Characterization of the *Rhodobacter capsulatus* Housekeeping RNA Polymerase

**IN VITRO TRANSCRIPTION OF PHOTOSYNTHESIS AND OTHER GENES**

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Paul J. Cullen‡, Charles K. Kaufman§, William C. Bowman, and Robert G. Kranz¶

From the Department of Biology, Washington University, St. Louis, Missouri 63130

To begin to characterize biochemically the transcriptional activation systems in photosynthetic bacteria, the *Rhodobacter capsulatus* RNA polymerase (RNAP) that contains the $\sigma^{70}$ factor (*R. capsulatus* RNAP/$\sigma^{70}$) was purified and characterized using two classical $\sigma^{70}$-type promoters, the bacteriophage T7A1 and the RNA I promoters. Transcription from these promoters was sensitive to rifampicin, RNase, and monoclonal antibody 2G10 (directed against the *Escherichia coli* $\sigma^{70}$ subunit). Specific transcripts were detected *in vitro* for *R. capsulatus* cytochrome $c_5$ (cyaA) and fructose-inducible (fruB) promoters and genes induced in photosynthesis (*puf* and *puc*) and bacteriochlorophyll biosynthesis (*bchC*). Alignment of these natural promoters activated by *R. capsulatus* RNAP/$\sigma^{70}$ indicated a preference for the sequence TTGAC at the $-35$ region for strong *in vitro* transcription. To test the $-35$ recognition pattern, the *R. capsulatus* nifA1 promoter, which exhibits only three of the five consensus nucleotides at the $-35$ region, was mutated to four and five of the consensus nucleotides. Although the nifA1 wild type promoter showed no transcription, the double mutated type promoter exhibited high levels of *in vitro* transcription by the purified *R. capsulatus* RNAP/$\sigma^{70}$ enzyme. Similarities and differences between the RNAPs and the promoters of *R. capsulatus* and *E. coli* are discussed.

As elucidated by many *in vitro* studies during the last 30 years, bacterial transcription requires a core RNA polymerase (RNAP)$^*$ enzyme and $\sigma$ factors that recognize specific promoter elements. RNAP has been purified from a variety of bacterial species but has been characterized most thoroughly in *Escherichia coli* (for review, see Ref. 1). RNAP from the photosynthetic bacterium *Rhodobacter sphaeroides* has been purified and shown to have a subunit composition similar to that of other Gram-negative bacteria (2). A report of a partially purified preparation of *Rhodobacter capsulatus* RNAP was published a decade ago (3), but from that study it was not possible to determine definitively which, if any, was the housekeeping $\sigma^{70}$ subunit nor to evaluate promoter recognition determinants. *In vitro* transcription of *R. sphaeroides* RNAP has been demonstrated from templates that contain *E. coli* $\sigma^{22}$ and $\sigma^{70}$ type promoters and from an *R. sphaeroides* rrn promoter (4). In the present report we describe *in vitro* studies on the transcription apparatus of *R. capsulatus* and promoters that are recognized by this system.

Most of the studies to date on gene regulation in *R. capsulatus* have concerned genetic characterization of signal transduction pathways or the *in vivo* analysis of mRNAs. From these investigations unique activators and repressors are theorized to regulate $\sigma^{70}$-dependent transcription at a variety of promoters in photosynthetic bacteria. In *R. capsulatus*, operons involved in photosynthesis are regulated by light and oxygen (for review, see Ref. 5). For example, *puc*, *puf*, and *puh* encode the structural polypeptides for the photosynthetic complexes, and *bch* encodes bacteriochlorophyll biosynthetic enzymes. The promoters of some of these genes have been studied by deletion and *in vivo* primer extension analysis (e.g. 6–8). Although *bch* and *puc* are thought to be transcribed by the *R. capsulatus* RNA polymerase/$\sigma^{70}$ holoenzyme, based on promoter sequences, it is unclear what $\sigma$ factor(s) recognize the *puf* and *puh* genes. Site-directed mutational analysis of the *bchC* promoter demonstrated that nucleotides typical of $\sigma^{70}$ promoters in the $-10$ and $-35$ hexamers upstream of the transcriptional start site are important for *in vivo* transcription (9). Light-dependent stimulation of transcription from the *puf* and *puh* operons requires the *hurA* gene (10). The oxygen-regulated *puf*, *puh*, and *puc* operons require the *regAregB*-encoded two-component system, although it is unknown whether these promoters are directly activated by such proteins (11, 12). The recently discovered CrtJ is required for aerobic repression of the *puf*, *puc*, *puh*, and *bch* operons by an unknown mechanism (13). In some cases cis-DNA elements upstream of these promoters have been proposed to mediate light and oxygen regulation by binding of the regulatory proteins (e.g. 9, 11). In the present study it is shown that the cytochrome $c_5$ gene (cyaA) and the fructose-inducible gene *fruB* (14) may also be activated by the *R. capsulatus* RNAP/$\sigma^{70}$. Other signal transduction pathways in *R. capsulatus* elucidated mainly by genetic studies include nitrogen sensing (for review, see Refs. 15 and 16) and the FnrL regulon.

