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Transcription of the *Rhodobacter sphaeroides cycA* P1 Promoter by Alternate RNA Polymerase Holoenzymes

BARBARA J. MACGREGOR, RUSSELL K. KARLS, AND TIMOTHY J. DONOHUE*

Department of Bacteriology, University of Wisconsin—Madison, Madison, Wisconsin 53706

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These experiments sought to identify what form of RNA polymerase transcribes the P1 promoter for the *Rhodobacter sphaeroides* cytochrome c_2 gene (*cycA*). In vitro, *cycA* P1 was recognized by an RNA polymerase holoenzyme fraction that transcribes several well-characterized *Escherichia coli* heat shock (σ^{32}) promoters. The in vivo effects of mutations flanking the transcription initiation site (+1) also suggested that *cycA* P1 was recognized by an RNA polymerase similar to *E. coli* Eσ^{32}. Function of *cycA* P1 was not altered by mutations more than 35 bp upstream of position +1 or by alterations downstream of −7. A point mutation at position −34 that is towards the *E. coli* Eσ^{32} −35 consensus sequence (G34T) increased *cycA* P1 activity ~20-fold, while several mutations that reduced or abolished promoter function changed highly conserved bases in presumed −10 or −35 elements. In addition, *cycA* P1 function was retained in mutant promoters with a spacer region as short as 14 nucleotides. When either wild-type or G34T promoters were incubated with reconstituted RNA polymerase holoenzymes, *cycA* P1 transcription was observed only with samples containing either a 37-kDa subunit that is a member of the heat shock sigma factor family (Eσ^{37}) or a 38-kDa subunit that also allows core RNA polymerase to recognize *E. coli* heat shock promoters (Eσ^{38}) (R. K. Karls, J. Brooks, P. Rossmeissl, J. Luedke, and T. J. Donohue, J. Bacteriol. 180:10–19, 1998).

In order to survive, all cells must continue to generate energy in response to a wide variety of environmental changes and signals. While viability in response to such challenges requires the coordinated induction and repression of numerous genes, relatively little is known about how the expression of energy-generating functions is altered by these stimuli. To analyze the control of proteins that participate in biological energy generation, we are studying transcriptional regulation of the cytochrome c_2 gene (*cycA*) from the facultative phototroph *Rhodobacter sphaeroides.*

We know that *cycA* contains several promoters (16, 21, 22). The downstream *cycA* promoter (*cycA* P1) provides sufficient cytochrome c_2 to support growth, but its output is not altered by conditions that alter levels or demand function of this electron carrier (16, 21, 22). In contrast, activity of the upstream promoter (*cycA* P2) is increased ~10-fold under photosynthetic conditions where cytochrome c_2 function is required (16, 21, 22). Activity of a third promoter (*cycA* P3) increased dramatically in cells containing the *trans*-acting *chr-4* mutation (21, 22).

A key unresolved issue is the nature of the RNA polymerase holoenzyme that recognizes each of the three *cycA* promoters (16, 21, 22). A previous study noted the similarity of DNA sequences upstream of *cycA* P1 and P2 to promoters recognized by the major eubacterial sigma factor, σ^{70} (16). Thus, one possibility was that *cycA* P1 and P2 were each recognized by the previously described *R. sphaeroides* RNA polymerase holoenzyme (Eσ^{D}) that transcribes the *Escherichia coli* σ^{70}-dependent *lacUV5* and ColE1 RNA promoters in vitro (10). Alternatively, *cycA* promoter recognition by minor RNA polymerase holoenzymes (5, 13, 14) could link cytochrome c_2 synthesis to conditions that increase or decrease function of alternate sigma factors. This appears to be the case for *cycA* P3, since the *chr-4* mutation appears to inactivate a negative regulator of an *R. sphaeroides* member of the σ^{E} family of alternative eubacterial sigma factors (18a, 21, 22).

These experiments sought to analyze function of *cycA* P1. We focused on *cycA* P1 because sequences within ~60 bp of the transcription start site (+1) are sufficient for function under both respiratory and photosynthetic conditions (16, 21, 22). In vitro transcription assays indicated that *R. sphaeroides* Eσ^{D} (10) did not recognize *cycA* P1. By analyzing a series of mutant *cycA* P1 promoters fused to a promoter-less *E. coli* β-galactosidase (*lacZ*) gene in vivo, we found that sequences within a presumed RNA polymerase binding site were sufficient for activity. Other promoter mutations identified elements related to the *E. coli* heat shock (Eσ^{32}) consensus sequence that were necessary for *cycA* P1 function. Reconstituted RNA polymerases were used to demonstrate *cycA* P1 transcription by a holoenzyme containing either a 37-kDa protein (σ^{37}) related to *E. coli* σ^{32} or a 38-kDa protein (σ^{38}) that also recognizes heat shock promoters from enteric bacteria (10, 11). We suggest that one reason why *cycA* P1 might be transcribed by alternate RNA polymerase is to ensure that cytochrome c_2 levels can change when its function in energy generation is required to provide ATP for chaperone activity. Consistent with this suggestion, *cycA* P1 function is elevated after an increase in temperature when eubacterial chaperones are commonly needed for cells to mount a heat shock response (11).

MATERIALS AND METHODS

Construction of individual *cycA* transcriptional fusions. To produce *cycA*::*lacZY′* operon fusions, an *E. coli-R. sphaeroides* shuttle plasmid (pBM4A) was constructed. In the first step, a promoterless *lacZY′* operon from pRS415 (23) was isolated (~3-kb *Eco*RI-*Dra*I restriction fragment) and cloned between the *Eco*RI and *Hpa*I restriction endonuclease sites of pKT231 (1), with *lacZY′* and *kan* transcription in opposite directions, to give pBM12. To place a transcription terminator upstream of *lacZY′*, the ΩSp cartridge from pHP45Ω (19) was isolated as an *Eco*RI restriction fragment and cloned into the same site of pUC6S (25) with *spc* transcription in the same direction as *bla*, yielding pUC6SΩA. Plasmid pUC6SΩA was then partially digested with *Spe*I and cloned into the *Eco*RI site of pBM12 that had been made blunt by treatment with the Klenow fragment of DNA polymerase. The resulting plasmid, pBM4A, has *Kpn*I

* Corresponding author. Department of Bacteriology, University of Wisconsin—Madison, 1550 Linden Dr., Madison WI 53706. Phone: (608) 262-4663. Fax: (608) 262-9865. E-mail: tdonohue@bact.wisc.edu.

and *Stu*I restriction endonuclease sites for directional cloning of DNA between the ΩSp cassette and *lacZY′* (the direction of *spc* transcription in pBM4A is opposite that of *lacZY′*). IncQ plasmids like pBM4A are present at ~4 to 10 copies per *R. sphaeroides* cell (4).

