cis-Acting Regulatory Elements Involved in Oxygen and Light Control of **puc** Operon Transcription in *Rhodobacter sphaeroides*

JEONG K. LEE AND SAMUEL KAPLAN*

Department of Microbiology and Molecular Genetics, The University of Texas, Medical School at Houston, P.O. Box 20708, Houston, Texas 77225

Received 20 September 1991/Accepted 13 December 1991

Transcriptional expression of the **puc** operon in *Rhodobacter sphaeroides* is highly regulated by both oxygen and light. The ≈600 bp of DNA upstream of the 5′ ends of the two **puc**-specific transcripts encompasses two functionally separable cis-acting domains. The upstream regulatory region (URS) (−629 to −150) is responsible for enhanced transcriptional regulation of **puc** operon expression by oxygen and light. The more proximal upstream region (downstream regulatory region [DRS]), containing putative promoter(s), operator(s), and factor binding sites (−150 to −1), is involved in unenhanced transcriptional expression of the **puc** operon under aerobic and anaerobic conditions. Thus, the DRS shows normal derepression of **puc** operon expression when cells are shifted from aerobic to photosynthetic growth conditions in terms of percent change but does not show the potential range of expression that is only observed when elements of the URS are present. Because of these observations, we have made a distinction between anaerobic control (describing the shift) and oxygen control (describing the magnitude of derepression). Promoter(s) and/or activator function(s) of the **puc** operon is associated with a 35-bp DNA region between −92 and −57. Homologous sequences at −10 to −27 and −35 to −52 appear to involve additional regulatory elements: mutations at −12 (A to C) and −26 (G to A) result in partial derepression of **puc** operon expression under conditions of high aeration. Both point mutations require the upstream regulatory region (−629 to −150) to be present in cis for partial derepression of **puc** operon transcription under aerobic conditions. Immediately upstream of the promoter and/or activator region are overlapping consensus sequences for IHF (integratin host factor) and FNR (fumarate nitrate reductase) (−105 to −129). This region appears to be essential for enhanced expression of the **puc** operon. Thus, these two regulatory domains (URS and DRS) appear to involve approximately seven unique regulatory elements. In addition, the data reveal a direct interaction between the URS (−629 to −150) and the DRS (−150 to −1).

Together with the B875 and reaction center complexes, the B800-850 light-harvesting (LH) complex is one of three major bacteriochlorophyll-protein complexes housed within the intracytoplasmic membrane of *Rhodobacter sphaeroides* (5, 6, 27). In addition to the presence of a typical gram-negative cell envelope, the intracytoplasmic membrane is induced when the partial pressure of oxygen is lowered below specific threshold values, e.g., 2.5% (4, 17). The relative abundance of the two LH complexes in the intracytoplasmic membrane is a function of the incident light intensity. The ratio of the B800-850 complex to the B875 complex varies inversely with the incident light intensity, such that the B800-850 complex is regarded as a component of the variable photosynthetic unit (11, 18, 19), while the ratio of the B875 complex to the reaction center complex is fixed at approximately 12:1 to 15:1, irrespective of the incident light intensity. Thus, the B875 and the reaction center complexes are referred to as the fixed photosynthetic unit (1, 5).

The **puc** operon of *R. sphaeroides* comprises the **pucBA** structural genes (encoding the B800-850-β and -α polypeptides, respectively) as well as an additional gene(s) which is within the 1.8-kb DNA immediately downstream of **pucBA** and which encodes functions essential for the posttranscriptional expression of the B800-850-β and -α polypeptides and for the formation of the B800-850 LH complex (21, 24). In the related bacterium *Rhodobacter capsulatus*, the involvement of the gene products encoded by the genes **pucCDE** downstream of **pucBA** in the formation of the B800-850 LH complex has also been reported (35).

In *R. sphaeroides*, the **puc**-specific transcripts are 0.5 and 2.3 kb in size, and both transcripts have the same 5′ end, which has been mapped to 117 nucleotides (nt) upstream of the start of **pucB** (24). The smaller transcript is ≈200-fold more abundant than the larger transcript. The 0.5-nt transcript encodes the **pucBA** structural genes (21), while the 2.3-kb transcript extends approximately 1.8 kb further downstream of the end of the smaller transcript and presumably encodes functions involved in posttranscriptional control (24). Previously, we demonstrated that transcription of the **puc** operon is highly regulated by oxygen as well as light (21). In *R. capsulatus*, two 5′ ends were reported for the 0.5-nt **puc** transcript; these were localized approximately 125 and 110 nt upstream of the start of **pucB** (37).

In an attempt to understand the role of oxygen and light in **puc** operon expression, we have examined the cis-acting regulatory element(s) involved in **puc** operon control. We have investigated cis-acting regulatory control of **puc** operon expression by using **puc**:lacZ transcriptional fusions containing serial 5′ or internal deletions of DNA sequences upstream of **pucBA** as well as through the selection of cis-acting mutations by using the transcriptional expression of Km with a **puc**:aph (coding for aminoglycoside-3′-phosphotransferase) transcriptional fusion in the presence of O2. The upstream DNA was found to contain at least two functionally separable cis-acting regulatory domains. These

* Corresponding author.
studies, together with an analysis of trans-acting mutations which are also involved in the transcriptional control of puc operon expression, have revealed a complexity of interacting regulatory signals which, together with the posttranscriptional control of puc operon expression, ensure the continuous expression of the B800-850 complex in response to O2, light, and a reducing environment. (A preliminary report of this work was presented at Pseudomonas 91 in Trieste, Italy, June 1991.)

MATERIALS AND METHODS

Bacteria, plasmids, and cell growth. All bacterial strains and plasmids used in this study are described in Table 1. R.
sphaeroides wild-type strain 2.4.1 and its derivatives were grown as previously described (12). When appropriate, tetracycline, kanamycin, streptomycin, and spectinomycin were added to Sistrom’s minimal medium to final concentrations of 1, 20 to 25, 50, and 50 μg/ml, respectively. Photoheterotrophic growth of R. sphaeroides in the presence of tetracycline was accomplished as described previously (9). Cell growth was monitored by use of a Klett-Summerson colorimeter (no. 66 filter) as previously described (33).