As an important step toward *in vitro* reconstitution of these signal transduction pathways, the *R. capsulatus* housekeeping RNAP holoenzyme was characterized. $\sigma^{70}$-Dependent tran-

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¶ To whom correspondence should be addressed: Dept. of Biology, Campus Box 1137, Washington University, One Brookings Dr., St. Louis, MO 63130. Tel.: 314-935-4278; Fax: 314-935-4432; E-mail: Kranz@wustl.wustl.edu.

The abbreviations used are: RNAP, RNA polymerase; $\sigma^{70}$, sigma 70 factor; Rif$^\beta$ and Rif$^\delta$, rifampicin-resistant and -sensitive, respectively; PCR, polymerase chain reaction; bp, base pair; PEG, polyethylene glycol; DTT, dithiothreitol; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography.

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Zeilstra-Ryall, J. H., Gabbert, K. K., Mouncey, N. J., Kaplan, S., and Kranz, R. G. (1997) _J. Bacteriol._, in press.
scription from a variety of promoters using linear or supercoiled templates was demonstrated. A consensus for optimal σ70 type promoters in this bacterium was tested further by mutating the nitrogen-regulated nifA1 promoter (18) toward the consensus and engineering each mutant promoter upstream of a transcriptional terminator on a supercoiled plasmid. Results of in vitro transcription studies on these mutant promoters confirmed the importance of specific –35 recognition elements for the holoenzyme.

EXPERIMENTAL PROCEDURES

Strains and pfoB Plasmids—The bacterial strains and plasmids used in this study are shown in Table I. Plasmids pUC:SBrif and pUC:BT10rif contain the sequences that encode the rifampicin binding domain of pfoB strains SB1003 (Rif +) and B10 (Rif +), respectively. The plasmids were constructed by polymerase chain reaction (PCR) of chromosomal DNA of the two strains using primers described in Table II. The R. capsulatus pfoB sequence for design of primers was provided by Dr. Robert Haselkorn (University of Chicago). The PCR products were digested with BamHI and PstI and cloned into pUC118. The pfoB RNAP β subunit fragments were sequenced using the Sequenase enzyme according to company protocols (Amersham Corp.).

Plasmids for Supercoiled Templates—Linear templates for transcription reactions were created by PCR using the primers indicated in Table II. For supercoiled templates, all DNA fragments that contained putative promoters were cloned into pUC118 directly upstream of a strong transcriptional terminator. The fruB promoter was cloned by PCR of SB1003 chromosomal DNA using primers described in Table II, based on Ref. 14. The 300-bp fruB promoter fragment was digested with KpnI and EcoRI and cloned into pUC118 to create pUC:fruB. Plasmid pUC:pT77 that contains 150 bps of the T7A1 promoter was made by excision of the T7A1 promoter fragment with BamHI and EcoRI from plasmid pCL185 (19) and ligation into pUC118. pUC:pT7 that contains 150 bps of the T7A1 promoter was made by excision of the T7A1 promoter fragment with BamHI and EcoRI from plasmid pCL185 (19) and ligation into pUC118. pUC:pT7 that contains 150 bps of the T7A1 promoter was made by excision of the T7A1 promoter fragment with BamHI and EcoRI from plasmid pCL185 (19) and ligation into pUC118. pUC:pT7 that contains 150 bps of the T7A1 promoter was made by excision of the T7A1 promoter fragment with BamHI and EcoRI from plasmid pCL185 (19) and ligation into pUC118. The transcriptional terminator was cloned downstream of each of the promoters by excision of the 125-bp terminator from pUC with PstI and HindIII and into pUC:pucC, pufQ, bchC, to create the supercoiled templates pUC:pucC, pufQ, bchC. The pUC:pucC was made by excision of the pufQ promoter region from pUC:pufQ with EcoRI and PstI and ligation into pUC. The pUC:nifA1 was created by excision of the nifA1 promoter from pA1-P16 with SalI and PstI followed by ligation into pUC. Templates were confirmed by restriction and sequence analysis and purified in CsCl gradients for all reactions.

