ChrR Positively Regulates Transcription of the *Rhodobacter sphaeroides* Cytochrome c_2 Gene

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Received 19 October 1994/Accepted 6 February 1995

Transcription of the *Rhodobacter sphaeroides* cytochrome c_2 gene (*cycA*) is negatively regulated by both the presence of oxygen and intermediates in tetrapyrrole biosynthesis. A mutation responsible for uncoupling *cycA* transcription from tetrapyrrole availability was localized to a gene (*chrR*) that encodes a 357-amino-acid protein. Analysis of a defined *chrR* null mutation indicated that this protein positively regulated *cycA* transcription. From this and other results, it appeared that the positive action of ChrR on *cycA* transcription is blocked by altering the availability of either heme or some intermediate in tetrapyrrole biosynthesis. A single missense mutation which substitutes an Arg for a Cys at residue 182 of ChrR (C182R) was shown to be necessary and sufficient for the increased *cycA* transcription seen in the mutant strain Chr4. Thus, it appears that this C182R substitution generated an altered-function form of ChrR. In addition, by analyzing *cycA* transcription in Δ ChrR strains, we showed that ChrR was not required for increased *cycA* transcription under anaerobic conditions. Instead, our results indicated that ChrR and the response regulator PrrA (J. M. Eraso and S. Kaplan, J. Bacteriol. 176:32–43, 1994) functioned independently at the upstream *cycA* promoter that is activated under anaerobic conditions.

Rhodobacter sphaeroides cytochrome c_2 (cyt c_2) is a heme-containing electron transfer protein (25) that functions in a branch of the aerobic respiratory chain similar to that found in mitochondria (2, 38, 39). In addition, under anaerobic conditions cyt c_2 is an essential component of the photosynthetic electron transport chain of this bacterium (9). While much is known about the structure and function of several members of the cyt c_2 family, little information is available about how their synthesis is regulated. To address this problem, we have been studying how the activity of individual promoters for the *R. sphaeroides* cyt c_2 structural gene (*cycA*) responds to different input signals.

When the expression of structural genes for electron carriers is considered, a common regulatory strategy whereby transcription is coupled to the availability of the ligand required for function appears to emerge (3–5, 12, 23, 40). For example, we have previously shown that transcription initiation at multiple *cycA* promoters was rapidly blocked by the addition of δ -aminolevulinic acid (ALA) (28). Since ALA is the first committed precursor in heme biosynthesis, this response suggested that ligand availability regulated cyt c_2 synthesis. In addition, a class of cobaltic heme-resistant (Chr) mutants in which transcription from several *cycA* promoters was resistant to the negative effects of ALA addition have been isolated (28).

In this paper, we describe the gene product (ChrR) responsible for the altered *cycA* transcription in one such mutant (Chr4). Evidence that ChrR acts positively comes from the fact that a defined *chrR* null mutation reduced β -galactosidase (LacZ) levels from *lacZYA* operon fusions under control of different *cycA* promoters. When viewed in light of our previous data (28), the positive action of wild-type ChrR on *cycA* transcription could be blocked by conditions that increase the

availability of either heme or some intermediate in tetrapyrrole biosynthesis.

In addition to responding to changes in ligand availability, *cycA* transcription increases under anaerobic conditions where function of cyt c_2 is required to support photosynthetic growth (19, 28). The increase in total *cycA* transcription that occurs under anaerobic conditions results primarily from differential activation of the upstream control region (–484 to –104 relative to the start of *cycA* translation [28]). Eraso and Kaplan (11) recently described a global response regulator (PrrA) which controls transcription of *cycA* and several other genes encoding proteins required for photosynthetic growth. Our results indicate that maximal transcription from the upstream *cycA* control region required a functional copy of both ChrR and PrrA and that these proteins functioned independently. In addition, mutant analysis indicates that PrrA acted only at this upstream *cycA* promoter, while ChrR regulated transcription from additional downstream *cycA* promoters. Thus, our results suggested that ChrR acts to link transcription from multiple *cycA* promoters to changes in ligand availability that could occur in the presence or absence of oxygen.

MATERIALS AND METHODS

Bacterial strains and plasmids. The plasmids and bacterial strains used are listed in Table 1. *Escherichia coli* DH5 α was used as a plasmid host, and S17-1 was used as a donor for conjugal DNA transfer into *R. sphaeroides*.

Growth of bacteria and genetic procedures. Derivatives of *R. sphaeroides* 2.4.1 were grown aerobically or photosynthetically in Sistrom's minimal medium A (30) as previously described (8). Cell growth was monitored turbidimetrically (35), and cells were harvested during exponential-phase growth (5×10^8 to 1×10^9 cells per ml). Antibiotics used to select for chromosomal or plasmid-encoded drug resistance markers in *R. sphaeroides* included kanamycin (25 μ g/ml), tetracycline (1 μ g/ml), spectinomycin (25 μ g/ml), and trimethoprim (30 μ g/ml). To minimize tetracycline photo-oxidation in photosynthetic cultures, a Carolina Biological far-red 750 filter was placed in front of the light source (15).