Escherichia coli JM109, DH5a, and S17-1 were grown at 37°C in Luria medium (29). Ampicillin, tetracycline, kanamycin, streptomycin, and spectinomycin (final concentrations, 50, 20, 25, 50, and 50 μg/ml, respectively) were added to the growth medium for E. coli strains carrying plasmids encoding these drug resistance genes. Plasmids pUC18, pUC19, and pBS were used for cloning.

DNA manipulation and sequence analysis. Large-scale plasmid DNA was prepared by use of chloramphenicol-amplified Triton X-100 lysates of E. coli and two successive equilibration CsCl gradients (21). Small-scale plasmid DNA was prepared by alkaline sodium dodecyl sulfate lysis (29) or by the Brij (polyethylene glycol hexadecyl ether) lysis method previously described by Summerton et al. (32). Treatment of DNA with restriction enzymes and other nucleic acid-modifying enzymes was performed in accordance with manufacturer specifications. DNA fragments were analyzed on agarose gels or polyacrylamide gels, and restriction fragments were isolated as previously described (10).

DNA sequencing was performed with phage M13 clones as described previously (12, 21) or with plasmid pBS clones and T3 or T7 primers by the chain termination method used for double-stranded DNA (29).

Conjugation techniques. Plasmid pRK415- or RSFl010-derived plasmids were mobilized into R. sphaeroides by previously described procedures (9).

Preparation of cell extracts and assay of β-galactosidase. R. sphaeroides cultures used for the measurement of β-galactosidase activities were grown chemoheterotrophically or photoheterotrophically by sparging with gas mixtures as described previously (9). Cells grown chemoheterotrophically were harvested at a cell density of 1.0 × 10⁸ to 3.0 × 10⁹ cells per ml, and cells grown photoheterotrophically were harvested at a cell density of 4.0 × 10⁹ to 1.0 × 10⁵ cells per ml. Cell breakage with a French press, preparation of crude extracts, and β-galactosidase assays (at 30°C for 5 min) with o-nitrophenyl-β-D-galactoside hydrolysis were performed as described previously (34). All experiments involving β-galactosidase assays were performed at least three times. When the activities being measured were higher than 200 U/mg of protein, the results were reproducible to within ±15%. Results for activities lower than 200 U/mg of protein were reproducible to within ±20 to 25%.

Construction of pLV106. To incorporate multiple cloning sites at the PstI restriction site of pUI511 (34), we undertook the following cloning steps. A BamHI restriction fragment of the ω cartridge containing Sm'/Sp' from pHP45Ω (28) was cloned into the BamHI restriction site of pUC4K following partial digestion with BamHI. From the isolated clone, the EcoRI restriction fragment incorporating BamHI-HindIII-PstI-Km'-PstI-HindIII-BamHI-HindIII-Ω Sm'/Sp'-HindIII-BamHI was cloned into the EcoRI restriction site of pUC19. The PstI restriction fragment containing Ω Sm'/Sp' and restriction sites flanking the Ω cartridge from the above-described pUC19 construction was placed into the PstI restriction site of pUI511 to generate pUI511:Ω/mcs. Finally, the HindIII restriction fragment of Ω Sm'/Sp' was removed from pUI511::Ω/mcs, and another EcoRI restriction site originally present between tet and oriV of pUI511 was interrupted after partial digestion of the plasmid with EcoRI accompanied by filling in of the overhangs with the Klenow fragment of E. coli DNA polymerase I. The resulting plasmid, pLV106, has new, unique EcoRI, KpnI, Smal, and XbaI restriction sites flanked by the PstI restriction site of pUI511.

Cloning of the puc-lacZ transcriptional fusion constructs into pLV106. To clone the transcriptional fusions of puc-lacZ within the restriction sites of pLV106, we used the following cloning steps. Firstly, each of the puc upstream DNA fragments containing serial 5' or internal deletions was cloned into the multiple cloning region of pUC19, pUC18, or pBS. Secondly, a transcription-translation stop cartridge, Ω, carrying the Sm'/Sp' gene from pHP45Ω (28), was cloned upstream of each of the R. sphaeroides inserts but within the multiple cloning site of each of the above-listed plasmids. Thirdly, each of the DNA fragments containing an Sm'/Sp' cartridge fragment was selected by virtue of pUC upstream DNA was cloned into the EcoRI, Smal, or BamHI restriction site within the multiple cloning region of pRS415 (31) to orient the puc upstream DNA to the same transcriptional direction as that of the lacZYA genes on pRS415. Fourthly, EcoRI (within the multiple cloning region of pRS415)-NrdI (within the lacI gene of pRS415)-generated DNA fragments containing the Ω Sm'/Sp' cartridge, the puc regulatory region, and lacZYA of pRS415 was cloned into the EcoRI-Smal restriction sites of pLV106.

Materials. Restriction endonucleases and nucleic acid-modifying enzymes were purchased from Bethesda Research Laboratories Life Technologies, Inc., Gaithersburg, Md., or New England BioLabs, Inc., Beverly, Mass., and used as specified by the manufacturer. The Klenow fragment of E. coli DNA polymerase I, proteinase K, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside were obtained from Boehringer Mannheim Biochemicals, Indianapolis, Ind. [α-35S]dATP was obtained from Amersham Corp., Arlington Heights, Ill. Isopropyl-β-D-thiogalactopyranoside and o-nitrophenyl-β-D-galactoside were obtained from Sigma Chemical Co., St. Louis, Mo. The DNA sequencing kit Sequenase was obtained from United States Biochemicals, Cleveland, Ohio, and the T7 Sequencing kit was obtained from Pharmacia, Piscataway, N.J. Molecular biology-grade phenol was purchased from Fisher, Pittsburgh, Pa. All other chemicals used in this work were reagent grade.