A pool of oligonucleotides (spanning from position −76 to +16 relative to the *cycA* P1 transcription start site) with approximately one random mutation per molecule was generated at the University of Wisconsin Biotechnology Center (Madison, Wis.). To synthesize complementary strands, a 16-mer (C2MUTP2, 5′-CCTCCCAGGCCTTGTA-3′; prepared at the University of Wisconsin Biotechnology Center) was annealed to the oligonucleotide pool (at 37 or 45°C) and extended at 70°C for 1 h with *Taq* DNA polymerase (Promega Inc., Madison, Wis.). The resultant double-stranded DNA molecules contained *Kpn*I and *Stu*I restriction endonuclease sites at the upstream and downstream ends, respectively, along with flanking sequences (TCCCGG and GGGAGG, respectively) to allow endonucleolytic cleavage and cloning into *Kpn*I- and *Stu*I-digested pBM4A. These plasmids were transformed into *E. coli* S17-1, conjugated into *R. sphaeroides* 2.4.1 (3), and screened for LacZ activity on Sistrom's minimal medium plates (15) containing 40 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside ml^{-1} and 25 μg (each) of kanamycin and spectinomycin ml^{-1}. For sequencing, *R. sphaeroides* plasmid DNA was transformed into *E. coli* DH5α (2), isolated, and extended with *Taq* DNA polymerase and *lacZ*-specific primers. *E. coli* strains were grown at 37°C in L broth (17) with 25 μg (each) of kanamycin and spectinomycin ml^{-1}.

A control reporter plasmid with *lacZY′* under control of the wild-type *cycA* P1 was designated pCM241; derivatives containing mutated *cycA* P1 fragments were named to describe their lesion. For example, reporter plasmids with *cycA* P1 deletions are named by the missing bases; 73Δ67 lacks promoter DNA from positions −73 to −67. Plasmids with single point mutations or small insertions upstream of +1 are named for the changed bases (for example, G34T has a G-to-T change at position −34 and 29C28 has a C inserted between coordinates −29 and −28). Reporter plasmids with multiple mutations are named M plus a number (e.g., M1). Several mutations (G34T, 29C28, T12A, and A11C) were introduced by site-directed mutagenesis (17) (oligonucleotides from Genosys Inc., The Woodlands, Tex.). When independent isolates of plasmids containing the same mutation were analyzed, only one set of results is reported, since in every case analyzed, the LacZ levels were indistinguishable (data not shown).

LacZ and primer extension assays. LacZ activity was measured in exponential-phase cells grown in Sistrom's minimal medium at 30°C (21). For aerobic cells, 5-ml cultures were grown on a rotary shaker in 125-ml flasks. Duplicate cultures were assayed at least three times for each strain. Primer extension assays (9) used a *lacZ*-specific primer.

Proteins used for in vitro transcription assays. Either aerobically grown wild-type or ΔRpoH cells (11) were used to obtain RNA polymerase preparations diminished for $E\sigma^D$ by Q-Sepharose chromatography (10). *E. coli* core RNA polymerase was purchased from Epicentre Technologies, Inc. (Madison, Wis.). Core *R. sphaeroides* RNA polymerase was obtained from an RpoH null strain (11) by affinity chromatography (10) (~5 g [wet weight] of cells) on a 0.5-ml bed of resin containing the 4RA2 monoclonal antibody against the α-subunit of *E. coli* RNA polymerase (provided by N. Thompson and R. Burgess). After the column was washed with 10 volumes of TE (0.1 M Tris [pH 7.9], 0.1 mM EDTA) plus 0.5 M NaCl buffer, core RNA polymerase was eluted with TE supplemented with 0.75 M NH_4SO_4, 40% 2,3-butanediol (24a). Prior to storage at −20°C, the eluted proteins (1 ml) were dialzyed twice against 500 ml of TE containing 0.1 M NaCl, 50% glycerol, and 1 μM dithiothreitol (DTT).

Proteins to be tested for sigma factor activity were eluted from sodium dodecyl sulfate (SDS)-polyacrylamide gels by slight modifications to existing protocols (7). All steps were performed in polypropylene microcentrifuge tubes. Prior to electrophoresis, RNA polymerase samples were concentrated by a 60-min precipitation in ice-cold 10% trichloroacetic acid. After centrifugation (15 min, 12,000 × *g*, 4°C), the precipitated proteins were washed twice with 800 μl of 80% acetone–20% 10 mM Tris-Cl (pH 7.9) and once with 80% acetone (with 5 min of centrifugation between washes). Concentrated proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (slab gel dimensions 6 by 8.8 by 1/16 in.) along with molecular weight standards (Bethesda Research Laboratories, Gaithersburg, Md.). After electrophoresis (75 V, 13 to 15 h), the gel was cut into ~0.5-cm-thick slices starting 1.0 cm below the stacking gel. Gel slices were rinsed twice with 800 μl of 1 mM DTT, overlaid with 400 μl of elution buffer (7), and ground with a conical tipped pestle. After the addition of 200 μl of elution buffer and 1 h of incubation at room temperature with occasional mixing, acrylamide particles were removed by centrifugation and the supernatant (~450 μl) was removed. Eluted proteins were precipitated with 4 volumes of 80% acetone (10 min, 4°C), harvested by centrifugation, and washed twice (with centrifugation between each wash) in 800 μl of 80% acetone–20% 10 mM Tris-Cl (pH 7.9). After residual acetone was removed by evaporation at room temperature, the precipitated proteins were incubated in 20 μl of dilution buffer containing 6 M guanidine chloride (7) for 1 h at room temperature. To renature the proteins, 50 volumes of dilution buffer were added. Samples were incubated for 12 h at room temperature prior to testing for sigma factor activity or storage at −80°C.

Amino-terminal hexahistidine-tagged *R. sphaeroides* σ^D (His-σ^D) was obtained by amplifying the *sigA* (*rpoD*) (6) gene and cloning it into pQE30 (Qiagen, Inc., Chatsworth, Calif.). The manufacturer's protocol was used to isolate *R. sphaeroides* His-σ^D from *E. coli*. Prior to in vitro transcription reactions, the purified protein was treated with guanidine and renatured (see above). To conform with the nomenclature suggested by Lonetto et al. (13), the protein previously referred to as σ^{93} (8, 10) will hereafter be called σ^D, as DNA sequence analysis of the *sigA* (*rpoD*) gene (6) shows that it is most closely related to *E. coli* RpoD.

