *E. coli* (Fig. 5 C) may be a partial product of S1 digestion. The size of the untreated control probe (Fig. 5 D) was about 500 bp, and the size of the S1 nuclease-resistant fragments were approximately 380 bp. No protected DNA fragments were observed with RNA prepared from *E. coli* carrying plasmid pUC8:17 which lacks the *vgb* promoter (Fig. 5 A).

Expression of the *vgb* promoter in organisms other than *E. coli*

It has been observed that VtHb provides a growth advantage to cells under certain situations (Fig. 1, I, and reference 18). The transfer of the globin gene to other organisms, especially those which are industrially or agriculturally important, may yield commercial benefits. To test whether the *vgb* promoter is

expressed and regulated in other organisms besides *Vitreoscilla* and *E. coli*, we constructed promoter fusion vector pKD-49, which has the *vgb* promoter fused with *xylE* gene and contains a broad host range replicon. The promoter activity can be indirectly assessed by the rate and intensity of color development in colonies harboring this plasmid. Some gram-negative bacteria in which the level of *vgb* promoter expression has been measured are listed in Table 3 which shows that the *vgb* promoter is active in several other microorganisms.

DISCUSSION

In this study, we have analysed the regulation of the *Vitreoscilla* globin gene expression by characterizing its regulatory region. Experiments using transcriptional fusion and direct measurements have revealed the oxygen-responsive nature of the *vgb* promoter, although the mechanism involved in transducing the signals to the *vgb* promoter is not yet clear. We have assessed and compared the *vgb* promoter activity through two different transcriptional fusions in two different types of plasmids in *E. coli* and they showed similar responses in terms of the oxygen-dependent induction of the *vgb* promoter. It was shown previously that the biosynthesis of hemoglobin in *Vitreoscilla* is regulated at the level of transcription (8,9) and that the *vgb* promoter is efficiently recognised in *E. coli* (3,6). The present study indicates that it is regulated similarly in response to oxygen in both organisms. The *vgb* regulatory region when fused with reporter genes, encoding chloramphenicol acetyl transferase (CAT), or catechol 2–3 dioxygenase (CDO) in two different constructions, efficiently expressed these fusion gene products in *E. coli*, and production of both reporter gene products increased at oxygen levels below 5%. Other genetic loci which are regulated by oxygen in prokaryotes are positively regulated by *fnr* or *oxyR* gene products (19,20,21), but the *vgb* gene promoter sequence (6) does not show any protein binding domain for these oxygen induced positive regulators. Cytochrome *d* of *E. coli*, which like VtHb is synthesized in response to low oxygen, also shows transcriptional regulation and does not require the *fnr* gene product (22). Similarly, in *Rhizobium meliloti* expression of the *nifA* gene is induced maximally under microaerobic conditions (23). The molecular mechanisms by which the free oxygen concentration is sensed by these promoters is unknown.

One recent model for the general control of gene expression for aerobic and anaerobic metabolism postulates that a change in DNA superhelicity leads to repression or derepression of transcription. Yamamoto and Droffner (24) suggest that the transition from an aerobic to an anaerobic environment results

Fig. 5. S1 nuclease protection analysis of *vgb* transcripts. RNAs isolated from *Vitreoscilla* and *E. coli* carrying pUC8:16 and pUC8:17 were used to protect the 5'-end labelled HindIII-MluI fragment (Fig. 2, B), containing the *vgb* promoter and part of the coding sequences as described in Materials and Methods. A 123 bp DNA ladder (BRL) was used as the size standard. The S1 protected fragment from A, pUC8:17 control; B, *Vitreoscilla*; C, pUC8:16; D, unprotected and untreated control fragment.

Table 3. Expression of *vgb* promoter in heterologous hosts as measured by the reporter gene protein, CDO.