RESULTS

Construction of plasmid pLV106 for the mobilization of puc-lacZ transcriptional fusions into R. sphaeroides. In a previous investigation, we determined that the cis-acting regulatory sequences involved in puc operon expression in R. sphaeroides were located within an ~600-bp region immediately upstream of the start site of puc-specific mRNA transcription, which itself is 117 nt upstream of pucB (24). To more accurately define these regulatory elements, we required a mobilizable vector for the introduction of puc-lacZ transcriptional fusion constructs into R. sphaeroides. It was necessary that the plasmid be stably maintained in R. sphaeroides and preferable that it be free of any portion of lacZ to preclude any possible intramolecular recombination within the final plasmid construction.

Plasmid pUI511 (7.5 kb) (34) was modified by placing the unique EcoRI-KpnI-Smal and XbaI restriction sites downstream of the tet gene to generate pLV106 (IncQ or IncP4)

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FIG. 1. Restriction map of pLV106 (IncQ or IncP4). This plasmid was derived from pUI511 by placing the unique EcoRI and Smal restriction sites downstream of the tet gene (see Materials and Methods). EcoRІ interrupted EcoRI site. Not all restriction sites are shown on the map.

(Fig. 1). Details of the construction of pLV106 are given in Materials and Methods.

Although all of the puc-lacZ transcriptional constructs have the same transcriptional direction as the tet gene on pLV106, the presence of ω Sm74/Sp7 prevents transcriptional readthrough of the tet gene. All of the derived plasmid constructions involving pLV106 and the different puc-lacZ fusions are in the range of 14.6 to 15.4 kb, depending on the precise length of the puc upstream DNA.

cis-acting regulatory region responsible for transcriptional regulation of puc operon expression by light. During photoheterotrophic growth, the steady-state level of puc-specific transcripts was shown to be inversely related to incident light intensity (21). To examine whether the regulation of puc operon expression by light is exerted through a regulatory site(s) located within the 629-bp DNA upstream of the 5' end of the puc-specific mRNA start site (Fig. 2A), we measured the β-galactosidase activities of two puc-lacZ transcriptional fusions, pCF200(−629) and pCF250(−92), placed in trans in wild-type R. sphaeroides 2.4.1 during photoheterotrophic (10 W/m²) growth. The number in parentheses following the named plasmid designates the distance upstream from the start site of transcription. pCF200(−629) contained the entire 629-bp puc upstream DNA from the 5' end of the transcripts to the PstI restriction site, while in pCF250(−92) the 536-bp PstI-XmaІІ restriction fragment of the puc upstream DNA was deleted, leaving only the proximal 92-bp puc upstream DNA (Fig. 2A). The β-galactosidase activity of pCF200(−629) at 10 Klett units (KU) was =8.2 U/ml, a level similar to that of pCF250(−92) (=7.2 U/ml) at 11 KU (Fig. 2B). By using increasing cell densities at moderate illumination (10 W/m²) as a means of limiting the light incident to each culture, we found that the β-galactosidase activity of pCF200(−629) rapidly increased to somewhat less than 50 U/ml of culture when the culture reached ~40 KU, whereas in pCF250(−92), we observed less than 25 U/ml at ~40 KU. Thereafter, the β-galactosidase activity of pCF200(−629) increased at an accelerated rate to ~200 U/ml of culture as the cell density increased to 129 KU. On the other hand, pCF250(−92) showed a steady increase in β-galactosidase activity to approximately 70 U/ml of culture as the cell density increased to 135 KU. Although these data reveal the differential increase in β-galactosidase activity with decreasing light intensity in pCF200(−629) relative to pCF250(−92) at constant cell densities, the insert in Fig. 2B more dramatically reflects these changes. Here we have plotted the specific activity of β-galactosidase versus cell growth. It is readily apparent that the β-galactosidase levels recorded for pCF250(−92) remained low and independent of decreasing incident light intensity, whereas for pCF200 (−629) there was a steady increase in the expression of the puc operon as light intensity decreased. At constant cell densities with decreased light availability, pCF200(−629) showed an accelerating increase in β-galactosidase activity relative to pCF250(−92). By using an incident light intensity of 10 W/m², we chose a level that was neither too high (100 W/m²) nor too low (3 W/m²); either of these levels would minimize the anticipated differences in unshaded and shaded cultures, although the absolute levels of enzyme activity would be expected to be lower and higher at the higher and lower light intensities, respectively. These results indicate that the 536-bp puc upstream DNA lying between the PstI and XmaІІ restriction sites contains some or all of the cis-acting regulatory site(s) involved in the regulation of puc operon transcription by light intensity.

Since pCF250(−92) in the wild-type background showed a constant level of β-galactosidase during photoheterotrophic growth, although independent of light intensity and cell density, but a substantially elevated enzyme level relative to that in aerobically grown cells (see below), the 92-bp puc upstream DNA may contain some or all of the cis-acting regulatory site(s) which is involved in aerobic versus anaerobic expression of puc operon transcription.

Deletion analysis of the 5' puc upstream regulatory DNA transcriptionally fused to the lacZ gene. In a previous analysis (24), we demonstrated genetically that DNA sequences upstream of the puc operon to the PstI restriction site (Fig. 3) are sufficient for apparently normal expression of the puc operon in the formation of the B800-850 LH complex. For further localization of the cis-acting regulatory site(s) involved in transcriptional regulation of the puc operon by oxygen and/or light, a number of 5'-to-3' serial deletions as well as internal deletions extending 3' to 5' from the XmaІІ site were constructed and transcriptionally fused to the lacZ gene. Each fusion is illustrated in Fig. 3, as are the β-galactosidase activities resulting from the fusions when present in wild-type R. sphaeroides 2.4.1 grown both chemoheterotrophically and photoheterotrophically.