In vitro transcription assays. With the following exceptions, conditions and templates for in vitro transcription assays have been described previously (10). Either RNA polymerase holoenzyme (0.4 pmol plus 4 μl of dilution buffer) or reconstituted enzyme (0.4 pmol of core plus 4 μl of renatured sample to be tested for sigma factor activity) was incubated at room temperature for 1 h and added to the transcription mixture (20 nM plasmid template, 40 mM Tris-Cl [pH 7.9], 2 mM EDTA, 5 mM $MgCl_2$, 100 mM KCl, 1 mM DTT, 0.5 mM ATP, 0.5 mM CTP, 0.5 mM GTP, 0.05 mM UTP, 1 μCi of [α-^{32}P]UTP) in a final volume of 20 μl. Assays were performed for 20 min at 30°C, Sequenase stop solution (U.S. Biochemical Corp., Cleveland, Ohio) was added, samples were heated to 90°C and then resolved on 6% polyacrylamide–7 M urea gels (10). Transcripts were visualized on dried gels with X-ray film and quantitated using a PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, Calif.).

The wild-type *cycA* P1 template (pRKK139) contained DNA from positions −76 to +16 cloned in the *Kpn*I-*Hinc*II sites of pRKK137 (16). Site-directed mutagenesis (12) was used to place the G34T mutation in an otherwise wild-type promoter (pRKK138). pRKK137 lacks *cycA* P1 sequences; it contains the *Stu*I-*Bam*HI multiple cloning sequence from pUC21 (25) cloned in the *Hinc*II-*Bam*HI sites of pUC19spf (10).

RESULTS

Transcription of the *cycA* P1 promoter by an alternate RNA polymerase holoenzyme. To identify the *R. sphaeroides* RNA polymerase holoenzyme that recognizes *cycA* P1, in vitro transcription assays were performed with enzyme samples that recognized different classes of eubacterial promoters (10). A sample enriched in the *R. sphaeroides* homolog of *E. coli* $E\sigma^{70}$, $E\sigma^D$ (8, 10), did not produce a transcript from a *cycA* P1 template extending from positions −76 to +16 relative to the transcription initiation site (Fig. 1A). However, this enzyme sample produced a $E\sigma^{70}$-dependent RNA1 transcript from a promoter near the plasmid DNA replication origin (Fig. 1A). The failure of this $E\sigma^D$ sample to transcribe *cycA* P1 was apparently not due to a limitation for σ^D, because a transcript was not observed when an excess of a recombinant *R. sphaeroides* His-σ^D preparation that stimulated RNA1 formation was added to *E. coli* core RNA polymerase (Fig. 1A).

Instead, *cycA* P1 was transcribed by an RNA polymerase sample that contained a protein (RpoH or σ^{37}) related to *E. coli* σ^{32} (10, 11) (Fig. 1A). Several observations indicate that this transcript was derived from *cycA* P1. First, it was absent when a control template (pRKK137) that lacks *cycA* P1 sequences was used (Fig. 1A). The product was also of the expected size after the distance between *cycA* P1 and a downstream transcription terminator was considered. This set of results suggest *cycA* P1 is transcribed by an alternate RNA polymerase holoenzyme. Since σ^{37} was reasonably abundant in the enzyme sample that transcribed *cycA* P1 (see below; 10), it was possible this promoter was recognized by a holoenzyme, $E\sigma^{37}$, that transcribed *E. coli* σ^{32}-dependent genes (10).

Function of *cycA* P1 from a reporter gene containing 76 bp of upstream DNA. To test the previous concept, we sought to analyze the in vivo effects of mutations on *cycA* P1 function. The normal *cycA* P1 transcription initiation site (16) was found in primer extension assays with RNA from cells containing wild-type *cycA* P1 (from positions −76 to +16) fused to *lacZ* (data not shown). LacZ levels in cells harboring this *cycA* P1::*lacZY′* fusion (pCM241) were ~10-fold higher than those when a promoter-less version of this plasmid (pBM4A) was present (Fig. 2, top). We present only promoter activity in aerobic cells because control experiments confirmed the prediction (16, 21, 22) that LacZ levels in photosynthetic cultures harboring wild-type or selected mutant *cycA* P1 reporter plasmids were identical (data not shown). These and other obser-

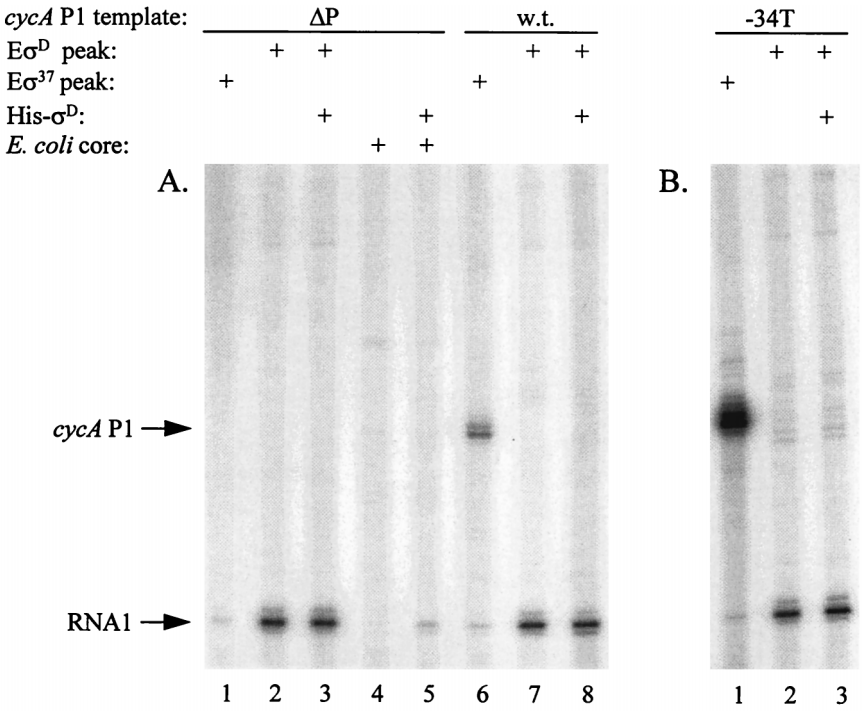

FIG. 1. In vitro transcription of *cycA* P1 templates by different RNA polymerase holoenzymes. (A) Transcription assays were performed as described in Materials and Methods with plasmid templates which lack (ΔP) or contain the wild-type *cycA* P1 promoter (w.t.) cloned upstream of the *spf* transcriptional terminator. The RNA polymerases used (+) are indicated above each set of samples. $E\sigma^D$ and $E\sigma^{37}$ are different *R. sphaeroides* holoenzymes that can be separated by Q-Sepharose chromatography (10). Where indicated, recombinant *R. sphaeroides* His-σ^D was added to increase the saturation of RNA polymerase preparations. The arrows indicate the positions of RNA1 and *cycA* P1 transcripts. (B) Analogous assays were performed with a template containing the G34T mutant *cycA* P1 promoter (−34T).

vations verify that the LacZ activity from these low-copy-number plasmids is a valid indicator of *cycA* P1 function.