E. coli strains were grown in Luria broth (20). Antibiotic concentrations used to maintain plasmids in *E. coli* were as follows: kanamycin, 25 μ g/ml; tetracycline, 10 μ g/ml; spectinomycin, 25 μ g/ml; trimethoprim, 100 μ g/ml; and ampicillin, 50 μ g/ml.

Plasmid DNA was mobilized into *R. sphaeroides* by either filter (9) or liquid

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Description	Reference or source
<i>R. sphaeroides</i>		
2.4.1	Wild type	Laboratory strain
Chr4	Cohemin-resistant, <i>chr-4</i> derivative of 2.4.1	28
TF18	<i>chrR</i> null mutation in 2.4.1	This study
Chr4TpA	<i>chrR</i> null mutation in Chr4	This study
PRRA1	<i>prpA</i> null mutation in 2.4.1	11
Chr4PR	<i>prpA</i> null mutation in Chr4	This study
TpAPR	<i>prpA</i> and <i>chrR</i> null mutations in Chr4	This study
<i>E. coli</i>		
DH5 α	<i>supE44</i> Δ <i>lacU169</i> (ϕ 80 <i>lacZ</i> ΔM15) <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i>	Bethesda Research Laboratories (Gaithersburg, Md.)
S17-1	C66::RP4 2-(Tc::Mu) (Kn::Tn7) <i>thi pro hsdR hsdM⁺ recA</i>	29
Plasmids		
pUC18	Ap ^r	36
pUC19	Ap ^r	36
pSP64	Ap ^r	Promega
pSUP202	Ap ^r Tc ^r Cm ^r Mob ⁺ pBR325 derivative	29
pLA2917	Kn ^r Tc ^r RK2 derivative, <i>cos</i> site	1
pChr66	Tp ^r Ap ^r	27
pBS5	Sp ^r Kn ^r <i>cycA</i> (−484 to −124):: <i>lacZYA</i>	28
pBS6	Sp ^r Kn ^r <i>cycA</i> (−104 to +94):: <i>lacZYA</i>	28
pBS6K [−]	pBS6 with Kn ^r removed by <i>Pst</i> I digestion	This study
pUI8089	Tc ^r ~29-kb fragment with <i>chrR</i> from 2.4.1 on pLA2917	This study
pBS12	Tc ^r 3.7-kb <i>Sal</i> I fragment containing <i>chrR</i> from 2.4.1 on pLA2917	This study
pBS13	Tc ^r 1.4-kb <i>Stu</i> I- <i>Sst</i> I fragment containing <i>chrR</i> from 2.4.1 on pLA2917	This study
pBS15	3.7-kb <i>Sal</i> I fragment containing <i>chrR</i> cloned into pUC19 in same orientation as P _{lac}	This study
pBS16	1.8-kb <i>Sal</i> I- <i>Sst</i> I fragment containing <i>chrR</i> cloned into pUC19 in same orientation as P _{lac}	This study
pBS17	1.8-kb <i>Sal</i> I- <i>Sst</i> I fragment containing <i>chrR</i> in opposite orientation of pBS16	This study
pBS18	1.4-kb <i>Stu</i> I- <i>Sst</i> I <i>chrR</i> fragment cloned into pUC18 in same orientation as P _{lac}	This study
pTF1	Internal <i>Eco</i> RV- <i>Nru</i> I fragment of pBS15 replaced by 1.6-kb <i>Pvu</i> II Tp ^r gene fragment from pChr66	This study
pTF2	4.8-kb fragment containing <i>chrR</i> and Tp ^r from pTF1 cloned into pSUP202	This study
pBS19	pSP64 + 1.4-kb <i>Stu</i> I- <i>Sst</i> I <i>chrR</i> fragment from 2.4.1	This study
pBS20	pSP64 + 1.4-kb <i>Stu</i> I- <i>Sst</i> I <i>chrR</i> fragment from Chr4	This study
pBS21	pSP64 + 1.3-kb <i>Xmn</i> I- <i>Sst</i> I <i>chrR</i> fragment from 2.4.1	This study
pBS22	pSP64 + 1.3-kb <i>Xmn</i> I- <i>Sst</i> I <i>chrR</i> fragment from Chr4	This study
pBS23	pBS19 with stop linker cloned in <i>Sfi</i> I site	This study
pBS24	Tc ^r 3.7-kb <i>Sal</i> I fragment containing <i>chrR</i> gene from Chr4 on pLA2917	This study
pUI1625	<i>prpA</i> ::Ω Sp ^r 2.2-kb fragment in pSUP203	11

(26) matings. Mating pairs were plated aerobically on Sistrom's minimal medium A containing the appropriate antibiotics to obtain isolated exconjugants.

