Analysis of the *Rhodobacter capsulatus* *puf* Operon

**LOCATION OF THE OXYGEN-REGULATED PROMOTER REGION AND THE IDENTIFICATION OF AN ADDITIONAL *puf*-ENCODED GENE**

(Received for publication, August 31, 1987)

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In an attempt to identify features of an oxygen-regulated promoter, we have determined the location of transcription initiation for the *puf* operon. The position for the oxygen-regulated promoter was demonstrated by several independent means to be located 699 base pairs (bp) upstream from the *pufB* structural gene. DNA sequence analysis of the promoter region demonstrates the presence of a 26-base pair region of dyad symmetry followed by a sequence containing homology to promoters which use the RNA polymerase σ^70 subunit (ntrA) for recognition of DNA. In addition to the oxygen-regulated promoter, a region responsible for low-level constitutive expression of the *puf* operon was shown to initiate transcription 511 bp upstream from the *pufB* gene. In contrast to the oxygen-regulated promoter, this second promoter contains no obvious secondary structure nor sequence homology to ntrA-dependent promoters. DNA sequence analysis demonstrates the existence of an additional open reading frame (designated as *pufQ*) that is located between the promoters and the *pufB* structural gene. A translational fusion of *pufQ* to *lacZ* was used to demonstrate that *pufQ* is efficiently translated and regulated in a manner analogous to a translational fusion of *pufM* to *lacZ*. Finally, we also demonstrate that *puf* operon transcription initiation and regulation does not involve any *puf*-encoded gene products.

Purple non-sulfur photosynthetic bacteria are facultative anaerobes that synthesize their photosynthetic apparatus only under conditions of reduced oxygen tension. *Rhodobacter capsulatus* synthesizes three membrane-bound pigment-protein complexes. The light harvesting I and II complexes (LH-I and LH-II, respectively) are antennas which absorb light and transmit the energy to a third pigment-protein complex, the reaction center (RC) that serves as the electron donor during photosynthesis. The LH complexes are each composed of two small membrane-spanning polypeptides, \( \alpha \) and \( \beta \), that function as scaffolding to anchor bacteriochlorophyll and carotenoids. The RC is composed of three membrane-spanning polypeptides L, M, and H that also function to bind bacteriochlorophyll and carotenoids as well as quinones. Recently, it has been demonstrated that operons which encode these pigment-binding polypeptides, such as the *puc* operon that encodes the LH-II \( \alpha \) and \( \beta \) polypeptides (*pucA* and *B*), the *puh* operon that encodes the RC-H polypeptide (*puhA*) as well as the *puf* operon that encodes for the LH-I \( \alpha \) and \( \beta \) polypeptides (*pufA* and *B*) and the RC-L and M polypeptides (*puFL* and *M*) are all transcriptionally repressed by molecular oxygen (1–4). The mechanism(s) whereby oxygen regulates the expression of these and other genes is unknown.

In order to determine what cis-acting regions are involved in oxygen regulation of transcription, it is important to rigorously determine the location of transcription initiation for oxygen-regulated operons. Towards this goal we have undertaken a "functional analysis" of the *puf* operon promoter region using deletion and insertion (interposon) mapping techniques as well as mung bean nuclease mapping of mRNA transcripts hybridized to DNA probes. The results of this study demonstrate that oxygen-regulated transcription initiation for the *puf* operon occurs 699 bp upstream from the *pufB* gene. This oxygen-regulated "photosynthetic promoter" contains a sequence that closely resembles the consensus sequence recognized by the \( \sigma^70 \) (ntrA) subunit for RNA polymerase which is used in a variety of bacteria for genes involved in nitrogen metabolism. In addition, this study also provides evidence that a second site for transcription initiation occurs 188 bp downstream from the position of primary transcription initiation and that this second promoter is responsible for low-level constitutive expression observed during aerobic growth.