Activity of *cycA* P1 requires sequences between positions −34 and −6. After analyzing function of an extensive library of mutant *cycA* P1 promoters, no significant change in LacZ levels was produced from mutant reporter plasmids with either single-base substitutions or a 1-bp deletion downstream of position −7 or point mutations and small deletions between −71 and −39 (data not shown). Thus, it appeared that *cycA* P1 activity was independent of these sequences.

LacZ levels from a set of mutant *cycA* P1 promoters with large deletions upstream of position −39 divide them into two groups. Cells containing mutant *cycA* P1 promoters with deletions ending upstream of −34 produced normal LacZ levels (a subset are shown in Fig. 2A) without altering the transcription initiation site (the primer extension product of the 69Δ49 promoter was indistinguishable from that of a wild-type reporter gene; data not shown). In contrast, LacZ levels from any mutant reporter gene with a deletion that extends downstream of −34 (74Δ32 plus others not shown) were significantly lower than those produced by a wild-type control (Fig. 2A). By comparing LacZ levels produced by cells harboring 41Δ35 or 74Δ32 reporter gene (Fig. 2A), it appeared that sequences downstream of −34 contributed to *cycA* P1 activity.

Point mutations that alter *cycA* P1 function map to regions similar to the *E. coli* σ^{32} consensus promoter. Several alterations between positions −34 and −7 significantly alter *cycA* P1 activity. Although some of these mutant promoters also contain other lesions, the results of our analysis of a large number of point mutations or deletions in these regions suggest that these additional changes do not alter *cycA* P1 function (see above).

Of all the mutant *cycA* P1 promoters isolated, only a substitution at position −34 (G34T) that improves the match to the *E. coli* σ^{32} consensus −35 element (5) increased its function (~20-fold [Fig. 2B]). This mutation appears to simply increase *cycA* P1 function because the G34T transcription initiation site is indistinguishable from a wild-type promoter (in vivo data not shown; see below for in vitro analysis). Other changes at position −34 had either no effect or decreased *cycA* P1 function (Fig. 2B), so the base preference at this site appears to be T>G≈A>C. Mutations at several other conserved bases in the *E. coli* σ^{32} −35 consensus sequence reduced *cycA* P1 function (Fig. 2B). For example, LacZ levels were reduced by placing an A (T33A) or G (M1) at position −33, placing an A at −32 (G32A), or placing a C at −31 (M3) (Fig. 2B). In contrast, LacZ levels from the sole *cycA* P1 promoter with a substitution at position −29 (T-to-A change in M9) were indistinguishable from a wild-type reporter plasmid (Fig. 2B). The analogous position is variable in the *E. coli* σ^{32} −35 consensus (5), so the failure of the T29A mutation to alter LacZ levels is not surprising.

Several mutations in a region related to the *E. coli* σ^{32} −10 consensus sequence also decreased *cycA* P1 function (Fig. 2C). The reduction in promoter activity caused by mutations at position −12 (T12A) or −11 (A11C or A11T) are expected, since they lowered the match of *cycA* P1 to conserved positions in the *E. coli* σ^{32} consensus −10 element (10). In contrast, a *cycA* P1 promoter with a C at −9 (M15) or either A (C7A) or T (M18) at −7 had essentially wild-type activity (Fig. 2C). The positions analogous to −9 and −7 are nonconserved in the *E. coli* σ^{32} −10 consensus (5), so it is not surprising that these later mutations changes did not significantly alter *cycA* P1 function.