A mobilizable cosmid bank of *R. sphaeroides* DNA (10) was screened initially in groups of 10 and then individually to identify a cosmid that reduced LacZ levels from a specific *cycA*::*lacZ* reporter plasmid (pBS6K[−]) in Chr4. When LacZ levels from *cycA*::*lacZ* operon fusions were screened, solid media were supplemented with 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside per ml. To prevent recombination between the *kan* genes on the vector (pLA2917) and the reporter plasmid used in the complementation test, the pBS6 drug resistance gene was removed by *Pst*I digestion to generate pBS6K[−]. When complementation by smaller pieces of DNA was tested, the pLA2917 *kan* gene was removed by *Hind*III-*Hpa*I digestion. For these experiments, candidate restriction fragments were isolated from pUC derivatives (Table 1) with *Hind*III and *Sma*I or *Hinc*II prior to cloning into *Hind*III- and *Hpa*I-digested pLA2917.

Construction of ChrR and PrpA null mutants. To construct a *chrR* allele, an internal 850-bp *Eco*RV-*Nru*I restriction fragment (see Fig. 1) of *chrR* was replaced with a 1.7-kb *Pvu*II restriction fragment containing a trimethoprim resistance gene from pChr66 (27). The resultant plasmid (pTF1) was digested with *Hind*III and *Sma*I to generate a restriction fragment carrying the interrupted *chrR* gene. The *Hind*III site was filled with Klenow fragment DNA polymerase before cloning into a filled-in *Eco*RI site of the mobilizable suicide plasmid pSUP202 (29). This plasmid (pTF2) was used to construct *R. sphaeroides* ΔChrR strains by homologous recombination (9). Trimethoprim-resistant colonies were screened for the loss of plasmid sequences (i.e., gene replacement) by testing for tetracycline sensitivity. Southern blot analysis confirmed the exchange of wild-type *chrR* with a trimethoprim resistance gene (data not shown).

PrpA null mutants were constructed with the suicide plasmid pUI1625 (11). In this case, gene replacement was identified by selecting for spectinomycin resistance and testing for tetracycline sensitivity.

Cloning a mutant *chrR* gene. To clone *chr-4*, agarose molds containing *R. sphaeroides* genomic DNA (31, 32) from strain Chr4 were treated with *Sal*I (34). Approximately 3.8-kb restriction fragments were purified with Gene Clean (Bio 101, Inc., La Jolla, Calif.). After cloning into *Sal*I-digested pUC18, transformants were screened by hybridization with an internal *chrR* probe on Colony/Plaque screen filters (New England Nuclear, Boston, Mass.) by using the manufacturer's recommendations and a prewash step prior to hybridization (20). Plasmid DNA from candidate colonies was analyzed by restriction enzyme digestion to identify one containing *chrR*.

Nucleic acid analysis. DNA was sequenced on both strands with *Taq* DNA polymerase (Promega, Inc., Madison, Wis.) by the manufacturer's protocols with either standard or deaza reagent kits. Most of the DNA sequence was obtained from double-stranded pUC-derived plasmids with *lac*-specific primers. Some single-stranded gaps were completed with synthetic primers derived from the sequence provided in this paper. A list of plasmids and internal primers used is available upon request. Genetics Computer Group software (Madison, Wis.) was used to analyze the DNA sequence (7).

Primer extensions were performed on RNA samples (8) as described previously (16, 28). An oligonucleotide (coordinates 622 to 639 in Fig. 2) specific for the noncoding strand (Genosys, The Woodlands, Tex.) was used for primer extensions on *chrR* mRNA. Primers used to analyze *cycA* 5' ends and conditions for Northern (RNA) blot analysis have been described previously (19).

Protein and enzyme analysis. Radiolabelled protein was synthesized in vitro with an SP6 transcription kit, a wheat germ translation system (Promega), and ³⁵S-Transmet (Amersham Corp., Arlington Heights, Ill.). Translation products were separated on sodium dodecyl sulfate–12% polyacrylamide gels, stained, treated with an autoradiography enhancer (Research Products International, Mount Prospect, Ill.), and exposed to X-ray film.

LacZ activity was measured (22) with sodium dodecyl sulfate and chloroform

FIG. 2. The nucleotide sequence of *chrR* and flanking sequences from *SalI* to *SstI*. The additional restriction enzyme sites listed were used in specific experiments in the text. Italicized DNA sequences (coordinates 501 to 506 and 557 to 560) show two potential ribosome binding sites. The DNA sequence used to design a complementary primer for mapping the 5' ends of *chrR*-specific mRNA (coordinates 622 to 639) is underlined. Boldface asterisks indicate the major 5' ends for *chrR* transcripts (see Fig. 5). Amino acids within a putative ligand binding site (residues 179 to 183) are printed in boldface italics. The Cys-to-Arg substitution (residue 182) found in the *chr-4* allele is shown in boldface. DNA sequences in underlined italics (coordinates 1608 to 1640) highlight an inverted repeat with similarity to factor-dependent transcription terminators.

extends to the upstream *StuI* site (pBS19), the in vitro-synthesized gene product had an apparent molecular size (~25 kDa) indistinguishable from that accumulated in *E. coli* (Fig. 3). The lower-molecular-weight proteins (Fig. 3B, lane —) probably result from premature translation termination or ChrR degradation in vitro. If the ~25-kDa gene product is full-length ChrR, then the difference in size from that predicted by the DNA sequence is most likely due to anomalous migration of this protein on sodium dodecyl sulfate-polyacrylamide gels.