Besides evidence for newly defined promoter regions, we also demonstrate the existence of an additional gene encoded by the *puf* operon (*pufQ*) that is located between the promoters and the *pufB* structural gene. A translational fusion of *pufQ* to the *Escherichia coli lacZ* gene was used to demonstrate that *pufQ* is efficiently expressed and regulated in a manner analogous to the *pufM* gene. Finally, we also demonstrate that *puf* operon expression and regulation does not involve any gene products encoded by the *puf* operon.

**EXPERIMENTAL PROCEDURES**

**RESULTS**

Deletion Mapping of the Promoter Region—In order to determine the extent of DNA that is required for *puf* operon expression, a nested set of deletions were constructed in the region of DNA upstream from the *pufB* gene. To facilitate
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**FIG. 1. Deletion analysis of the puf operon.** A nested set of deletions were constructed on plasmid-encoded copies of the puf operon that contained either a pufM::lacZ or a pufQ::lacZ translational fusion. Deletions were constructed at the restriction sites, EcoRI (+), XhoI (W), FspI (O), XmaIII (O), NcoI (V), and SalI (O) (see Fig. 4 for sequence analysis of this region). The phenotypic effect of the deletions on expression of the puf operon (lacZ) is shown on the table to the right. The units of β-galactosidase activity are expressed as micromoles of O-nitrophenyl-β-D-galactoside hydrolyzed/min/mg protein.

| Deletion             | β-galactosidase activity |
|----------------------|--------------------------|
|                      | -O₂ | +O₂ | -O₂/±O₂ |
| pCB552               | 1758 | 60  | 29.3    |
| pCB552:del1          | 1,493 | 57  | 26.2    |
| pCB552:del2          | 58   | 48  | 1.2     |
| pCB552:del3          | 13   | 12  | 1.1     |
| pCB552:del4          | 11   | 6   | 1.8     |
| pCB552:del5          | <1   | <1  | -       |
| pCB532               | 2,417 | 81  | 29.8    |
| pCB532:del6          | <1   | <1  | -       |
| pCB532:del13         | <1   | <1  | -       |

**FIG. 2. Insertion (interposon) mapping of the puf operon.** A, using homologous recombination, a restriction fragment encoding Spc' flanked by transcription/translation termination sites (Q) was inserted at various distances (790, 691, and 407 bp) upstream from the pufB gene. B, a spectral analysis of crude chromatophores showing the puf-encoded RC and LH-I absorbance peaks at 865 and 876 nm, respectively. The upper most spectrum is that of chromatophores obtained from strain MW442 containing Q inserted at position 1. In contrast, insertion of Q at position 2 shows only slight absorbance in this region (middle peak) and insertion of Q at position 3 results in the absence of absorbance in this region (bottom line). Note, in order for the Q 1 LH-I peak to be on scale, the chromatophore preparation from this strain was diluted 2.5X relative to the chromatophore preparation obtained from strains containing Q 2 and 3.
Fig. 3. Mung bean nuclease mapping of the 5'-mRNA. Autoradiograms A and B show the results of mung bean nuclease mapping analysis for transcription initiation using DNA probes A and B, respectively. Probe A is a 799-bp NcoI-EcoRI restriction fragment from the EcoQ region that is 5'-end-labeled at the NcoI site located 29 bp upstream from pufQ. This probe shows two sites of mRNA protection (Punj and PunR). Probe B, used to resolve the PunR transcript to a nucleotide level, is a 645-bp FspI-EcoRI restriction fragment 5'-end-labeled at the FspI site. The mRNA-protected DNA was separated on a denaturing 8% polyacrylamide gel next to G+A and C+T chemical cleavage reactions (18) performed on aliquots of the probes.

These results demonstrate that a region of DNA spanning from 560 to 802 bp upstream from pufB encodes sequence information necessary for anaerobic induction of transcription, whereas a region of DNA spanning from 407 to 560 bp upstream from pufB encodes sequence information responsible for low-level expression observed under aerobic conditions.