	-70 -60 -50 -40 -30 -20 -10 +1 +10	LacZ activity
pCM241	ATCCAAATGTCATGCATGATCCGGAACGCGCGGCCCGCAGTA GTGATT GTGTGCCGGCGGCACC TATATC TTTACCACCATCTACCCATACA	26.5 ± 8
pBM4A		*3.4 ± 1
A. Upstream deletions.		
73Δ67	gggtAccatcCATGCATGATCCGGAACGCGCGGCCCGCAGTA GTGATT GTGTGCCGGCGGCACC TATATC TTTACCACCATCTACCCATACA	26.5 ± 3
76Δ57	AattcgAgcTCccGggTaccCCGGAACGCGCGGCCCGCAGTA GTGATT GTGTGCCGGCGGCACC TATATC TTTACCACCATCTACCCATACA	26.5 ± 3
69Δ49	gaattcgaGctcccgggtAcCatccAaaCGCGGCCCGCAGTA GTGATT GTGTGCCGGCGGCACC TATATC TTTACCACCATCTACCCATACA	21.2 ± 3
76Δ49	ATCCccggGaatTcgAgctcCCGGgtacCGCGGCCCGCAGTA GTGATT GTGTGCCGGCGGCACC TATATC TTTACCACCATCTACCCATACA	26.5 ± 5
41Δ35	gggtAccatcCAaatgTcATgCatgAtcCGgaaCgCGCgGcc GTGATT GTGTGCCGGCGGCACC TATATC TTTACCACCATCTACCCATACA	23.8 ± 5
74Δ32	caatcAATcaCcgGatccccggGaAttcgagctCCCGggtac catATT GTGTGCCGGCGGCACC TATATC TTTACCACCATCTACCCATACA	*4.2 ± 1
B. -35 Element Mutations.		
G34T	ATCCAAATGTCATGCATGATCCGGAACGCGCGGCCCGCAGTA tTGATT GTGTGCCGGCGGCACC TATATC TTTACCACCATCTACCCATACA	‡465.9 ± 53
34ΔG	ATCCAAATGTCATGCATGATCCGGAACGCGCGGCCCGCAGT. aTGATT GTGTGCCGGCGGCACC TATATC TTTACCACCATCTACCCATACA	15.9 ± 3
M35	ATCCAAATGTCATGCATGATCCGGAACGCGCGGCCCGCAtTA cTGATT GTGTGCCGGCGGCACC TATATC TTTACCACCATCTAC..ATACA	* 4.5 ± 1
T33A	ATCCAAATGTCATGCATGATCCGGAACGCGCGGCCCGCAGTA GaGATT GTGTGCCGGCGGCACC TATATC TTTACCACCATCTACCCATACA	*10.6 ± 3
M1	ATCCAAATGTCATGCATGATCCGGAACGCGCGGCCCGCAGTA GgGATT GTGTGCCGGCGGCACC TATATC TTTACtACCATCTACCCATACA	*13.3 ± 3
G32A	ATCCAAATGTCATGCATGATCCGGAACGCGCGGCCCGCAGTA GTaATT GTGTGCCGGCGGCACC TATATC TTTACCACCATCTACCCATACA	*9.0 ± 1
M3	gTCCAAATGTCATGCATGATCCGGAACGCGCGGCCCGCAGTA GTGcTT GTGTGCCGGCGGCACC TATATC TTTACCACaATCTACCCATACA	*13.3 ± 3
M9	ATCCAAATGTCATGCATGATCCGGAACGCGCGGCCCGCAGTA GTGATa GTGTGCCGGCGGCACC TATATC TTTACCACCATCgACCCATACA	18.5 ± 3
C. -10 Element Mutations.		
T12A	ATCCAAATGTCATGCATGATCCGGAACGCGCGGCCCGCAGTA GTGATT GTGTGCCGGCGGCACC aATATC TTTACCACCATCTACCCATACA	*2.9 ± 1
A11C	ATCCAAATGTCATGCATGATCCGGAACGCGCGGCCCGCAGTA GTGATT GTGTGCCGGCGGCACC TcTATC TTTACCACCATCTACCCATACA	*2.4 ± 1
A11T	ATCCAAATGTCATGCATGATCCGGAACGCGCGGCCCGCAGTA GTGATT GTGTGCCGGCGGCACC TtTATC TTTACCACCATCTACCCATACA	*13.2 ± 3
M15	ATCCAAATGTCATGCATGATCCGGAACGCGCGGCCCGCAGTA GTGATT GTGTGCCGGCGGCACC TATcTC TTTACCACCATCcACCCATACA	15.9 ± 3
M18	ATCCAAATGTCATGCATGATCCGGAACGCGCGGCCCGCAGTc GTGATT GTGTGCCGGCGGCACC TATATt TTTACCACCATCTACCCATACA	*10.6 ± 3
C7A	ATCCAAATGTCATGCATGATCCGGAACGCGCGGCCCGCAGTA GTGATT GTGTGCCGGCGGCACC TATATa TTTACCACCATCTACCCATACA	21.2 ± 3
D. Spacer mutations.		
29C28	ATCCAAATGTCATGCATGATCCGGAACGCGCGGCCCGCAGTA GTGATTgcTGTGCCGGCGGCACC TATATC TTTACCACCATCTACCCATACA	*10.3 ± 1
25ΔT	ATCCAAATGTCATGCATGATCCGGAACGCGCGGCCCGCAGTA GTGATT GTG.GCCGGCGGCACC TATATC TTTACCACCATCTACCCATACA	29.1 ± 5
16ΔC	ATCCAAATGTCATGCATGATCCGGAACGCGCGGCCCGCAGTA GTGATT GTGTGCCGGCGG.ACC TATATC TTTACCACCATCTACCCATACA	26.5 ± 5
13ΔC	ATCCAAATGTCATGCATGATCCGGAACGCGCGGCCCGCAGTA GTGATT GTGTGCCGGCGGCAC. TATATC TTTACCACCATCTACCCATACA	34.4 ± 3
M24	ATCCAAATGTCATGCATGATCCGGAACGCGCGGCCCGCAGTA GTGATT GTGTGCCGG..GCACC TATATC TTTACCACCAgCTACCCATACA	15.9 ± 3
C13A	ATCCAAATGTCATGCATGATCCGGAACGCGCGGCCCGCAGTA GTGATT GTGTGCCGGCGGCACa TATATC TTTACCACCATCTACCCATACA	*10.6 ± 3