The removal of sequences downstream of the XmaІІ site, as in pCF252(−32), resulted in negligible background levels of β-galactosidase activities when cells were grown either chemoheterotrophically or photoheterotrophically, as in the two negative controls, pCF260(70) and pCF100, with deletions extending into the 5' leader region of the transcripts and into the pucB gene, respectively. The HinII restriction site limiting the puc upstream DNA on pCF252(−32) is located 32 bp upstream of the 5' end of the puc-specific transcripts and contains one of two sequences of dyad symmetry, TGTCA-8-bp-TGACA, which resembles a recog-
tion site for DNA-binding regulatory proteins involved in transcriptional regulation (14). pCF250(−92) contains an additional 60 bp of DNA immediately upstream of the HinfI restriction site and showed substantially increased β-galactosidase activities, as well as anaerobic control of puc operon expression, when cells were shifted from aerobic to anaerobic growth, i.e., an approximate 10-fold increase in puc operon expression. However, as described earlier, such cells lacked light regulation as well as the full range of puc operon expression. The inclusion of the region from XmaIII to StuI [pCF240(−225)] resulted in high-level expression of LacZ under either chemoheterotrophic or photoheterotrophic growth conditions when compared with that in pCF250(−92) and, as with pCF250(−92), anaerobic control was still manifest in pCF240(−225). Thus, it appears that the region between XmaIII and StuI contains enhancer or activator sequences because of the very high levels of puc operon expression.

Equally striking is the fact that the absence of upstream sequences in pCF240(−225) resulted in the virtual absence of O₂ repression as well as an enhancement in the light response. One possible explanation for these results is that O₂ and light regulatory regions exist upstream of the StuI site. The fact that both O₂ and light control were returned to normal in pCF220(−563) led us to conclude that an important region of O₂ control lies between the BamHI site and the most downstream XhoI site, because when this region is absent but all other downstream regions are intact, there is a

FIG. 2. (A) Restriction map of puc upstream DNA and two lacZ transcriptional fusions, pCF200(−629) (a) and pCF250(−92) (b). (B) β-Galactosidase activities of pCF200(−629) (○) and pCF250(−92) (●) in trans in R. sphaeroides 2.4.1 during photoheterotrophic growth (10 W/m²).
loss of O₂ repression, as shown in pCF230(−348) and pCF240(−225). Conversely, the presence of the region from the HgaI to StuI sites appears to be required for increased expression at 10 W/m² when O₂ regulation is lost. Thus, a possible activator region involved in light regulation may be present immediately upstream of the HgaI site. When O₂ regulation is lost because of the absence of additional upstream sequences, it appears that light regulation is decoupled, so the regions for O₂ and light control may be functionally interactive although physically separable.

To further support these interpretations, we monitored β-galactosidase levels as described in the legend to Fig. 2B, i.e., continuously monitoring enzyme levels at constant cell densities as a function of increased cell growth resulting in diminished light intensity (Fig. 4). It is readily apparent that the inclusion of the region from StuI to the proximal XhoI site in pCF230(−348) resulted in fully active light regulation. Similarly, light regulation of puc operon expression was also present in pCF240(−225) (HgaI-StuI), but it was not as pronounced as with pCF230(−348). As a control, pCF250(−92) showed no light regulation. Thus, the DNA sequence including the HgaI site to the StuI site appears to be required for enhanced light control of puc operon expression and the region from XhoI to BamHI appears to be essential for the repression of puc operon expression by O₂. It is evident that a more thorough analysis of the region from HgaI to BamHI is warranted.

These results are further supported by the β-galactosidase levels observed when pCF210(Δ130), pCF213(Δ255), and pCF214(Δ414) were present in R. sphaeroides 2.4.1 grown chemoheterotrophically and photoheterotrophically (Fig. 3). With pCF210(Δ130), we observed relatively normal aerobic versus anaerobic regulation, as is expected when the region downstream of the XmaIII site is intact. We also saw near-normal O₂ control as well as light control when cells were grown at 10 W/m², because these putative elements are believed to be intact and brought closer to the region downstream of the XmaIII site. However, it remains to be determined whether the full range of enhanced expression due to limiting light was still present. The importance of the 130-bp region between XmaIII and StuI is best illustrated by the results obtained with pCF216(130 inversion) (Fig. 3). This construction contains the XmaIII-StuI enhancer DNA region, which has been inverted without a change in the length of the overall regulatory fragment. This construction showed the same levels of β-galactosidase activity as did pCF250(−92) when grown photosynthetically, as was expected because the region downstream of XmaIII was unaltered. However, in contrast to the results for pCF200(−629), we did not observe enhanced anaerobic expression, a result which may indicate a diminished interaction between the upstream regulatory region (URS) (−629 to −150) and the downstream regulatory region (DRS) (−150 to −1). O₂ control, however, was shown to be partially derepressed in pCF216(130 inversion) for the same reasons. Thus, the enhancer region, XmaIII-StuI containing overlapping con-
sensus sequences for IHF (integration host factor) and FNR (fumarate nitrate reductase) (see below), appears to be required for normal O₂ and anaerobic control. This model is partially supported by the results observed when pCF214(A414) was placed in trans in the wild type. Most upstream regulatory signals were absent, yet we did not see highly derepressed expression of the puc operon in the presence of O₂. This result could be explained by the absence of the enhancer region or by the existence of a second O₂-responsive element in the region of the distal XhoI site. However, we did see near-normal light and anaerobic control, a result which is difficult to reconcile with the information presently available. The fact that anaerobic control was near normal is consistent with the region downstream of XmaIII being intact.

Finally, how do we reconcile the results observed when pCF213(Δ255) was placed in trans with those described above? At face value, the presence of the purported O₂ regulatory region lying upstream of the proximal XhoI site would have been expected to result in low, wild-type levels of β-galactosidase when cells were grown chemoheterotrophically, but to the contrary, the highest level of LacZ activity was observed. Two reasonable explanations come to mind: (i) there is a position or spacing effect; (ii) the O₂ regulatory region remains intact but, when it is positioned immediately upstream of the normal downstream sequences bounded at their 5' ends by the XmaIII site, protein binding to the O₂ regulatory region now serves the role of the missing enhancer region, thus resulting in high-level expression of the puc operon under aerobic conditions. Clearly, the results derived from the analysis of internal deletions are much more difficult to interpret, undoubtedly because of the difficulties encountered when the correct spacing of putative cis-acting elements is affected.