The nifA1 promoter mutants A1Mut1, A1Mut2, and A1Mut3 were generated by PCR of plasmid pA1-P16 using the primers described in Table II. The PCR products were digested with MluI and PstI and cloned into pUC-nifA1 to create pA1M1, pA1M2, pA1M3. The mutations were confirmed by sequence analysis, and the plasmids were purified in CsCl gradients for in vitro transcription reactions.

RNA Polymerase Purification, Properties, and Stability—R. capsulatus RNAP was purified from 12 liters of R. capsulatus cells (strain SB1003) grown aerobically to mid exponential phase (–17 h, A540 = 2.0) in RCV medium (21) at 34 °C. These conditions, although aerobic, still allowed the synthesis of some photosynthetic pigments, albeit at lower levels than fully anaerobic, light-grown cells. The rifampicin-sensitive strain R. capsulatus B10 was used for some experiments, where noted. Otherwise, R. capsulatus RNAP refers to the enzyme from SB1003. Cells were harvested by centrifugation and stored as a cell pellet at −80 °C. Purification was based on procedures described previously (22, 23) with modifications. Cell lysis was performed on ice, and the purification was at 4 °C unless otherwise noted. Cells (~200 g, wet weight) were lysed by resuspension in 105 ml of sucrose solution (10 mM Tris-HCl, pH 8, 25% sucrose, 100 mM NaCl) for 15 min, followed by the addition of 0.24 ml of lysozyme solution (300 mM Tris-HCl, pH 8, 100 mM EDTA, and 4 mg/ml lysozyme) for 5 min and addition of 135 ml of lysis solution (1 mM NaCl, 20 mM EDTA, 110 mg of sodium deoxycholic acid). Lysis was allowed to proceed for 10 min at 10 °C. Following cell lysis, the RNA polymerase-nuclease acid complexes were precipitated by the addition of 380 ml of PEG solution (17% polyethylene glycol 8000 (PEG, Sigma), 157 mM NaCl, 1 mM DTT), followed by centrifugation at 7,000 rpm for 10 min in a Sorvall centrifuge. The supernatant was removed, and proteins were eluted from the PEG pellet by the addition of 60 ml of high salt solution (10 mM Tris- HCl, pH 8, 5% PEG, 2 mM NaCl, 1 mM DTT). The PEG supernatant containing the R. capsulatus RNAP was diluted in 430 ml of column buffer (10 mM Tris-HCl, pH 8, 10 mM MgCl2, 1 mM EDTA, 1 mM DTT, and 7.5% glycerol) to 500 mM NaCl and loaded onto 5 × 4 ml heparin-agarose (Sigma) columns that were run at −0.5

### Table I

| Strain/plasmid | Description | Ref. |
|---------------|-------------|-----|
| E. coli TB1   | F' araΔ(lac-proAB)RpsL(Str−) [Δ80dlacZM15hsdR Str− m−] | 43 |

R. capsulatus

| Strain/plasmid | Description | Ref. |
|----------------|-------------|-----|
| B10            | Wild type   | 44 |
| SB1003         | Rif + mutant of B10 | 44 |

Plasmid

| Strain/plasmid | Description | Ref. |
|----------------|-------------|-----|
| pUC118, 119    | Amp′, M13 intergenic region | 45 |
| pHP45          | Amp′, Spec′, cassette with flanking transcri transplantional terminators | 20 |

pC42

| Strain/plasmid | Description | Ref. |
|----------------|-------------|-----|
| Amp′, 500-nt PCR insert of cyaA in pUC119 | 46 |

pCL185

| Strain/plasmid | Description | Ref. |
|----------------|-------------|-----|
| Amp′, 2.2-kb T7A1 promoter in pBR322 | 19 |

pRPSLH2

| Strain/plasmid | Description | Ref. |
|----------------|-------------|-----|
| Amp′, pucCBA genes EcoRI fragment in pBR322 | 17 |

pCB7011

| Strain/plasmid | Description | Ref. |
|----------------|-------------|-----|
| Amp′, pPBHα-lacZ fusion | 8 |

p1255ex

| Strain/plasmid | Description | Ref. |
|----------------|-------------|-----|
| Amp′, pufQ EcoRI fragment in pBR322 | 6 |

pDAY23

| Strain/plasmid | Description | Ref. |
|----------------|-------------|-----|
| Amp′, spec′ bchC gene | This study |