FIG. 2. Upstream or downstream mutations that do or do not alter *cycA* P1 activity. The sequence of wild-type *cycA* P1 (plasmid pCM241) is shown at the top with potential −10 and −35 elements set off by spaces. Bases that differ in individual mutant promoters are shown in lowercase letters and in bold type. Small deletions are indicated by dots. Vector sequences contributed by the deletions are underlined twice in panel A. Bases that are identical to those of the wild-type *cycA* P1 sequence are shown in uppercase letters. The reported β-galactosidase (LacZ) activities are averages of at least three independent cultures and are shown with standard deviations. LacZ activities that are significantly lower than the wild-type activity (*) or significantly higher than a wild-type reporter gene (‡) are indicated.

Other mutations consistent with recognition of *cycA* P1 by a member of the σ^{32} family. Creating a 17-bp spacer (29C28) decreased *cycA* P1 function slightly, but a mutant promoter with a 15-bp (25ΔT, 16ΔC, or 13ΔC) or 14-bp (M24) spacer had wild-type activity (Fig. 2D). Function of *cycA* P1 also requires sequences upstream of −10, since changes at −13 (C13A) decreased LacZ levels (Fig. 2D). The *E. coli* σ^{32} consensus includes three conserved C residues in an extended −10

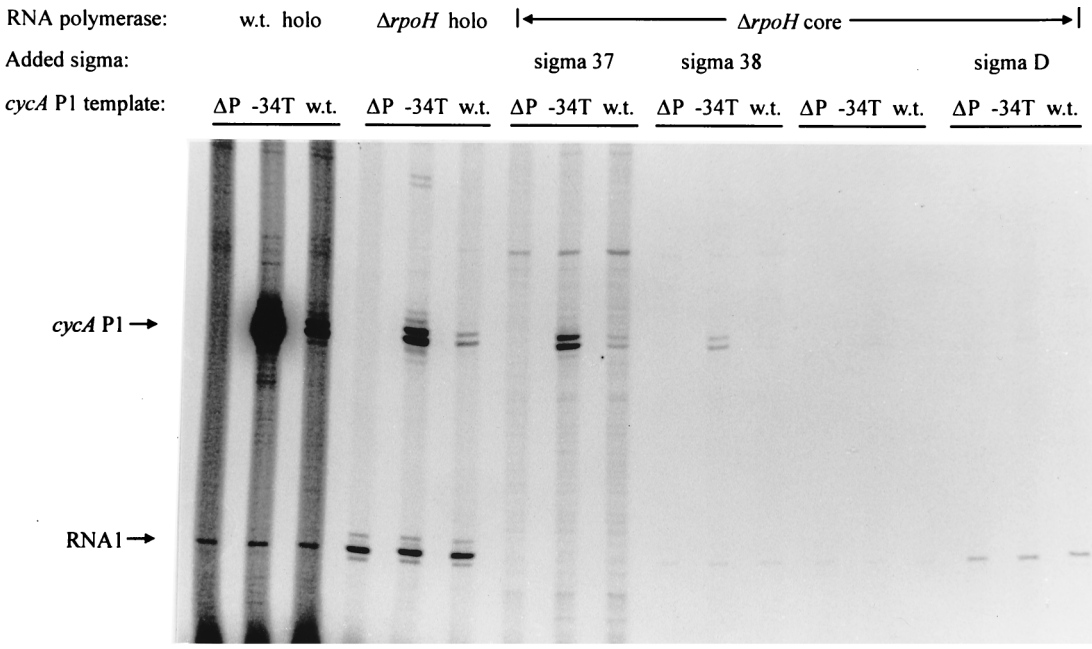

FIG. 3. Transcription of wild-type *cycA* P1 and G34T templates by individual RNA polymerase holoenzymes. Templates which lack the *cycA* P1 sequence (ΔP), the wild-type *cycA* P1 promoter (w.t.), or the G34T (−34T) mutation in the *cycA* P1 promoter were used as indicated directly above the gel. Above each set of lanes (ΔP, −34T, and w.t.) is indicated the source of RNA polymerase holoenzyme (w.t. holo, holoenzyme preparations from wild-type cells; Δ*rpoH* holo, analogous material from a ΔRpoH mutant [11]; Δ*rpoH* core, core RNA polymerase from a ΔRpoH mutant). For assays where core RNA polymerase was reconstituted with potential sigma subunits, σ^{37} and σ^{D} were obtained from a wild-type RNA polymerase sample (fractions 9 and 19, respectively, in Fig. 4A). σ^{38} was obtained from the ΔRpoH RNA polymerase sample (fraction 20 in Fig. 4B). The positions of transcripts (arrows) are indicated as follows: RNA1, the $E\sigma^{D}$-dependent transcript from the *oriV* promoter present on all templates (10); *cycA* P1, the specific *cycA* transcript produced by templates that contain wild-type or G34T mutant promoters.

element (5), so loss of the C at position −13 is a simple explanation for the lower activity of the C13A and M9 mutant promoters.

Transcription of the G34T promoter by individual *R. sphaeroides* holoenzymes. The ability of an $E\sigma^{37}$-containing fraction to transcribe *cycA* P1 in vitro and the in vivo behavior of several mutant promoters suggested that *cycA* P1 was transcribed by an alternate RNA polymerase holoenzyme. Because it is difficult to obtain homogenous samples of alternate RNA polymerase holoenzymes by chromatography, our $E\sigma^{37}$ fraction probably contained additional sigma factors. To assess what holoenzyme transcribed *cycA* P1, we sought to perform in vitro transcription assays after core RNA polymerase was mixed with potential sigma factors that were isolated from SDS-polyacrylamide gels. These assays initially used the G34T mutant promoter that exhibited increased activity in vivo in order to increase our chances of detecting an in vitro product with reconstituted RNA polymerase holoenzymes. Control experiments showed that the G34T promoter was transcribed by the same *R. sphaeroides* RNA polymerase holoenzyme preparations that recognized *cycA* P1 (Fig. 1B). The size of the transcript from the G34T template was also indistinguishable from that produced by wild-type *cycA* P1. Of equal significance, the approximately eightfold increase in abundance of the G34T product using an RNA polymerase sample from wild-type cells indicates that this mutation alters transcription in a minimal in vitro system (see Fig. 3 for a direct comparison).

Our core *R. sphaeroides* RNA polymerase lacked detectable σ^{37} and contained only small amounts of σ^{D} (Fig. 4A). This explains why little transcription of the G34T, wild-type *cycA* P1, and the σ^{70}-dependent RNA1 promoter was observed with this sample (Fig. 3). When individual proteins were reconstituted with this core RNA polymerase, the RNA1 transcript was synthesized when either an ~93-kDa protein (i.e., *R. sphaeroides* σ^{D}; 10) or recombinant His-σ^{D} was added (Fig. 4A). In contrast, the G34T transcript was generated when fraction 18 or 19 (containing ~37- or 38-kDa proteins) was added to core RNA polymerase (Fig. 4A). One explanation for synthesis of the *cycA* P1 transcript in two reconstitution mixtures is the presence of the same sigma factor in both fractions. However, when the abundance of the transcripts in these two reaction mixtures is compared to the levels of the 37-kDa and 38-kDa polypeptides in the RNA polymerase sample used as a source of sigma factors (fractions 18 and 19 [Fig. 4A]), it is also possible that different RNA polymerase holoenzymes ($E\sigma^{37}$ and $E\sigma^{38}$) transcribe G34T.

A second RNA polymerase holoenzyme transcribes the G34T *cycA* P1 promoter. One indication that more than one RNA polymerase holoenzyme transcribes *cycA* P1 was the ability of RNA polymerase from a strain lacking σ^{37} (ΔRpoH holo) to recognize both the wild-type and G34T promoters (Fig. 3). To identify the sigma factor within the ΔRpoH enzyme preparation that recognizes these promoters, this holoenzyme was fractionated by SDS-PAGE, reconstituted with core RNA polymerase, and tested for activity with the G34T promoter (Fig. 4B). Of the proteins in the ΔRpoH holoenzyme preparation that were reconstituted with core RNA polymerase, only the ~38-kDa protein produced a G34T-specific transcript (Fig. 4B). Thus, we conclude that *cycA* P1 is transcribed by two distinct RNA polymerase holoenzymes, $E\sigma^{37}$ and $E\sigma^{38}$.

Wild-type *cycA* P1 is also transcribed by two alternate RNA polymerase holoenzymes. When wild-type *cycA* P1 was tested with the same reconstituted holoenzymes, only fractions containing σ^{37} or σ^{38} enabled core RNA polymerase to recognize this promoter (Fig. 3). Note that the *cycA* P1 transcripts are