To determine where *chrR* translation initiated, an *XbaI* amber stop codon linker (Pharmacia LKB Biotechnology, Milwaukee, Wis.) was cloned into an *SfiI* restriction site 24 bp upstream of the first methionine codon (coordinate 514 in Fig. 2). If *chrR* translation began at this first ATG, no in vitro protein product should be detected from this template on this gel system because protein synthesis would terminate after only 9 amino acids. In contrast, insertion of an amber stop

codon at this position should not affect ChrR translation if the second in-frame ATG was used (amino acid 18 in Fig. 2) since the *SfiI* restriction site is 29 bp upstream of this methionine codon. The fact that synthesis of the ~25-kDa protein was lost (Fig. 3) from this later template indicates that translation begins at the first methionine codon indicated in Fig. 2.

Computer analysis of the deduced ChrR gene product failed to shed light on its function since no significant homology was found between this 357-amino-acid protein and other polypeptides in the database. ChrR appears to be soluble because it contains no obvious stretches of hydrophobic regions which could represent transmembrane domains. In addition, the lack of significant similarity between the N terminus of ChrR and procaryotic signal peptides suggests that this protein is probably not exported. However, ChrR appears to be enriched for histidine (9 residues) and other charged amino acids, which could explain its aberrant migration on sodium dodecyl sulfate-

FIG. 3. In vitro translation products generated from *chrR* plasmids containing the indicated DNA fragments. (A) Two potential initiator codons for *chrR* and the location of an *SfiI* site where an *XbaI* amber stop codon linker was inserted (see text). (B) Protein products obtained when in vitro-synthesized mRNA from pBS19 (wild-type *chrR* template) (lane -) and pBS23 (containing the amber stop codon linker at the *SfiI* site) (lane +) were independently translated in wheat germ extracts in the presence of 35 S-Transmet. The migration of molecular weight standards (MW) is indicated in thousands.

polyacrylamide gels. In addition, ChrR has 5 cysteine residues whose potential function will be discussed later.

The *chr-4* allele is partially dominant. To quantitate the effects of *chrR* on *cycA* transcription in the Chr4 mutant, LacZ levels were measured in cells carrying the *cycA::lacZ* reporter plasmid (pBS6) and a second compatible plasmid (pBS13) containing *chrR* (Table 2). Placing a *chrR* plasmid in Chr4 reduced LacZ activity approximately ninefold to levels comparable to those found in wild-type cells (Table 2). Previous experiments showed that the mutation in Chr4 increased levels of several wild-type *cycA* transcripts and led to accumulation of a transcript with a 5' end at -52 (relative to the start of *cycA* translation) that is not detectable in wild-type cells (28) (Fig. 4, lanes 1 and 5). When primer extension analysis was used to independently assess complementation, the *chrR* plasmid reduced the abundance of several genomic *cycA* transcripts in Chr4 (Fig. 4, lane 6). The *chrR* plasmid also reduced the level of the -52 transcript that is unique to Chr4. However, there were still slightly elevated levels of the -28 transcript in Chr4 cells containing the *chrR* plasmid. This result suggested that the *chr-4* mutation in Chr4 is not totally recessive to the wild-type *chrR* gene on pBS13.

ChrR positively regulates *cycA* transcription. To determine whether ChrR negatively or positively regulates *cycA* transcription, we constructed strains in which a trimethoprim resistance gene was inserted into *chrR*. Placing a Δ *chrR* allele in Chr4 (Chr4TpA) decreased transcription from the downstream *cycA* reporter plasmid (pBS6) ~10-fold (Table 2). Indeed, the LacZ levels measured from this reporter plasmid in Chr4TpA were

FIG. 4. *cycA*-specific primer extension products from the wild type (2.4.1), the Chr4 mutant, and two different ChrR null mutants (TF18 and Chr4TpA). +, presence of a wild-type copy of the *chrR* gene on a plasmid; *, presence of a *chrR* gene containing the C182R substitution on a plasmid; -, no plasmid present. Equivalent amounts of RNA (25 μ g) were assayed. The numbers on the right indicate the positions of these 5' ends relative to the start of translation (at +1).

virtually identical to those obtained when a Δ *chrR* allele was placed in a wild-type background (TF18; Table 2). The decreased expression from the downstream *cycA* reporter plasmid observed when a Δ *chrR* allele is placed in either Chr4 or wild-type cells indicates that ChrR normally acts positively.