pA1-P16

| Strain/plasmid | Description | Ref. |
|----------------|-------------|-----|
| Amp′, 300-bp R. capsulatus nifA1 promoter | 18 |

pU19

| Strain/plasmid | Description | Ref. |
|----------------|-------------|-----|
| Amp′, PCR terminator PstI-HindIII in pUC118 | This study |

pUC:SBrif

| Strain/plasmid | Description | Ref. |
|----------------|-------------|-----|
| Amp′, PCR Rif β-subunit domain of SB1003 | This study |

pUC:BT110rif

| Strain/plasmid | Description | Ref. |
|----------------|-------------|-----|
| Amp′, Rif β-subunit domain of B10 | This study |

pUC:BT7

| Strain/plasmid | Description | Ref. |
|----------------|-------------|-----|
| Amp′, T7A1 promoter from pCL185 in pUC118 | This study |

pUC:bchC

| Strain/plasmid | Description | Ref. |
|----------------|-------------|-----|
| Amp′, bchC promoter from pDAY23 in pUC118 | This study |

pUC:pucC

| Strain/plasmid | Description | Ref. |
|----------------|-------------|-----|
| Amp′, pucC promoter from pRPSLH2 in pUC118 | This study |

pUC:pufQ

| Strain/plasmid | Description | Ref. |
|----------------|-------------|-----|
| Amp′, pufQ promoter from p1255ex in pUC118 | This study |

pUC:fruB

| Strain/plasmid | Description | Ref. |
|----------------|-------------|-----|
| Amp′, fruB promoter from SB1003 in pUC118 | This study |

pUC:nifA1

| Strain/plasmid | Description | Ref. |
|----------------|-------------|-----|
| Amp′, terminator PstI-HindIII cloned downstream of pUC:fruB, pucC, pufQ, bchC, fruB, nifA1 constructs | This study |

a nt, nucleotide.

b kb, kilobase.
Rhodobacter capsulatus RNA Polymerase and Promoters

**RESULTS AND DISCUSSION**

**Purification of the R. capsulatus RNA Polymerase α970 Holoenzyme**—The R. capsulatus RNAP was purified to characterize α970 type promoters and to study activator- and repressor-regulated transcription in R. capsulatus. The R. capsulatus RNAP protein was purified by PEG precipitation followed by heparin-agarose and DEAE-Sepharose chromatography to at least 95% homogeneity by SDS-PAGE analysis (Fig. 1). The purification was assayed by nonspecific transcription assays (see below) and SDS-PAGE. Analysis of the R. capsulatus RNAP by SDS-PAGE showed subunits that by molecular weight correspond to an α, β, β′, and θ, as well as the 90-kDa major (housekeeping) σ970 (Fig. 1, lane 6). The E. coli RNA polymerase (E. coli RNAP, strain TB1) was purified using the same procedure (Fig. 1, see lanes 1–3), which was compared with the HPLC-purified E. coli RNAP970 holoenzyme (Fig. 1, lane 7). A doublet at approximately 150 kDa was confirmed by SDS-PAGE analysis of underloaded DEAE-Sepharose pure R. capsulatus RNAP fraction; moreover, a Bio-Rex 70 column was also able to separate at least two 150-kDa bands (data not shown). The phosphoamidase of the R. capsulatus RNAP α polypeptides to the β and β′ bands corresponded to 2:1:1 stoichiometry, similar to the E. coli RNAP (Fig. 1, compare lane 3 with lane 6). An ω subunit occasionally purified with the core E. coli RNAP (27) but has no known function in vivo (28). The polypeptide observed by SDS-PAGE at approximately 15 kDa may be the R. capsulatus ω subunit homolog, but this has not been investigated further.