Localization of the puc promoter(s) to a 35-bp DNA sequence between −57 and −92. As described above, puc promoter activity is associated with a 60-bp DNA region (−32 to −92) between the XmaIII and Hinfl restriction sites limiting pCF250(−92) and pCF252(−32), respectively. pCF252(−32) contains one of two similar regions of dyad symmetry, TGTCG-8 bp-TGACA (Fig. 5), located at −10 to −27. The second, very similar sequence is located at −35 to −52 (Fig. 5B) and is situated immediately upstream of the Hinfl restriction site limiting pCF252(−32). Since the most downstream region of dyad symmetry present on pCF252(−32) was shown not to be associated with promoter function, we examined whether the upstream region of dyad symmetry (−35 to −52) or both regions taken together possessed promoter function. We did this by constructing another set of plasmid-localized transcriptional fusions, pCF450(−92), pCF460(+36), and pCF500(−57) (Fig. 6). These fusions contained the lacZ gene fused to the DraII restriction site of the puc DNA, which is located 70 nt downstream of the 5' end of the puc transcripts in the untranslated leader region of the transcripts. These fusions were constructed in the same way as the other puc-lacZ fusions, which employed the XmnI restriction site within the pucB gene.
FIG. 5. (A) DNA sequence of 148 bp immediately upstream of the 5' ends of the puc-specific transcripts. The DNA region designated by the open arrow (b) was protected by purified E. coli IHF in a DNase I footprinting analysis (13a). An overlapping box designates the putative FNR-like sequence (a). Promoter(s) function was shown to be associated with a 35-bp DNA region between the XmaIII and HaeIII restriction sites. The putative promoter sequence is shown (c). Two regions of dyad symmetry are shown (d and e). The cis mutations at -26 and -12 are identified by asterisks over the corresponding bases within region e. (B) DNA sequence homology between dyad symmetry regions d and e.

pCF500(-57) limited by the HaeIII restriction site contained both regions of dyad symmetry but yielded only background levels of β-galactosidase activity when present in cells grown under both chemoheterotrophic and photoheterotrophic conditions (Fig. 6). pCF460(+36), which contained puc upstream DNA limited by the BstNI restriction site (36 bp downstream of the 5' end of the puc transcript), served as a negative control (Fig. 6). pCF450(-92), defined by the XmaIII restriction site, showed, like pCF250(-92), ~9-fold induction of β-galactosidase activity when present in cells grown under photoheterotrophic conditions, compared with the activity observed during chemoheterotrophic growth. This result strongly indicates that oxygen-regulated puc promoter(s) function is tightly associated with the 35-bp DNA region between the XmaIII(-92) and HaeIII(-57) restriction sites (Fig. 5A).

Additionally, the β-galactosidase activity of pCF450(-92) (Fig. 6) was fivefold lower than that of pCF250(-92) (Fig. 3) under both chemoheterotrophic and photoheterotrophic conditions, although the degree of anaerobic control (the fold derepression following a shift from aerobic to photosynthetic growth) was the same. This was true despite the fact that the two fusion plasmids shared the same 5' limit (XmaIII restriction site) of the puc upstream DNA. The higher
activity associated with pCF250(-92) may have been due to the additive effect of two regions of translation initiation, i.e., the two-cistron effect, as has been observed for other systems (25). The presence of a preceding cistron on pCF250 (-92) as opposed to pCF450(-92) results in increased transcriptional expression of the downstream cistron. However, our basic interpretation of the data is not altered.

Selection of cis-acting mutations giving rise to derepression of puc operon transcription in the presence of O₂. As an alternative approach to the isolation of cis-acting (relative to the reporter gene) regulatory sequences involved in the transcriptional regulation of the puc operon by oxygen, we fused the promoterless fragment of the aminoglycoside-3’-phosphotransferase gene from Tn903 (26) to the 699-bp PstI-DraII restriction endonuclease fragment described in Fig. 6. Wild-type cells containing the plasmid-derived fusion in trans cloned into the vector were plated in the presence of kanamycin (20 μg/ml) under chemoheterotrophic growth conditions (22). Although both trans-acting (chromosomal) and cis-acting (plasmid) spontaneous mutations were isolated, three cis-acting mutations were used for further analysis. Each of the 699-bp PstI-DraII restriction endonuclease fragments containing the mutations was cloned into the PstI-HincII sites of the multiple cloning region of pBS and sequenced by use of the T3 or T7 primers flanking the multiple cloning region. One mutation representing a transition, G to A, was found at -26, while the other two mutations were transversions of A to C and were localized to -12 (Fig. 7). These two separate cis-acting mutations, -26 and -12, were localized within the region of symmetry designated “e” in Fig. 5A. The nature of the trans-acting mutations are discussed in the accompanying paper (22).

To determine the physiological effects of the cis mutations at -26 and -12 on the transcriptional regulation of the puc operon, we transcriptionally fused the DNA fragments containing the mutations to the lacZ gene and measured the β-galactosidase activities of the fusion constructs in plasmid constructions placed in trans in R. sphaeroides under both chemoheterotrophic and phototrophetic growth conditions 2.4.1 (Fig. 8). Under chemoheterotrophic growth conditions, the cis mutation at -26, pCF300(-629), derepressed the puc operon 5-fold, while the β-galactosidase activities of pCF302(-629) and pCF304(-629), with cis mutations at -12, were increased 9- to 8-fold compared with that of the wild-type fusion construct, pCF400(-629), pCF350(-629), pCF352(-92), and pCF354(-92), derived from pCF300 (-629), pCF302(-629), and pCF304(-629), respectively, are limited by the XmaIII restriction site as the 5’ boundary of puc upstream DNA. Interestingly, pCF350(-92), pCF352 (-92), and pCF354(-92) showed levels of β-galactosidase activity similar to that of the corresponding wild-type fusion construct, pCF450(-92), under chemoheterotrophic or phototrophetic growth conditions. Therefore, the 536-bp PstI-XmaIII puc upstream DNA was required in cis for the mutations at -26 and -12 to derepress puc operon transcription in the presence of oxygen. This observation provides convincing evidence for the interaction of upstream DNA sequences (URS) with downstream sequences (DRS) in modulating puc operon expression.