To ensure that this reporter plasmid provided an accurate picture of *cycA* transcription, primer extension analysis of genomic *cycA* transcripts was used to independently monitor the effects of a Δ *chrR* allele. As expected, placing a Δ *chrR* allele in either Chr4 (Fig. 4, lanes 5 and 7) or wild-type cells (lanes 1 and 3) reduced the levels of genomic *cycA* transcripts under aerobic conditions. These results independently support the notion that increased *cycA* transcription in Chr4 required an uninterrupted *chrR* gene. Presumably, the *chr-4* allele in Chr4 alters but does not abolish function of either ChrR or some other protein.

***chrR* is altered in Chr4.** To determine if the increased *cycA* transcription in Chr4 was due to a mutation in *chrR*, the gene was cloned from this strain and placed in Δ ChrR strains (TF18 and Chr4TpA) on a plasmid (pBS24). LacZ levels from the downstream *cycA::lacZ* reporter plasmid (pBS6) were elevated ~10-fold in both strains (Table 2). In addition, primer extension analysis of genomic *cycA* transcripts from either ChrR null strain carrying the *chrR* gene from Chr4 on a plasmid showed elevated levels of both the wild-type products and the -52 species that is specific to Chr mutants (Fig. 4, lanes 4 and 8). A

FIG. 5. Analysis of *chrR* transcription. (A) Northern blot using a *chrR*-specific riboprobe (Fig. 1C) against the indicated amounts of RNA from wild-type or Chr4 cells. The size standards (nt, nucleotides) are from *Hind*III-digested lambda DNA. (B) *chrR*-specific primer extension products (25 µg of RNA) from wild-type cells grown aerobically (lane O₂) or photosynthetically (lane PS). The numbers on the right indicate the positions of these 5' ends relative to the start of translation (at +1).

simple conclusion from these data is that Chr4 cells contain a mutation in *chrR*.

Upon sequencing the *chrR* gene from Chr4, we found a single T→C transition, which causes a Cys-to-Arg substitution at amino acid 182 (coordinate 1057 in Fig. 2). As expected for a simple missense mutation, the *in vitro* transcription-translation product from this mutant *chrR* gene (hereafter referred to as C182R) has an apparent molecular weight indistinguishable from that of wild-type ChrR (data not shown).

To confirm that this T→C transition was sufficient for the altered *cycA* transcription in Chr4, an ~200-bp fragment containing this mutation was exchanged for the same region of wild-type *chrR*. When this hybrid *chrR* gene was placed in the ΔChrR strains on a plasmid, it produced the same effect on *cycA* transcription from the pBS6 reporter plasmid as the entire mutant gene isolated from Chr4 (data not shown).

Regulation of *chrR* expression. When Northern blot analysis with a *chrR*-specific probe (Fig. 1C) was used to assess if this gene was part of a larger operon, a major transcript of ~1,350 nucleotides was found (Fig. 5A). A transcript of ~1,300 nucleotides is predicted if it encoded only ChrR when one considers the locations of the 5' ends of the major *chrR*-specific transcripts (Fig. 5B), the size of the open reading frame (1,070 bp), and the location of a possible factor-dependent transcription terminator downstream of *chrR* (Fig. 2). Since the measured size of the transcript (~1,350 nucleotides) is in excellent agreement with that estimated for a monocistronic operon, it appears *chrR* is not cotranscribed with other genes.

However, Northern blot analysis revealed that *chrR* transcript levels are significantly increased in Chr4 (Fig. 5A). Primer extension analysis shows the same two *chrR*-specific products in Chr4 as in wild-type cells (data not shown). The increased abundance of *chrR*-specific transcripts in cells containing the altered-function C182R protein suggests that ChrR has the ability to positively regulate its own synthesis.

***chrR* is not linked to *cycA*.** Pulsed-field gel electrophoresis

TABLE 3. Retention of oxygen regulation of *cycA* transcription in ChrR null mutants

Strain ^a	<i>chrR</i> genotype ^b	LacZ activity from pBS5 ^c		Photosynthetic/aerobic activity ratio
		Aerobic	Photosynthetic	
2.4.1	+	114	1,322	8
TF18	—	53	617	9
Chr4	*	632	1,057	2
Chr4TpA	—	56	554	9

^a For descriptions of strains and plasmids, see Table 1.

^b *, presence of an altered-function *chrR* gene containing the C182R substitution; —, Δ*chrR* allele; +, wild-type *chrR*.

^c Data represent the averages from three independent experiments. Variation in independent experiments was ±10%. pBS5 contains the *cycA* promoter region from –484 to –124. LacZ activity units were calculated by using the formula provided in Materials and Methods.

and Southern blot analysis were used to show that *chrR* is not part of the *R. sphaeroides* photosynthetic gene cluster which contains *cycA* (34). Rather, *chrR* hybridized to the 910-kb *Ase*I restriction fragment of chromosome I. For further mapping, we took advantage of a unique *Ase*I site on the pTF2 suicide plasmid (Table 1) used to generate ChrR null mutants. An *Ase*I digest of DNA from cells containing pTF2 integrated into the genome placed *chrR* ~145 kb from one end of the 910-kb *Ase*I restriction fragment. Unfortunately, the sizes of the restriction fragments generated by a *Sna*BI-*Ase*I double digest did not allow us to determine the precise map position of *chrR* (data not shown). However, placement of *chrR* some 145 kb from either end of the 910-kb *Ase*I restriction fragment maps this gene either ~182 or ~798 kb from *cycA*.