Strains	Visual Expression level (a)	CDO activity U/mg protein
<i>E. coli</i> JM83	* * * *	124
<i>Pseudomonas putida</i>	* * * *	147
<i>Pseudomonas aeruginosa</i>	* * * *	144
<i>Azotobacter vinelandii</i> (ATCC 475)	* * *	— (b)
<i>Rhizobium japonicum</i>	* *	28

Fusion plasmid pKD-49 was transferred into different hosts by triparental filter mating (32) using *E. coli* HB101 harboring the helper plasmid pRK 2013. a. The yellow color of the highest intensity that was observed in recombinant colonies after spraying with catechol solution is indicated by * * * *. b. Not determined.

in gross changes in chromosomal superhelicity, which in turn activate expression of a large number of genes. Oxygen regulated transcription of several genes, for example *nif* genes in *Rhodospseudomonas capsulata* and *Klebsiella pneumoniae* (25) and photosynthetic genes in *Rhodobacter capsulatus* (26), may be mediated by changes in the DNA superhelix. Similarly, the *tonB* promoter is very sensitive to changes in DNA supercoiling which occur in response to oxygen availability (27). Further experimentation is required to determine if such a mechanism also governs *vgb* expression, or whether there is negative or positive regulation, governed by repressor or activator proteins, respectively.

One negative regulatory mechanism that affects the expression of *vgb* besides oxygen is suggested by the inhibition of the synthesis of globin and reporter gene proteins in the presence of glucose. In microorganisms, several genes are controlled through glucose repression (16) which operates by affecting the cellular level of cyclic AMP (17) and such a mechanism may also control *vgb* expression in *E. coli*. Independent evidence for the involvement of cyclic AMP or a cyclic AMP receptor protein, which are key elements in transcriptional control of the glucose sensitive operon (28), in *vgb* gene regulation has been reported recently (9). The transcriptional fusions described in this report will be helpful in extending the study in this direction.

In *Vitreoscilla* the biosynthesis of two enzymes, δ -amino-levalulinic acid synthetase, the first enzyme in the heme biosynthetic pathway, and NADH-methemoglobin reductase, which keeps the protein in its physiologically functional ferrous form, are also regulated by oxygen (8) as is *Vitreoscilla* catalase which has the probable function of protecting the cell from hydrogen peroxide produced by the autooxidation of VtHb (29). Whether these proteins are regulated coordinately by oxygen using the same or independent mechanisms is not yet known.

The S1 mapping analysis (Fig. 5) furnished additional evidence that both *Vitreoscilla* and *E. coli* use the same promoter. Only one S1-protected DNA fragment was observed in *Vitreoscilla* and it was identical to the size of the major fragment from *E. coli*. An additional minor band, about 35 bp larger and representing 20% of the total intensity, observed in the polyacrylamide gel for *E. coli* (Fig. 5 C) could be the result of incomplete S1 nuclease digestion. Alternatively, it could be interpreted as evidence for the existence of a second promoter which has been reported by others using primer extension analysis about 50 bp upstream of the first promoter (9). The length of the promoter region in our cloned *vgb* gene is at least as long as that used for the latter experiment (150 bp upstream of the structural gene). Since there is no evidence for a second promoter site in the S1 protected fragments from *Vitreoscilla*, either the promoter region is recognized differently in *E. coli* or the extra band observed is an experimental artifact.

Under hypoxic conditions, *E. coli* cells that can synthesize VtHb grow better than those cells that cannot. In *Rhizobium*, leghemoglobin has been shown to facilitate oxygen diffusion and respiratory activity under conditions of very low oxygen pressure (30). VtHb is closely associated with respiratory membranes in *Vitreoscilla* (1,31) and also in recombinant *E. coli* (31), which location may facilitate respiration by a similar mechanism.

The *vgb* promoter in the broad host range pKD-49 was also expressed in some other gram-negative bacteria with varying efficiency (Table 3). We have also transferred the entire *vgb* gene together with its own promoter into *Pseudomonas* where it is highly expressed and produces hemoglobin in relatively large

amounts. Manipulation of *vgb* and other genes in organisms besides *Vitreoscilla* and *E. coli* using the regulatory properties of this promoter appears very feasible.

ACKNOWLEDGEMENT

We are grateful to Dr. A.M. Chakrabarty for providing strains of *Pseudomonas* and *Azotobacter*, Dr. V. Deretic for plasmid pVDX18, and to Dr. Ben Stark for helpful advice. This work was supported by NIH grant GM27085.