Interposon Mapping of the Promoter Region—As an inde-
pendent “functional” assay for the in vivo location of the puff operon promoter, we inserted a translation/transcription terminator (interposon O) at various positions within the chromosome of strain MW442. We chose MW442 for this analysis since this strain contains a mutation within the puff operon that results in a loss of LH-II absorbance (23). Thus, this strain allows us to directly measure puff operon expression by observing RC and LH-I absorbance at 805 and 872 nm, respectively (Fig. 2B). As demonstrated in Fig. 2, insertion of O at a position 790 bp upstream from puffB (O 1) has no effect on puff operon expression. In contrast, insertion of O at position 2, located 692 bp upstream from puffB, has a marked reduction in puff operon expression. Finally, insertion of O at position 3, located 407 bp upstream from puffB, results in the complete absence of puff operon expression. These results, which agree with the deletion analysis, demonstrate that a region of DNA between 692 and 790 bp upstream from the puffB gene is required for oxygen-regulated transcription initiation, whereas a position between 407 and 692 bp is responsible for low-level expression of the puff operon.

**Mung Bean Nuclease Mapping of mRNA 5’ Ends**—In order to determine, at a nucleotide level, which base initiates transcription, we performed 5’-mRNA mapping by digesting P-labeled DNA probes that were hybridized to cellular mRNA with mung bean nuclease. Two probes were used to locate the start of transcription within the DNA maps of deletion and insertion analysis. Using a Neo1-EcoRI probe (see “Experimental Procedures”) we were able to observe two start sites for transcription initiation (Fig. 3A). One was a less intense region of protection that occurs 511 bp upstream from puffB (Pstart). This is within the region of DNA, shown by deletion analysis, to be responsible for low-level constitutive (aerobic expression). The other more intense band of protection observed with this probe occurs 511 bp upstream, in the region of DNA shown by deletion and insertion mapping to be responsible for oxygen-regulated transcription initiation. This primary transcript was resolved at a nucleotide level, to a position 692 bp upstream from puffB, using a FspI-EcoRI probe (Fig. 3B). Fig. 4 shows the position of transcription initiation mapped by this procedure relative to the positions mapped by deletion and insertion mutagenesis.

**Sequence Analysis of the Promoter Region and the Identification of an Additional puff Operon Gene**—Analysis of the DNA sequence located between the promoter region and the puffB structural gene (Fig. 4) shows the existence of an open reading frame (termed puffQ) that could potentially encode a protein composed of 74 amino acids. An excellent codon preference plot (a statistical program for identifying potential genes; Ref. 24) is generated for puffQ when using a codon table obtained from known R. capsulatus genes (Fig. 5). This would be expected for open reading frames efficiently translated by R. capsulatus. Furthermore, a plasmid containing a translational fusion of the E. coli lacZ gene to the 13th amino acid of puffQ (pCBS32) demonstrates a level of β-galactosidase activity and regulation by molecular oxygen that is comparable to what is observed with a plasmid containing a puffM::lacZ fusion (Fig. 1). These analyses, in conjunction with the promoter mapping studies described above, support our conclusion that puffQ is the first structural gene of the puff operon.

**Puf Operon Expression Is Not Dependent on puff Operon Gene Products**—The mechanism whereby the puff operon is regulated by molecular oxygen is unknown. Transcription could be directly or indirectly regulated by a puff operon encoded gene product(s). To test this possibility, we constructed a strain of R. capsulatus (SB1003:del,502) containing a deletion of the puff operon by replacing the puffQ through puffM genes with interposon O. A reporter plasmid for puff operon expression (pCBS32:Km) was then introduced into SB1003:del,502. This plasmid contains the puff operon promoter region followed by a translational fusion of puffQ to lacZ. Using this strain we can assay whether the absence of puffQ through puffM has an effect on the β-galactosidase activity expressed from the plasmid. The results of this analysis (Table 1) demonstrate that the absence of puff encoded gene products has no effect on the expression of the puffQ::lacZ fusion. Thus, transcriptional regulation of puff operon does not appear to directly or indirectly involve any puff encoded genes.

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2 Although insertion of O 1 into the chromosome 790 bp upstream from the puffB gene does not affect puff operon expression, both O 1 and O 2 disrupt the bacteriochlorophyll A (bchA) gene that overlaps the puff operon promoter region (D. A. Young, C. E. Bauer, and B. L. Marrs, manuscript in preparation). Thus, the spectral analyses shown in Fig. 2 are derivatives of MW442 that contain their respective O insertions as well as a plasmid-encoded translational addition of the bchA gene.