ChrR is not responsible for anaerobic induction of *cycA* transcription. Previous analysis indicated that the *chr-4* allele in Chr4 increased transcription from both the upstream and downstream *cycA* control regions (28). To determine if a Δ*chrR* allele had the expected negative effect on transcription from the upstream control region, we measured LacZ levels in ΔChrR strains containing a reporter plasmid (pBS5) with only the upstream *cycA* control region (–484 to –124) fused to *lacZ*. As expected, placing a Δ*chrR* allele in Chr4 (Chr4TpA) reduced transcription from the upstream *cycA* reporter plasmid ~10-fold under aerobic conditions (Table 3). Indeed, the LacZ levels produced from the upstream *cycA* reporter plasmid were identical in both TF18 (ΔChrR derivative of wild-type cells) and Chr4TpA (ΔChrR derivative of Chr4) (Table 3). When this observation is combined with the results obtained with the pBS6 downstream reporter plasmid (Table 2), it suggests that ChrR acts positively at both *cycA* control regions.

To test if ChrR was required for the increased transcription from this upstream *cycA* control region that occurs under anaerobic conditions (28), LacZ levels were also measured in photosynthetically grown cells. The results of this experiment showed that transcription from the upstream *cycA* control region was increased approximately ninefold when both ΔChrR strains (TF18 and Chr4TpA) were grown anaerobically by photosynthesis (Table 3). The magnitude of the increase in the ChrR null strains was comparable to that of wild-type cells (approximately eightfold). Therefore, ChrR is not responsible for increased *cycA* transcription under anaerobic conditions.

PrrA is also required for maximal transcription from the upstream *cycA* control region. Recently, Eraso and Kaplan (11) identified a global activator (PrrA) of *cycA* and several other genes which encode components of the *R. sphaeroides* photosynthetic apparatus. To determine if PrrA functions only

TABLE 4. Independent regulation of *cycA* transcription by ChrR and PrrA

Strain ^a	<i>prrA</i> ^b	<i>chrR</i> ^b	Aerobic LacZ activity from <i>cycA</i> reporter plasmid ^c	
			pBS5 (-484 to -124)	pBS6 (-104 to +94)
2.4.1	+	+	114	107
PRRA1	-	+	23	100
Chr4	+	*	632	703
Chr4PR	-	*	163	736
Chr4TpA	+	-	56	72
TpAPR	-	-	14	70

^a For descriptions of strains and plasmids, see Table 1.^b *, altered-function *chrR* gene that encodes the C182R protein; -, Δ *chrR* or Δ *prrA* allele; +, wild-type *chrR* or *prrA* gene.^c Data represent the averages from three independent experiments. Variation in independent experiments was $\pm 10\%$. The numbers in parentheses indicate the *cycA* control region fused to a promoterless *lacZ* gene (28). LacZ activity units were calculated by using the formula provided in Materials and Methods.

at the upstream *cycA* control region which is activated under photosynthetic conditions, we measured the effect of a Δ *prrA* mutation on transcription from both *cycA::lacZ* reporter plasmids.

When transcription from the upstream *cycA* reporter plasmid (pBS5) was monitored in a series of isogenic strains, the Δ *prrA* allele reduced LacZ levels approximately four- to fivefold under aerobic conditions (Table 4). If PrrA acted at the upstream *cycA* promoter(s), then primer extension analysis should reveal a specific reduction in the abundance of these transcripts in Δ *prrA* cells. As expected, there are reduced levels of genomic *cycA* transcripts with 5' ends at -137 and -155 in

Δ PrrA strains (PRRA1 and Chr4PR; Fig. 6A). Longer exposures of this gel show the expected reduction in the abundance of genomic *cycA* transcripts with a 5' end at -250 (data not shown). When a *lacZ*-specific primer was used to better examine the effect of PrrA on the abundance of the *cycA* 5' ends upstream of -124 (Fig. 6B), transcripts with 5' ends at -250 and -155 were present at significantly reduced levels in Δ PrrA strains. In contrast, *cycA* transcripts with 5' ends at -274 and -267 appear independent of PrrA since their levels were similar in Chr4 and Chr4PR. While such a finding suggests that only the promoter for the -267 *cycA* transcript requires the presence of C182R, such assignments require additional experiments.

In contrast, PrrA does not appear to function at the downstream *cycA* control region because the levels of transcripts with 5' ends at -52 and -28 are not significantly affected by the Δ *prrA* mutation in either PRRA1 or Chr4PR (Fig. 6A). As predicted from these results, LacZ levels from the downstream reporter plasmid (pBS6) were unaffected by a Δ *prrA* allele (Table 4), regardless of whether this mutation was present in wild-type cells (PRRA1), a ChrR null mutant (TpAPR), or cells containing C182R (Chr4PR).