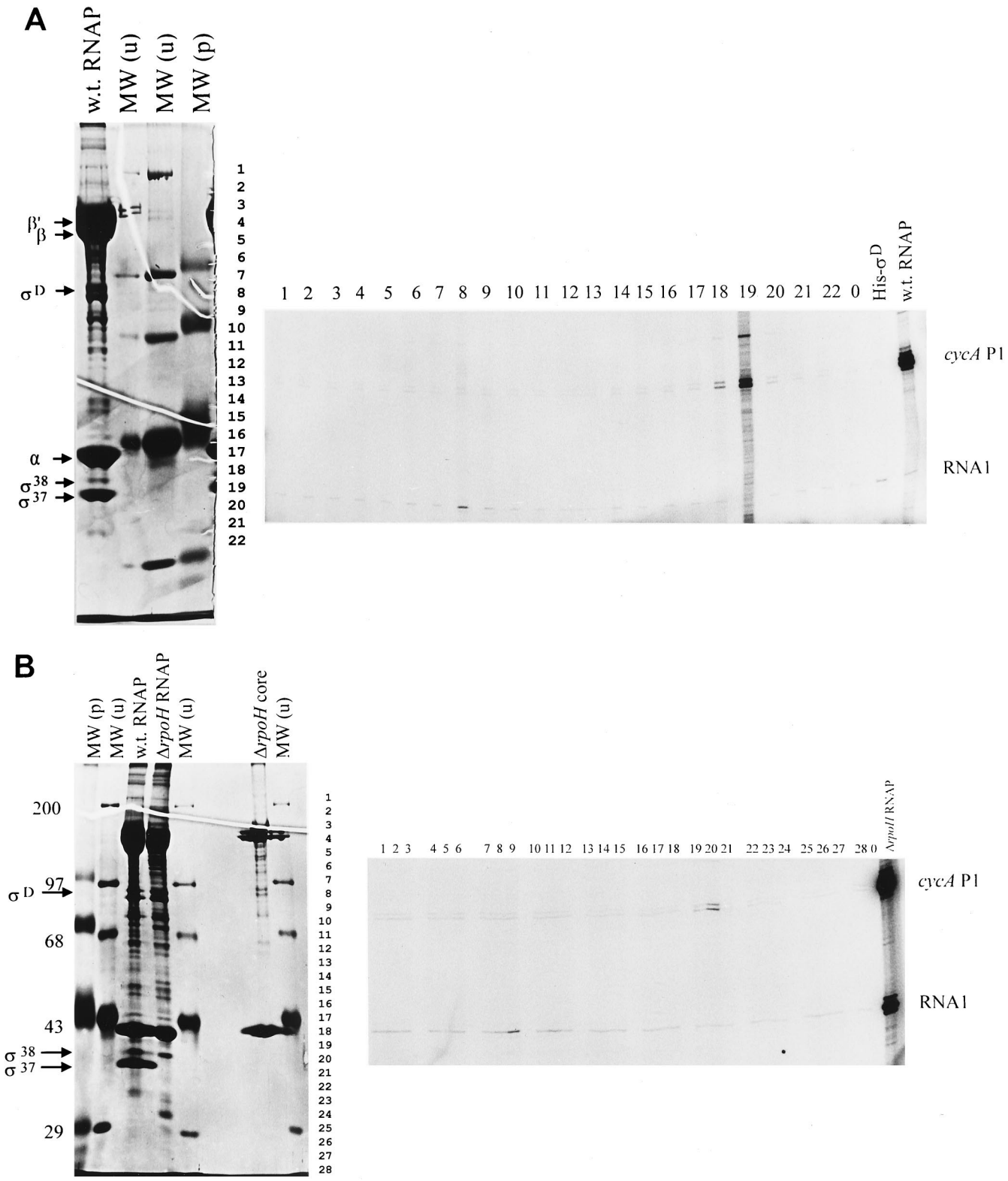

FIG. 4. Transcription of the G34T mutant promoter by reconstituted RNA polymerase holoenzymes. (A) Transcripts produced when the G34T promoter is incubated with different *R. sphaeroides* RNA polymerase samples. The positions of transcripts are indicated to the right of the rightmost gels as follows: RNA1, the Eσ^D-dependent *oriV* transcript from the transcription plasmid template (10); *cycA* P1, the product of the G34T promoter. Reactions in panel A used renatured proteins from a wild-type RNA polymerase sample (shown on the left) as a source of potential sigma factors for addition to core RNA polymerase (shown in panel B) from the ΔRpoH mutant (11). Reactions in panel B used renatured proteins from a RpoH null mutant as a source of potential sigma factors (11) for reconstitution with the same ΔRpoH core RNA polymerase. In panels A and B, numbers above the rightmost gels denote the SDS-polyacrylamide gel fraction that was added to core RNA polymerase as a source of potential sigma factor. Lane 0 contains transcripts produced by core RNA polymerase alone. Lane His-σ^D in panel A shows transcripts produced when recombinant *R. sphaeroides* His-σ^D is added to core RNA polymerase. To the left of the leftmost gels in panels A and B is shown the polypeptide

identical in size to those produced from G34T regardless of whether reconstituted $E\sigma^{37}$ (generated with the 37-kDa protein from wild-type RNA polymerase preparations) or $E\sigma^{38}$ (prepared with the 38-kDa protein from the RpoH mutant; Fig. 3) was used. In addition, the G34T transcript produced by each reconstituted holoenzyme is more abundant than the product of wild-type *cycA* P1 (~5-fold more for $E\sigma^{37}$ and ~3-fold more for $E\sigma^{38}$; Fig. 3). These results suggest that the G34T mutation increases promoter recognition by both $E\sigma^{37}$ and $E\sigma^{38}$.

DISCUSSION

Previous studies have shown that *cycA* P1 function is independent of signals that control genes for other *R. sphaeroides* energy-generating proteins (16, 21), but it was not known whether its transcription required proteins in addition to RNA polymerase. In addition, the exact *cycA* P1 promoter was not known, since this region contains several appropriately positioned elements with limited similarity to those recognized by eubacterial RNA polymerase holoenzymes (16). From our in vivo and in vitro analysis of *cycA* P1 function, we can make several conclusions regarding DNA sequences, RNA polymerase holoenzymes, and proteins required for transcription.

A minimal promoter region is sufficient for *cycA* P1 function. The wild-type LacZ levels produced by *cycA* P1 promoters with mutations upstream of position −34 or downstream of −7 suggested these regions are not target sites for positive or negative regulators. If a transcription factor binds upstream of −34, a significant fraction of the deletions in this region should have altered *cycA* P1 function either by preventing this interaction or by changing the orientation of this potential regulator and RNA polymerase. Thus, it appears unlikely that the RNA polymerase holoenzymes that recognize *cycA* P1 require other DNA binding proteins for activity.

Sequences required for *cycA* P1 function. In contrast, elements between positions −34 and −7 are required for *cycA* P1 function, since several mutations reduced transcription. The spectrum of *cycA* P1 mutations analyzed allows us to rule out a role for previously noted hexamers with similarity to the *E. coli* σ^{70} −35 consensus sequence ($^{-36}$TAGTGA^{-31} and $^{-30}$TTGTGT^{-25}) in promoter function (16).

We also found that *cycA* P1 function was altered by point mutations in elements similar to the −35 ($^{-34}$G**TGA**TT^{-29}) and −10 ($^{-12}$**TATA**TC^{-7}) consensus sequences for both *E. coli* $E\sigma^{32}$ (boldfaced bases) and $E\sigma^{70}$ (underlined bases). The high degree of amino acid sequence identity in regions 2.4 and 4.2 of both *R. sphaeroides* σ^{D} (6) and σ^{37} (11) to their *E. coli* counterparts predicts that these sigma factors will recognize similar promoter elements. Therefore, conclusions about which holoenzyme transcribes *cycA* P1 could not have been made solely from an in vivo analysis because our mutant promoter bank did not contain every possible change at all positions. When the in vitro and in vivo results are considered together, we can conclude that *cycA* P1 is transcribed by alternate *R. sphaeroides* RNA polymerase holoenzymes that recognize *E. coli* heat shock promoters (Fig. 5 summarizes data from salient *cycA* P1 alleles).