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**Fig. 4. DNA sequence of the puff operon region located upstream from the puffB gene.** The DNA sequence is numbered relative to the start of the puffB gene that was arbitrarily assigned a value of 0. Deletions and insertions were constructed between the bases indicated. The start sites for transcription initiation, as mapped by mung bean nuclease, are indicated with a solid bar. The most probable base for transcription initiation, which was determined as the furthest base protected by mRNA with various concentrations of nuclease, is highlighted by reverse contrast. Direct repeats and inverted repeats located upstream from the puffB site of transcription initiation are indicated by straight arrows. The location of the puffQ gene and the start site of the puffB gene are indicated by the predicted amino acid sequence. The solid bar below the DNA sequence denotes the probable Shine-Dalgarno ribosome binding site.
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**FIG. 5.** Codon preference plot of the *pufQ* gene. The codon preference plot was generated using a program written by Gribskov et al. (24). This program identifies efficiently translated genes as peaks above open reading frames (open boxes below peaks) that contain few rare codons (dashes below the open reading frames). A codon frequency table used to produce the plot was obtained from previously published *pufB*, *A*, *L*, *M*, *pucB*, *A*, and *puM* genes (33, 43). The window size and rare codon threshold were set at 25 and 0.1, respectively.

**TABLE I**

| Strain                        | Low O₂ | High O₂ | Low O₂/high O₂ |
|-------------------------------|--------|---------|----------------|
| SB1003/pCB532:Km              | 2417   | 81      | 29.8           |
| SB1003 del₅₁₀²/pCB532:Km      | 2760   | 87      | 31.7           |

* Units are expressed as micromoles of O-nitrophenyl-β-D-galactoside hydrolyzed/min/mg protein.

**DISCUSSION**

*Features of the* *puf* Operon *Promoters*—In this investigation, we have undertaken a study to determine the location of transcription initiation for the *puf* operon. By employing a functional analysis of the *puf* operon through deletion and insertion mutagenesis, as well as a physical analysis of the mRNA length by Mung bean nuclease mapping, we have mapped the site for oxygen-regulated transcription initiation to a position 699 bp upstream from the *pufB* gene. Since it had previously been presumed that *pufB* was the first structural gene of the *puf* operon (25), the extended distance of the *Pₚₕₜₖ* promoter region from the *pufB* structural gene was at first surprising. However, sequence information we obtained upstream from the *pufB* gene demonstrates the existence of an additional *puf* operon gene (*pufQ*; see below) which is located between the promoter region and *pufB*. Therefore, in retrospect, it is not surprising that transcription initiation occurs within the region mapped. It should also be noted that an earlier attempt at S1 nuclease mapping of the *puf* operon 5′-mRNA suggested that transcription initiation or a site of mRNA processing occurred just upstream (approximately 100 bp) from the *pufB* gene (25). However, our deletion analysis shows no evidence for transcription initiating from this region (Fig. 1). Since the previous S1 nuclease mapping study does not correlate with the functional length of the *puf* operon, we presume the discrepancy is due either to mRNA processing and/or the use of probes which did not extend into the promoter region. Additional studies on the stability of the *puf*-encoded mRNA will have to be undertaken to determine if the region -100 bp upstream from the *pufB* gene is indeed a position of mRNA processing.