ChrR and PrrA independently regulate *cycA* transcription. Table 4 also shows that Δ PrrA cells containing C182R (Chr4PR) had approximately sevenfold higher LacZ levels from the upstream *cycA* reporter plasmid (pBS5) than a isogenic strain containing wild-type ChrR (PRRA1). A simple explanation for this result is that ChrR and PrrA control *cycA* transcription independently. If this were true, cells containing null mutations in both *chrR* and *prrA* (TpAPR) should have lower LacZ levels from the upstream *cycA* reporter plasmid (pBS5) than an isogenic *prrA*⁺ strain (Chr4TpA). As predicted, LacZ levels from this upstream *cycA* reporter plasmid in cells lacking both PrrA and ChrR (TpAPR) were approximately fourfold lower than those in a strain lacking only ChrR (Chr4TpA). However, cells lacking both PrrA and ChrR (TpAPR) still had approximately twofold higher levels of LacZ from this upstream *cycA* reporter plasmid than a wild-type strain containing a promoterless version of this reporter plasmid (28). This difference probably reflects residual transcription from the upstream *cycA* control region in the absence of PrrA and ChrR.

DISCUSSION

Previously, we reported that *cycA* transcription was coupled to availability of the heme ligand required for assembly of a functional *cyt c₂* protein (28). The notion that *cycA* transcription was linked to ligand availability came in part from the observation that the addition of ALA (the first committed precursor in this pathway) to cells rapidly and effectively reduced new transcription initiations at several independent *cycA* promoters. In addition, the activities of several *cycA* promoters in mutants such as Chr4 were resistant to concentrations of ALA that effectively blocked transcription in wild-type cells (28). Taken together, these observations led us to suggest that the mutation in Chr4 prevented the productive interaction of a regulator of *cycA* transcription with a signal molecule derived from tetrapyrrole biosynthesis.

ChrR positively regulates *cycA* transcription. When one considers that a defined *chrR* null mutation reduced *cycA* expression, it appears that ChrR positively regulates *cyt c₂* synthesis. In addition, ChrR appears to control the activities of several *cycA* promoters since a Δ *chrR* allele reduced transcription from both the upstream and downstream control regions. A wild-type copy of *chrR* on a plasmid decreased *cycA* tran-

FIG. 6. *cycA*-specific primer extension products. (A) Results obtained with a *cycA* primer located downstream of the *cycA* initiation codon (28); (B) results obtained with a *lacZ* primer downstream of the *cycA::lacZYA* cloning junction of pBS5. Each lane used 25 μ g of RNA. Molecular weights (in thousands) are indicated on the right. The numbers on the right indicate the positions of these 5' ends relative to the start of translation (at +1).

scription in both the Chr4 mutant and several other independently isolated mutants with similar phenotypes (data not shown). Thus, *chrR* is either the major or sole locus affected when mutants were isolated by screening cobaltic heme-resistant (Chr) cells for increased *cycA* transcription (28). However, the deduced protein sequence of ChrR did not provide us with any clear insights into the function of this gene product. Therefore, additional experiments are required to determine if ChrR is a transcription factor or whether it acts elsewhere within a signal transduction chain.

If ChrR positively regulates *cycA* transcription, then the phenotypic difference between the Chr4 mutant and a Δ ChrR strain suggests that the *chr-4* allele generates an altered function protein that is insensitive to the signal(s) generated by the addition of ALA. If ChrR normally functioned as an oligomer, then formation of mixed oligomers with wild-type protein and an altered-function polypeptide like C182R could explain the partial dominance of the *chr-4* allele when Chr4 cells contain a wild-type *chrR* plasmid. A logical extension of this reasoning is that the increased *cycA* transcription under aerobic conditions found in cells containing the altered-function C182R protein reflects the fact that some fraction of wild-type ChrR is inactive under standard laboratory growth conditions. Thus, it appears that wild-type cells exist in a situation where ChrR is poised to respond to subtle changes in ligand availability.

What is the signal recognized by ChrR? Our previous analysis of *cycA* transcription could not distinguish if ChrR function is normally blocked by the accumulation of ALA, an intermediate in tetrapyrrole biosynthesis, or a product of this pathway, such as heme. Since the *chr-4* allele alters ChrR function, the amino acid substitution in this mutant protein may define domains of the wild-type protein that interact with either the regulatory ligand or some other component of a signal transduction chain. Upon sequencing of the *chr-4* allele, a Cys-to-Arg substitution (underlined below) was found within a motif (C-D-E-C-R) which is related to the consensus heme binding site for *c*-type cytochromes (C-X-Y-C-H). While this ChrR motif lacks the His residue (indicated in boldface above) usually present in covalent heme binding sites, a His-to-Arg change in the analogous position of *Saccharomyces cerevisiae* cyt *c* maintains ligand association and cytochrome function (33). If this C-D-E-C-R motif of wild-type ChrR does interact with either heme or some tetrapyrrole intermediate, then the C182R substitution would be expected to prevent the mutant protein from interacting with its regulatory ligand since an Arg substitution at the analogous Cys of yeast cyt *c* prevents heme binding (14). However, it is also possible that the C182R substitution indirectly prevents its productive interaction with some other protein that recognizes the signal produced by the addition of ALA.