Under phototrophetic growth conditions, the cis mutation at -26, pCF300(-629), yielded β-galactosidase activity similar to that of the wild-type fusion construct, pCF400(-629). However, the cis mutation at -12 yielded in excess of a twofold increase in β-galactosidase activity compared with that of pCF400(-629). Again, however, this effect was only observed when the 536-bp puc upstream DNA sequence was present in cis to the point mutations shown for pCF302(-629) and pCF304(-629). Thus, the presence of the upstream DNA sequences suggests an interaction with the downstream DNA sequences. The extent of the upstream regulatory sequences which are required to interact with these downstream regulatory sequences remains to be determined. Furthermore, this interaction serves to complicate the analysis of light and O₂ control.

**DISCUSSION**

It was previously shown (21) that the steady-state level of the 0.5-kb puc-specific transcript in R. sphaeroides 2.4.1 was both O₂ and light regulated, increasing approximately three- to sixfold under phototrophetic conditions at 10 and 3 W/m², respectively, compared with the level of the puc transcript in cells grown at 100 W/m². Additionally, we also demonstrated the presence of a second, less abundant 2.3-kb puc-specific transcript which has the same 5’ end (117 nt upstream of the start of pucB) and the same O₂ regulatory activity as the 0.5-kb transcript (24). The 2.3-kb puc-specific transcript spanning approximately 1.8 kb of puc DNA immediately downstream from the 3’ end of the 0.5-kb tran-
script encodes an additional gene(s) which is involved in the posttranscriptional expression of the pucBA gene products to productively assemble into a functional B800-850 LH complex (24). Recently, through the use of lacZ translational fusions to the pucB or pucA gene crossed into the chromosome of R. sphaeroides PUC-Pv, containing an interruption downstream of pucBA, we found that the pucBA genes are translated (23). Thus, the region downstream of pucBA appears to encode activities which are involved in the posttranslational regulation of the pucBA gene products.

We have also shown that the 629-bp DNA region upstream from the start site of puc operon transcription is apparently sufficient for the normal expression of puc operon transcription (24). To better delineate the role(s) of these upstream sequences in regulating puc operon transcription, we used a number of puc-lacZ transcriptional fusions placed in trans and also isolated cis-acting mutations (relative to the reporter gene) leading to the derepression of puc operon transcription in the presence of oxygen.

All of the studies described here were performed with an otherwise wild-type genetic background, in trans on a plasmid whose copy number is between four and six in R. sphaeroides 2.4.1 (34). However, because all of the constructions are internally consistent, our interpretation of these results is not likely to be affected by plasmid copy number, although this should always be a concern when working with plasmids.

For ease of discussion we have divided the upstream regulatory region into two functional domains, the most proximal extending from just upstream (−150) of the XmaIII site at −92 to the start of transcription. The observation that this region alone is sufficient for both puc operon expression and anaerobic control, i.e., approximately 10- to 20-fold derepression of puc operon transcription when photoheterotrophic cells are compared with chemoheterotrophic cells, is consistent with the earlier results of Kiley and Kaplan (21), who monitored puc operon expression in vitro. Furthermore, the results reported here also reveal that this region by itself is not sufficient for fully enhanced expression of the puc operon either by light or by the genetic removal of O₂ repression. However, this region is sufficient for fully repressed expression in the presence of O₂. Both the sigmoidal nature of the increase in puc operon expression with decreasing light availability at constant cell densities and the analysis of puc operon expression at constant light intensities with various deletion derivatives within the region upstream of the XmaIII site suggest the existence of a potential regulatory region involved in light control between −150 and the most proximal XhoI site. Although the data are

![FIG. 8. β-Galactosidase activities associated with transcriptional fusions of the lacZ gene to the puc upstream DNA containing the cis mutations at −26 and −12. pCF400 (−629) and pCF450 (−92) are wild-type fusion constructs containing the entire PstI-DraII sequence and the XmaIII-DraII sequence upstream of pucB, respectively. pCF460 (+36) contains the BstNI-DraII fragment upstream of pucB and was used as a negative control together with pCF100, which contains no puc upstream DNA. pCF300 (−629) and pCF350 (−92) correspond to pCF400 (−629) and pCF450 (−92), respectively, and contain the cis mutation at −26. From two independent isolates containing the −12 mutation, the following series of fusions were derived: (i) pCF302 (−629) (PstI-DraII) and pCF352 (−92) (XmaIII-DraII) and (ii) pCF304 (−629) (PstI-DraII) and pCF354 (−92) (XmaIII-DraII).]
still preliminary, one element of light regulation would appear to involve activation.

The data also support the conclusion that the promoter(s) for puc operon expression lies within the 35-bp region bounded by the XmaI(III) (−92) and HaeIII(−57) restriction sites. Within this region is contained the sequence (−84) TGGC-10 bp-TGGCA (−66). A similar sequence (TGGC-8 bp-CCGCA) is present at between −80 and −40 upstream of the start sites of the puc transcripts of R. capsulatus (36, 37). We have also found a sequence, (−86) CGCG-12 bp-TCGGT (−64), upstream of the start site for Q gene transcription in R. sphaeroides (22), as well as a comparable sequence (CGCG-8 bp-TGTT) located at −85 to −67 upstream of the start of Q gene transcription in R. capsulatus (3). A consensus sequence derived from these four sequences is T/CGGC-N8_12/T/CCGC/A/T.

Although we have suggested that the region from −84 to −66 in R. sphaeroides serves as the puc promoter, it is also possible that this region controls the binding of a specific transcriptional regulatory protein(s) involved in promoting local denaturation of the puc promoter located further downstream for transcription of the puc operon by RNA polymerase. Although there is a sequence, (−30) CTGACA (−35), which is similar to the −35 consensus sequence of the E. coli σ70-type promoter (16), no −10-like sequence was found. It is further unlikely that this −35-like sequence serves to regulate puc operon expression because puc-lacZ fusions containing this sequence up to −57 showed only background levels of β-galactosidase activity under different growth conditions.