The existence of a second low-level constitutive promoter (*Pₚₚₚ*) located 511 bp upstream from *pufB* (188 bp downstream from *Pₚₚₚ*) was demonstrated by both deletion and insertion analysis and further confirmed and mapped to a nucleotide level with mung bean nuclease mRNA mapping. By aligning *Pₚₚₚ* and *Pₚₚₚ* at the start of transcription (Fig. 6A), we observe a fair degree of homology in the -1 to -8 region (6/
obvious similarity to the canonical promoter sequence (26) present in the used by E. coli. One area of striking difference between P\text{pun} and P\text{punu} is the numerous regions of secondary structure, such as direct repeats from +1 to −14 and from −72 to −92 and inverted repeats from −25 to −53 and from −72 to −95, that are present in the more highly expressed P\text{pun} promoter absent in the P\text{punu} promoter (Fig. 4). It should also be noted that the existence of two promoters in highly regulated prokaryotic operons is not uncommon. For example, the seven ribosomal RNA operons from E. coli (rrn) are each thought to contain two tandem promoters separated by a distance of 110–120 bp (27–29). Furthermore, in analogy to what we observed for the puf operon, the upstream \textit{rrn} promoter(s) (P\text{r}) is highly expressed and regulated (by stringent response and growth rate dependence), whereas the downstream promoter(s) (P\text{d}) is weakly expressed and unregulated (30, 31). It remains to be seen whether other photosynthetic genes exhibit two promoters.

In an attempt to determine what features of the P\text{pun} promoter are important for expression and regulation, we scanned other oxygen-regulated genes from \textit{R. capsulatus} for similar promoter sequences. One such candidate is the puhA (RC-H) gene that is encoded by a 1400-base transcript that is expressed and regulated by oxygen and light intensity in a manner similar to the puf operon transcript (2). Since the RC-H structural gene encompasses 760-bp, the position of transcription initiation must occur no more than 640 bp upstream from the start of translation. Using a homology search program (32), we scanned a 3100-bp region of DNA upstream from the pufA gene (33) for homology to the P\text{pun} promoter. The result of this analysis demonstrates the existence of a region of DNA located 440 bp upstream from puhA that exhibits a striking degree of homology to the P\text{pun} promoter (Fig. 6B). Interestingly, conserved in both sequences is a region containing extensive homology to the consensus sequence for promoters using the ntrA σ subunit (34–36, 44). The ntrA σ subunit appears to be a constitutively expressed minor subunit of RNA polymerase that is present in a number of diverse gram-negative bacteria (for a review of ntrA promoters see Ref. 44). One interesting feature common to promoters using the σ\text{50} subunit is that they all appear to be highly induced by an element which binds upstream from the promoter. For example, genes involved in nitrogen metabo-

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**Figure 6.** Sequence conservation between the puf promoters and the identification of a putative pufA promoter. A, comparison of the P\text{pun} and P\text{punu} promoter sequences when aligned at the position of transcription initiation. The shaded boxes denote conserved regions in both promoters. B, comparison of the puf operon promoters to the putative pufA promoter. Bases containing homology to the ntrA consensus sequence (34–36) are highlighted by inverse contrast. C, a comparison of the sequence from the right-hand top strand and the left-hand bottom strand of the inverted repeats present in the P\text{pun} and the putative puhA promoter.

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**Figure 7.** \textit{R. sphaeroides} genome also contains the pufQ gene. The top line contains the \textit{R. capsulatus} DNA sequence extending from the carboxyl-terminal third of pufQ to the pufB gene. In contrast, the bottom line contains the \textit{R. sphaeroides} DNA sequence upstream from pufB as published by Kiley et al. (42). Amino acids conserved in both species are highlighted as shaded boxes, whereas amino acids sharing similar functions are boxed with dashed lines.
lism such as nitrogen fixation (37–39), amino acid transport (argTr, dhuA; Ref. 37), and catabolism of amino acids (hut, put, orn; Ref. 40) as well as genes in Pseudomonas spp. for the catabolism of aromatic hydrocarbons (xyICAB, xyIS; Refs. 35 and 41), pilin formation (45), and carboxypeptidase (26, 42 (46) have promoters using the ntrA subunit that are thought to require an upstream activator for expression. Indeed, if the puf (P₂₃₅₆) and pufA promoters are recognized by the σ⁶⁰ subunit, a potential region for binding such an upstream activator could be the conserved 26-bp region of diad symmetry located from −24 to −50 bp upstream from the start of transcription (Fig. 6C). Direct evidence for the involvement of one or more of these regions in regulating expression, however, will have to await the future isolation of cis- and trans-acting mutations that effect photosynthetic gene expression.