Alternate *R. sphaeroides* RNA polymerase holoenzymes transcribe *cycA* P1. The G34T mutation was particularly useful in demonstrating that alternate RNA polymerase holoenzymes transcribe *cycA* P1. Even though G34T is a change towards both the σ^{70} and σ^{32} −35 consensus sequence (Fig. 5), active preparations of *R. sphaeroides* $E\sigma^{D}$ failed to transcribe either this promoter or wild-type *cycA* P1 in vitro. Instead, both wild-type *cycA* P1 and a G34T template were recognized by *R. sphaeroides* RNA polymerase holoenzymes containing either a 37-kDa ($E\sigma^{37}$) or 38-kDa ($E\sigma^{38}$) subunit. *R. sphaeroides* σ^{37} is a member of the *E. coli* σ^{32} family of alternate sigma factors (10, 11). Recently, $E\sigma^{38}$ has also been shown to transcribe *E. coli* heat shock promoters (11). $E\sigma^{37}$ and $E\sigma^{38}$ must both contribute to *cycA* P1 activity in vivo, since its function in steady-state aerobic cells is not abolished by loss of σ^{37} (11).

Additional suggestions that *cycA* P1 is recognized by an alternate RNA polymerase holoenzyme came from analyzing how altering the spacer length or sequence changed promoter activity. Spacing of 17 or 18 bp between the −35 and −10 elements is optimal for *E. coli* $E\sigma^{70}$ promoters (18, 20, 24, 26). In contrast, *E. coli* σ^{32} promoters have spacer lengths of 12 to 17 bp (10). Thus, function of RNA polymerase holoenzymes with DNA recognition properties similar to $E\sigma^{32}$ could explain why decreasing the *cycA* P1 spacer length to 15 or 14 bp did not affect promoter activity. The reduction in *cycA* P1 activity caused by changing a conserved C (C13A [Fig. 5]) in a potential extended −10 element similar to *E. coli* heat shock promoters (5) is also consistent with transcription by *R. sphaeroides* RNA polymerase holoenzymes ($E\sigma^{37}$ and $E\sigma^{38}$) related to $E\sigma^{32}$.

Potential metabolic significance of *cycA* P1 transcription by alternate RNA polymerase holoenzymes. One can envision several reasons why *cycA* P1 is transcribed by $E\sigma^{37}$ and $E\sigma^{38}$. Other data indicate that *R. sphaeroides* $E\sigma^{37}$ and $E\sigma^{38}$ each transcribe *E. coli* heat shock genes (11). Thus, in addition to maintaining a pool of cytochrome c_2 in steady-state cells, *cycA* P1 transcription by these alternate RNA polymerase holoenzymes could increase levels of this electron carrier when cells need ATP for cytoplasmic chaperones to assemble or renature macromolecular assemblages (5). Indeed, the modest increase in *cycA* P1 function that occurs when aerobic wild-type cells are shifted to 42°C is reduced in a σ^{37} mutant (11).

The increased promoter function caused by the G34T mutation indicates that *cycA* P1 is not optimal for recognition by either $E\sigma^{37}$ or $E\sigma^{38}$ (Fig. 5). This, plus the lack of information on how mutations at individual positions in the *E. coli* σ^{32} consensus sequence alters its function (5), makes it difficult to decipher consensus promoters for $E\sigma^{37}$ and $E\sigma^{38}$ and ask if related sequences are present upstream of genes for other *R. sphaeroides* energy-generating proteins.

In summary, our results indicate that *cycA* P1 is transcribed by alternate forms of eubacterial RNA polymerase. Both wild-type *cycA* P1 and a mutant promoter with increased transcription in vivo (G34T) are transcribed in a minimal in vitro system consisting of either reconstituted $E\sigma^{37}$ or $E\sigma^{38}$. Recognition of *cycA* P1 by alternate RNA polymerase holoenzymes or its function without additional transcription factors is not in conflict with previous observations that activity of this promoter is

composition of RNA polymerase samples used for in vitro transcription reactions. These samples include RNA polymerase samples from wild-type cells (w.t. RNAP in panels A and B) or a ΔRpoH mutant (11) (Δ*rpoH* RNAP in panel B) and core RNA polymerase from an RpoH null strain (Δ*rpoH* core in panel B) separated alongside prestained [MW (p)] or unstained [MW (u)] protein molecular weight standards (molecular weights [in thousands] indicated on the left margin). Numbers to the right of the leftmost gel indicate how this gel was sliced to obtain proteins to be tested for sigma factor activity. Individual RNA polymerase subunits are indicated to the left.

E. coli σ^{70} consensus	TTGACA	17–18 bp	TATAAT	
				RELATIVE
E. coli σ^{32} consensus	**CTTGAAA**	11–16 bp	**CCCCAT T**	ACTIVITY
G34T	**T**			20.00
	↑			
Wild type *cycA* P1:	^{35}AG**TGA**TT	GTGTGCCGGCGGC	ACCT**ATA**T**C**$^{-7}$	TTTACCA 1.00
	↓||| |	(13bp)	||| | |	+1
G34A (34ΔG)	A||| |		||| | |	0.54
G34C (M35)	C↓|| |		||| | |	*<0.05
T33A	A|| |		||| | |	*0.31
T33G (M1)	G↓| |		||| | |	*0.42
G32A	A↓ |		||| | |	*0.24
A31C	C ↓		||| | |	*0.43
T29A	A		||| | |	0.65
			↓|| | |	
C13A			A↓| | |	*0.31
T12A			A↓ | |	*<0.05
A11T			T | |	*0.42
A11C			C ↓ |	*<0.05
A9C (M15)			C ↓	0.54
C7A			A	0.77

FIG. 5. Effects of point mutations on *cycA* P1 function. Below the consensus promoters for *E. coli* Eσ^{70} and Eσ^{32} (top) are listed mutations that increase (in bold type and above the wild-type promoter) or decrease (below the wild-type promoter) *cycA* P1 activity (normalized to wild-type activity of 1.00; see Fig. 2B to D for primary data). Relative activities were corrected for LacZ levels produced from a control plasmid lacking *cycA* P1 sequences (pBM4A [Fig. 2]). Asterisks indicate mutations that significantly alter promoter activity. In cases where the *cycA* P1 mutation is not identical to the name of the mutant promoter, its name is provided in parentheses. Bases shown in bold type in the *cycA* P1 sequence are identical to the bases in the *E. coli* Eσ^{70} and Eσ^{32} consensus promoters.

increased by the *trans*-acting *chr-4* allele (21, 22). Ongoing experiments suggest that the *chr-4* mutation increases function of an *R. sphaeroides* member of the eubacterial σ^E family (18a). By analogy to the situation where *E. coli* Eσ^E transcribes the σ^{32} gene (5), it seems likely that increased σ^E function caused by the *chr-4* mutation simply elevates activity of either Eσ^{37} or Eσ^{38} and thereby raises *cycA* P1 activity. From our current understanding of *cycA* P1, it seems clear that a further dissection of its function will increase our understanding of how synthesis of energy-generating electron transport proteins is altered under conditions of metabolic or environmental stress. It could also provide important insights into how gene expression is modulated by regulating function of different RNA polymerase holoenzymes that recognize overlapping promoters.

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