Further downstream of the region from −84 to −66 are two very similar sequences of dyad symmetry, TGTCG-N8-TGACA (Fig. 5B), which are characteristic of the recognition sites for the multi-mer A DNA-binding proteins involved in transcriptional regulation (14). In R. capsulatus, a similar sequence (one) of dyad symmetry (TGTAAN4-TTACA) is present between −65 and −20 (35, 36). No such palindromic sequences were found upstream of the Q genes in either R. sphaeroides (22) or R. capsulatus (3). However, a similar motif, TGTCART-N4-A-N4-TTACA (R. purine), has been reported for the 5′-flanking sequences of cregA, -T, -D, and -E within the creg gene cluster of R. capsulatus (2). There are additional sequences centered at −368 and −167, but only that at −167 contains dyad symmetry and strongly resembles the consensus sequence TGTCG-N8-TGACA. These could be involved in the interaction between the URS and the DRS.

Mutations isolated at −26 and −12 and leading to partial derepression of puc operon expression in the presence of O2 provide direct evidence for the importance of the most downstream of the two palindromic sequences in puc operon transcription. The fact that these mutations required intact upstream (−629 to −92) sequences for their expression in the presence of O2 as well as under photosynthetic growth conditions strongly indicates an interaction between these two domains, i.e., −629 to −150 and −150 to −1, for normal puc operon expression. Additionally, the fact that the mutation at −26 was only effective under chemoheterotrophic conditions while the mutation at −12 was effective under both aerobic and photosynthetic growth conditions further supports the idea that this downstream region interacts with a DNA sequence further upstream. Importantly, however, anaerobic control is similar and functional with both mutations, as revealed by the results obtained when only the downstream domain was present. Therefore, aerobic versus anaerobic control of puc operon expression appears to be readily separable from the full magnitude of enhanced derepressional regulation of gene expression which can be exerted on the puc operon.

At the most proximal end of the upstream domain involved in puc operon expression, between −117 and −105, is a putative IHF-binding site (13, 15), TTTCAA-N4-TTA. This region was protected by E. coli IHF in DNase I footprinting analysis (13a). Furthermore, there is also a consensus FNR-binding site (8) which overlaps the distal portion of the IHF-binding site. Thus, this region may serve to both bend the DNA, bringing the URS into a close approximation with the downstream domain, as well as enhance the transcription of the puc operon in both chemoheterotrophically and photoperheterotrophically grown cells beyond the levels observed when only the downstream domain is present. This enhancement is presumed to take place in conjunction with factor binding.

Although much more work is required to precisely define the DNA sequences within the upstream domain which appear to be involved in O2 repression and light control of puc operon expression, several points seem clear. The O2 regulatory region appears to be at least partially separable from and further upstream of the light regulatory region because relief from O2 repression occurs when the most distal sequences of the URS are removed and light regulation appears to require the presence of the more proximal sequences of the URS. We therefore tentatively suggest that O2 regulation involves a repressor(s) and light regulation involves an activator(s). It is further likely that these two regions have some form of hierarchical relationship, with O2 repression at the top of the hierarchy.

The region immediately upstream of and including the FNR- and IHF-binding sites appears to link the upstream and downstream domains and is involved in enhancing full transcriptional expression of the puc operon. Lastly, in light of the recent compilation and analysis of a large number of σ70 and σ54-regulated operons by Collado-Vides et al. (7), we propose that the regulatory region involved in puc operon expression has elements of both types of regulatory circuits. On the one hand, the presence of duplicated potential operator regions downstream of the proposed promoter, with perhaps a third operator upstream of the promoter element, shows a clear resemblance to a σ70 form of regulation. However, the presence of activator sequences, in particular, both IHF- and FNR-like sequences, as well as a clear requirement for an interaction(s) between the upstream and downstream sequences is more typical of a σ54 type of regulation. The model which we have developed is generally consistent with the results observed. However, we fully appreciate that as work develops in this and other systems, numerous iterations of the model will doubtlessly be necessary.

Thus, these studies strongly suggest that the regulation of puc operon expression by O2 and light is exceedingly complex and furthermore that trans-acting factors play a critical role in the expression of the puc operon. The studies described in the accompanying paper have documented the existence of some, but probably not all, of these trans-acting factors (22).