ChrR links *cycA* transcription to ligand availability. Our results suggest that conditions which cause accumulation of either heme or some tetrapyrrole intermediate block the function of ChrR as a positive regulator of *cycA* transcription. However, this ligand-dependent signal serves only to fine-tune *cycA* transcription since a Δ ChrR strain has no observable growth phenotype under either aerobic or photosynthetic conditions (data not shown). Thus, blocking ChrR activity in wild-type cells would only partially reduce the rate of cyt *c*₂ synthesis until the proper level or flux of intermediates through the tetrapyrrole biosynthetic pathway was restored (6, 18, 37). Thus, this negative-feedback loop ensures that synthesis of tetrapyrrole intermediates and tetrapyrrole-binding proteins such as cyt *c*₂ are homeostatically coupled.

What is a ChrR target site? The results presented in this and previous work (28) suggest that ChrR is a global regulator. The

increased abundance of *chrR*-specific transcripts in cells containing the altered-function C182R protein indicates that ChrR positively regulates its own synthesis. C182R also increased ALA synthase activity (28), suggesting that ChrR also acts positively at the *hemA* and/or *hemT* promoter (24). In this regard, it will be particularly interesting to see if ChrR is responsible for ALA-dependent changes in the levels of bacteriochlorophyll-protein complexes in the *R. sphaeroides* photosynthetic membrane (24). Unfortunately, a target site for ChrR-dependent regulation has yet to be identified since searches for sequence similarity between the *cycA*, *hemA*, *hemT*, and *chrR* regulatory regions do not reveal strongly conserved regions.

ChrR is not solely responsible for stimulating *cycA* transcription under photosynthetic conditions. From the negative effect of exogenous ALA on *cycA* transcription, the properties of Chr mutants, and the known oxygen-dependent changes in *R. sphaeroides* tetrapyrrole availability (6, 18), it seemed reasonable that ChrR could be solely responsible for increased cyt *c*₂ synthesis under anaerobic conditions when photosynthetic membrane assembly is induced (28). However, several results indicate that this is not the case. For example, we found that transcription from the upstream *cycA* control region in a Δ ChrR strain shows the same level of induction under photosynthetic conditions as in a wild-type strain. Rather, our results show that the response regulator, PrrA (11), is required for maximal transcription from this upstream *cycA* control region. Thus, the data indicate that PrrA and ChrR function independently at the upstream *cycA* control region.

Why couple *cycA* transcription to ligand availability? The use of multiple input signals to control the output from *cycA* promoters is in many respects reminiscent of how transcription of the *S. cerevisiae* cyt *c* structural gene (*cyc1*) is governed by both a heme-dependent activator (12) and an activation complex (12, 13) that responds to catabolite repression. The use of heme as an apparent transcriptional coactivator of HAP1 makes metabolic sense in *S. cerevisiae*, since this microbe synthesizes this ligand only under aerobic conditions (17, 21). In contrast, the positive action of ChrR on *cycA* transcription is apparently blocked by either heme or an unknown intermediate in tetrapyrrole biosynthesis. Thus, the metabolic role of ChrR is most likely as a modulator of target genes in response to relatively small changes in heme or tetrapyrrole availability that occur when different cytochrome-dependent respiratory or photosynthetic pathways generate energy. In many respects the subtle changes in tetrapyrrole availability experienced by *R. sphaeroides* (6, 18, 37) might be analogous to those in cells that always require cytochrome function to generate energy. Thus, it will be interesting to see if and how transcription of analogous genes is coupled to ligand availability in systems which do not experience the dramatic changes in tetrapyrrole availability found during the transition between fermentation and respiration in *S. cerevisiae*.

In conclusion, these experiments have identified both PrrA and ChrR as positive regulators of *cycA* transcription. Future experiments need to identify the regulatory ligand that controls ChrR activity, to determine if this protein is a transcription factor, and to ascertain if its target genes include additional proteins which either synthesize or require tetrapyrrole derivatives for function. Answers to these questions will provide important insights into how cells sense and regulate tetrapyrrole availability and how they couple synthesis of ligand-requiring proteins to changes in ligand availability that are likely to occur under defined physiological conditions.

ACKNOWLEDGMENTS

This work was supported by Public Health Service grant GM37509 to T.J.D. The work began while B.A.S. was a recipient of a National Science Foundation predoctoral fellowship. We thank Tracy Ferguson for assistance while she was supported by a University of Wisconsin—Madison Graduate School grant to promote research experiences for undergraduates from other campuses.

We also thank Patricia Kiley for editorial comments on the manuscript.

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