Identification of pufQ—Sequence data demonstrates the existence of an open reading frame between P₂₃₅₆ and pufB. Evidence for this open reading frame encoding an actual protein is presented by its excellent codon usage as well as by the β-galactosidase activity expressed from the pufQ fusion to lacZ that exhibits a level of activity and regulation analogous to that observed for the pufM fusion to lacZ (Fig. 1). Besides evidence for translation, we have additional evidence that the protein encoded by pufQ is required for bacteriochlorophyll biosynthesis. Thus, the puf operon appears to encode a protein required for bacteriochlorophyll biosynthesis as well as for proteins that bind bacteriochlorophyll to form the light harvesting and reaction center complexes.

Finally, sequence analysis for the Rhodobacter sphaeroides pufB and pufA genes along with several hundred base pairs of DNA upstream from the pufB gene has recently been published (42). Comparison of the DNA sequence from the pufQ gene obtained in this study with the R. sphaeroides sequence demonstrates the existence of the pufQ gene in this related organism (Fig. 7). Thus, the genomic organization for the puf operon in R. capsulatus also appears to be conserved in other purple non-sulfur bacterium. Presumably, additional sequence information and mRNA mapping data for the R. sphaeroides puf operon, as well as additional studies on oxygen-regulated promoters from other operons, should shed more light on the DNA sequences involved in the initiation and regulation of transcription in these organisms.

Acknowledgments—We thank M. Eleuterio and P. Scolnik for stimulating discussions and for careful proofreading of the manuscript. We especially thank T. Reed for technical assistance.

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EXPERIMENTAL PROCEDURES

Analysis of R. capsulatus puf Operon

Location of the Oxygen-Regulated Promoter Region and the Identification of an Additional Puf-Encoded Gene

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Sequence Analysis

The entire O2-1 to O2-5 restriction fragment (395 bp) was conveniently sequenced (Sanger et al., 1977) using the Maxam and Gilbert (1980) chemical sequencing method. In addition, the region spanning from the promoter region immediately 480 bp upstream of pufC through the pufG gene was confirmed by determining the sequence of one strand using the Sanger et al. (1977) dideoxy sequencing procedure.

Special Growth Conditions: Cells were grown at 30°C for 2-3 days in 30 ml of R. capsulatus medium growing at low oxygen (100 ppm) in a 50 ml Erlenmeyer flask. After harvesting by centrifugation at 12,000 g for 10 min, the cells were resuspended to 1/10 vol of 0.1 M NaCl, pH 7.5, 0.05 M EDTA and dialyzed at 0°C by rotation with two 30 min changes. After salting by centrifugation at 27,000 g for 15 min, the supernatant fraction was stored for absorbance from 400 to 900 nm using a Perkin-Elmer Lambda model 340 spectrophotometer. Alternatively, the migrations were scanned from the left to the right with sodium-methanol ($\text{H}_2\text{SO}_4$) as described by Chapeville (1975) and scanned for the spectra absorbance as described above.

Non-Collinear Measurement—Cells were grown in R. capsulatus medium grown under photosynthetic conditions by illuminating a filled 0.1 ml screw-cap tube in a 125 ml Erlenmeyer flask. After growth reached approximately 1 cm (0.1 mm), cells were collected at 0°C in a microcentrifuge tube and washed with sodium-methanol ($\text{H}_2\text{SO}_4$) as described by See et al. (1975). The absorbance was measured at 400 nm and the OD was recorded at 600 nm.

Mung Bean Nuclease Treatment—The DNA was treated by harvesting the RNA by adding the RNA to DNA and DNA overlap with mung bean nucleases using the conditions described by Brown et al. (21). The DNA template, 10 ml of cells grown phototrophically in PES and harvested and grown under aerobic conditions by harvesting in a microcentrifuge tube (380 g for 10 min) at 4°C in a 125 ml Erlenmeyer flask. After growth reached approximately 1 cm (0.1 mm) cells were collected at 0°C in a microcentrifuge tube and washed with sodium-methanol ($\text{H}_2\text{SO}_4$) as described by Chapeville (1975). The absorbance was measured at 400 nm and the OD was recorded at 600 nm.

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