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REFERENCES

1. Aagaard, J., and W. R. Sistrom. 1972. Control of synthesis of reaction center bacteriochlorophyll in photosynthetic bacteria. Photochem. Photobiol. 15:209–225.
2. Armstrong, G. A., M. Alberti, F. Leach, and J. E. Hearst. 1989. Nucleotide sequence, organization, and nature of the protein products of the carotenoid biosynthesis gene cluster of Rhodobacter capsulatus. Mol. Gen. Genet. 216:254–268.
3. Bauer, C. E., D. A. Young, and B. L. Marrs. 1988. Analysis of the Rhodobacter capsulatus puf operon. J. Biol. Chem. 263:4820–4827.
4. Chory, J., T. J. Donohue, A. R. Varga, L. A. Stachelin, and S. Kaplan. 1984. Induction of the photosynthetic membrane of Rhodopseudomonas sphaeroides: biochemical and morphological studies. J. Bacteriol. 159:540–544.
5. Chory, J., and S. Kaplan. 1983. Light-dependent regulation of the synthesis of soluble and intracytoplasmic membrane proteins of Rhodopseudomonas sphaeroides. J. Bacteriol. 153:465–474.
6. Cogdell, R. J., and J. P. Thornber. 1980. Light-harvesting pigment-protein complexes of purple photosynthetic bacteria. FEBS Lett. 122:1–8.
7. Collado-Vides, J., B. Magasanik, and J. D. Gralla. 1991. Control site location and transcriptional regulation in Escherichia coli. Microbiol. Rev. 55:371–394.
8. Colonna-Romano, S., W. Arnold, A. Schluter, P. Boistart, A. Puhler, and U. B. Priester. 1990. An Fnr-like protein encoded in Rhizobium leguminosarum biovar vicieae shows structural and functional homology to Rhizobium mellioti FixK. Mol. Genet. 223:138–147.
9. Davis, J., T. J. Donohue, and S. Kaplan. 1988. Construction, characterization, and complementation of a Puf mutant of Rhodobacter sphaeroides. J. Bacteriol. 170:320–329.
10. DeHoff, B. S., J. K. Lee, T. J. Donohue, R. I. Gumport, and S. Kaplan. 1988. In vivo analysis of puf operon expression in Rhodobacter sphaeroides following deletion of a putative intercistronic terminator. J. Bacteriol. 170:4681–4692.
11. Donohue, T. J., and S. Kaplan. 1986. Synthesis and assembly of bacterial photosynthetic membranes, p. 632–639. In L. A. Stachelin and C. J. Arntzen (ed.), Photosynthetic membranes. Encyclopedia of plant physiology, vol. 19 (new series). Springer-Verlag, New York.
12. Donohue, T. J., A. G. McEwan, and S. Kaplan. 1986. Cloning, DNA sequence, and expression of the Rhodobacter sphaeroides cytchrome c gene. J. Bacteriol. 168:962–972.
13. Friedman, D. I. 1988. Integration host factor: a protein for all reasons. Cell 55:545–554.
13a. Gardner, J., J. K. Lee, and S. Kaplan. Unpublished results.
14. Gicquel-Sanzy, B., and P. Cossart. 1982. Homologies between different probkaryotic DNA-binding regulatory proteins and between their sites of action. EMBO J. 1:591–595.
15. Hoover, T. R., E. Santero, S. Porter, and S. Kustu. 1990. The integration host factor stimulates interaction of RNA polymerase with NIFA, the transcriptional activator for nitrogen fixation operons. Cell 63:11–22.
16. Horwitz, M. S. Z., and L. A. Loeb. 1990. Structure-function relationships in Escherichia coli promoter DNA. Prog. Nucleic Acid Res. Mol. Biol. 38:137–164.
17. Kaplan, S. 1978. Control and development of photosynthetic membrane development, p. 809–839. In R. K. Clayton and W. R. Sistrom (ed.), The photosynthetic bacteria. Plenum Publishing Corp., New York.
18. Kaplan, S., and C. J. Arntzen. 1982. Photosynthetic membrane structure and function, p. 65–151. In Govindjee (ed.), Photosynthesis: energy conversion by plants and bacteria, vol. 1. Academic Press, Inc., New York.
19. Kaplan, S., B. D. Cain, T. J. Donohue, W. D. Shepherd, and G. S. L. Yen. 1983. Biosynthesis of the photosynthetic membranes of Rhodopseudomonas sphaeroides. J. Cell. Biochem. 22:15–29.
20. Keen, N. T., S. Tamaki, D. Kobayashi, and D. Trolinger. 1988. Improved broad-host range plasmids for DNA cloning in gram-negative bacteria. Gene 70:191–197.
21. Kiley, P. J., and S. Kaplan. 1987. Cloning, DNA sequence, and expression of the Rhodobacter sphaeroides light-harvesting B800-850-α and B800-850-β genes. J. Bacteriol. 169:3268–3275.
22. Lee, J. K., and S. Kaplan. 1992. Isolation and characterization of trans-acting mutations involved in oxygen regulation of puc operon transcription in Rhodobacter sphaeroides. J. Bacteriol. 174:1158–1171.
23. Lee, J. K., and S. Kaplan. Unpublished results.
24. Lee, J. K., P. J. Kiley, and S. Kaplan. 1989. Posttranscriptional control of puc operon expression of B800-850 light-harvesting complex formation in Rhodobacter sphaeroides. J. Bacteriol. 171:3391–3405.
25. Makoff, A. J., and A. E. Smallwood. 1990. The use of two-cistron construction in improving the expression of a heterologous gene in E. coli. Nucleic Acids Res. 18:1711–1718.
26. Oka, A., H. Sugisaki, and M. Takamine. 1981. Nucleotide sequence of the kanamycin resistance transposon Tn903. J. Mol. Biol. 147:217–226.
27. Okamura, M. Y., L. A. Steiner, and G. Feher. 1974. Characterization of reaction centers from photosynthetic bacteria. I. Subunit structure of the protein mediating the primary photochemistry of Rhodopseudomonas sphaeroides R-26. Biochemistry 13:1394–1403.
28. Prentki, P., and H. M. Kirsch. 1984. In vitro insertional mutagenesis with a selectable DNA fragment. Gene 29:303–313.
29. Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. Simons, R., U. Prierer, and A. Puhler. 1983. A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram-negative bacteria. Bio/Technology 1:37–45.
31. Simons, R. W., F. Houman, and N. Kleckner. 1987. Improved single and multicopy lac-based cloning vectors for protein and operon fusions. Gene 53:85–96.
32. Summerton, J., T. Atkins, and R. Bestwick. 1983. A rapid method for preparation of bacterial plasmids. Anal. Biochem. 133:79–84.
33. Tai, S. P., and S. Kaplan. 1985. Intracellular localization of phospholipid transfer activity in Rhodopseudomonas sphaeroides and a possible role in membrane biogenesis. J. Bacteriol. 164:181–186.
34. Tai, T. N., W. A. Havelka, and S. Kaplan. 1988. A broad-host range vector system for cloning and translational lacZ fusion analysis. Plasmid 19:175–188.
35. Tichy, H. V., B. Oberle, H. Stiehle, E. Schiltz, and G. Drews. 1989. Genes downstream from pucB and pucA are essential for formation of the B800-850 complex of Rhodobacter capsulatus. J. Bacteriol. 171:4914–4922.
36. Youvan, D. C., and S. Ismail. 1985. Light-harvesting II (B800-850 complex) structural genes from Rhodopseudomonas capsulata. Proc. Natl. Acad. Sci. USA 82:58–62.
37. Zacconi, A., and J. T. Beatty. 1988. Posttranscriptional regulation by light of the steady-state levels of mature B800-850 light-harvesting complexes in Rhodobacter capsulatus. J. Bacteriol. 170:877–882.