REFERENCES

- Webster, D.A. (1987) In Eichhorn, G.L. & Marzilli, L.G. (eds) *Advances in Inorganic Biochemistry*, Elsevier NY, Vol.7 pp. 245–265.
- Wakabayashi, S., Matsubara, H., and Webster, D.A. (1986) *Nature* 322, 481–483.
- Dikshit, K.L. and Webster, D.A. (1988) *Gene* 70, 377–386.
- Boerman, S.J. and Webster, D.A. (1982) *J. Gen. Applied Microbiol.* 28, 35–43.
- Lamba, P. and Webster, D.A. (1980) *J. Bacteriol.* 142, 169–173.
- Khosla, C. and Bailey, J.E. (1988) *Mol. Gen. Genet.* 214, 158–161.
- McClure, W.R. and Hawley, D.K. (1983) *Nucleic Acid Res.* 8, 2237–2254.
- Dikshit, K.L., Spaulding, D., Braun, A. and Webster, D.A. (1989) *J. Gen. Microbiol.* 135, 2601–2609.
- Khosla, C. and Bailey, J.E. (1989) *J. Bacteriol.* 171, 5995–6004.
- Maniatis, T., Fritsch, E.F., and Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY.
- Brosius, J. and Lupski, J.R. (1987) *Methods Enzymol.* 153, 54–68.
- Konyecsni, W.M. and Deretic, V. (1988) *Gene* 74, 375–386.
- Berk, R.J., and Sharp, P.A. (1977) *Cell* 12, 721–732.
- Shaw, W.V. (1975) *Methods Enzymol.* 43, 737–755.
- Bradford, M. (1976) *Analytical Biochem.* 72, 248–254.
- Magasanik, B. and Neidhardt, F.C. (1987) In *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*, Am. Soc. Microbiol., Washington, D.C. pp. 1313–1317.
- Hemphling, W.P. and Beeman, D.K. (1971) *Biochem. Biophys. Res. Commun.* 45, 924–930.
- Khosla, C. and Bailey, J.E. (1988) *Nature* 331, 633–635.
- Jamieson, D.J. and Higgins, C.F. (1986) *J. Bacteriol.* 168, 389–397.
- Jones, H.M. and Gunsalus, R.P. (1987) *J. Bacteriol.* 169, 3340–3349.
- Christman, M.F., Storz, G. and Ames, B.N. (1989) *Proc. Natl. Acad. Sci. USA* 86, 3484–3488.
- Georgiou, C.D., Dueweke, T.J. and Gennis, R.B. (1988) *J. Bacteriol.* 170, 961–966.
- Ditta, G.E., Palomeres, V.A. and Kim, C.H. (1987) *J. Bacteriol.* 169, 3217–3223.
- Yamamoto, N. and Droffner, M.L. (1985) *Proc. Natl. Acad. Sci. USA* 82, 2077–2081.
- Kranz, R.G. and Haselkorn, R. (1986) *Proc. Natl. Acad. Sci. USA* 83, 6805–6809.
- Zhu, Y.S. and Hearst, J.E. (1988) *Proc. Natl. Acad. Sci. USA* 85, 4209–4213.
- Dorman, C.J., Barr, G.C., Bhriain, N.N. and Higgins, C.F. (1988) *J. Bacteriol.* 170, 2816–2826.
- de Crombrughe, B., Busby, S. and Buc, H. (1984) *Science* 224, 831–838.
- Abrams, J. and Webster, D.A. (1990) *Arch. Biochem. Biophys.* (Accepted for publication).
- Wittenberg, J.B., Appleby, C.A. and Wittenberg, B.A. (1972) *J. Biol. Chem.* 247, 527–531.
- Khosla, C. and Bailey, J.E. (1989) *J. Mol. Biol.* 210, 79–89.
- Riess, G. and Puhler, A. (1984) In Puhler, A. & Timmis, K.N. (eds) *Advanced Molecular Genetics*, Springer-Verlag, Berlin, pp. 51–63.