**TABLE II**

| Primers for PCR | Upstream oligonucleotide (5′ to 3′) for PCR | Downstream oligonucleotide (5′ to 3′) for PCR |
|----------------|--------------------------------------------|---------------------------------------------|
| pA1-P16        | pA1M                                      | GCCAGCGCGTCGGAAGAATCGTCCGCTTTTGCGCCAT       |
| pA1-P16        | pA1M2                                     | AACAGCATGACCATTGAGACGCGCTCTCTCTCTCTCTCTCT   |
| pA1-P16        | pA1M3                                     | AACAGCATGACCATTGAGACGCGCTCTCTCTCTCTCTCTCT   |
| pCBADE         | pUCT-pucC                                  | AACTGCAGCTGGATCATGGAGACGCGCTCTCTCTCTCTCTCT |
| pCB701         | pUCT-puPHA                                 | AACTGCAGCTGGATCATGGAGACGCGCTCTCTCTCTCTCTCT |
| pDAY23         | pUCT-bchC                                  | AACTGCAGCTGGATCATGGAGACGCGCTCTCTCTCTCTCTCT |
| pT7A1          | pUCT-puMQ                                 | AACTGCAGCTGGATCATGGAGACGCGCTCTCTCTCTCTCTCT |
| Chromosome     | pUCT-fruitB                               | AACTGCAGCTGGATCATGGAGACGCGCTCTCTCTCTCTCTCT |
| pH45           | pUCT                                      | AACTGCAGCTGGATCATGGAGACGCGCTCTCTCTCTCTCTCT |
| Chromosome     | pUC8SBrif and                             | AACTGCAGCTGGATCATGGAGACGCGCTCTCTCTCTCTCTCT |
|                | pUCB10rif                                  | AACTGCAGCTGGATCATGGAGACGCGCTCTCTCTCTCTCTCT |

m/min using a peristaltic pump. The columns were washed with 20 column volumes (320 ml total) of column buffer that contained 300 mM NaCl, and RNA polymerase was eluted in 48 ml of column buffer in 450 mM NaCl. Fractions that contained peak RNA polymerase activity (~5 ml; see below) were diluted to 135 ml in column buffer to 150 mM NaCl and loaded onto 3 × 4 ml DEAE-Sepharose (Sigma) columns run at 0.6 ml/min. The columns were washed with 20 column volumes (320 ml total) of column buffer in 150 mM NaCl, and the RNA polymerase was eluted off of the column in 80 ml of column buffer in 300 mM NaCl. Fractions that contained the peak RNAP activity (~9 ml) were ammonium sulfate precipitated and stored at 2 mg/ml in 30% glycerol at ~80 °C. The E. coli RNAP was purified using the above procedure from 2 liters of cells (~10 g wet weight; strain TB1) which were grown aerobically for 17 h in Luria broth (24) at 37 °C. RNA polymerase from the Rif R. capsulatus strain B10 (2 liters grown to mid exponential phase at 34 °C) was purified by PEG precipitation and heparin-agarose chromatography as described above. Proteins were quantitated by BCA assays (Pierce) and SDS-PAGE analysis using serial dilutions of 2 mg/ml bovine serum albumin as a control. The E. coli RNAP HPLC-purified RNAPα70 holoenzyme was provided by Dr. Richard Burgess and Nancy Thompson (University of Wisconsin, Madison). Monoclonal antibody 2G10 was kindly provided by Dr. Richard Burgess and Nancy Thompson (University of Wisconsin, Madison). Antibodies to the R. capsulatus RNAP were generated by immunization of New Zealand White rabbits with the holoenzyme of greater than 95% purity. In vivo primer extension analysis was performed as described (26). The primer 5′-TGGTGAAATCTTCTTGCACGCGGCGG-3′ was used for the reverse transcription reaction for the cycA gene.
Rhodobacter capsulatus RNA Polymerase and Promoters

The approximately 90-kDa polypeptide observed by SDS-PAGE in the *R. capsulatus* RNAP DEAE-Sepharose fraction (Fig. 1, lane 6) was shown to be the major (housekeeping) *R. capsulatus* σ70 factor. Monoclonal antibody 2G10 is specific for a 15-amino acid epitope in region 3.1 of *E. coli* σ70 (29). 2G10 also cross-reacts with the major σ factor from a variety of bacterial species, including *R. sphaeroides* (4). Western analysis demonstrated that 2G10 cross-reacts with the 90-kDa subunit observed by SDS-PAGE analysis of the *R. capsulatus* RNAP purification fractions (not shown).

Initially, transcriptional activities of *R. capsulatus* RNAP and *E. coli* RNAP enzymes at sequential purification steps were measured by a nonspecific transcription assay that determines the accumulation of RNA product using salmon sperm DNA as template and radiolabeled UTP (Table III). The *R. capsulatus* RNAP purification resulted in a 140-fold enrichment (150% yield) and a higher yield (75%), which was comparable to results from *E. coli* RNAP (from SB1003) was resistant to a high level of rifampicin (50 μg/ml) similar to RNAP from a particular class of Rif* E. coli* mutants (30).

![Image](59x257 to 296x373)

**FIG. 1.** Purification of the *R. capsulatus* RNAP and *E. coli* RNAP. A 10% SDS-PAGE gel loaded with 10 μg of sample/lane is shown (unless otherwise noted). Size standards are shown on the left (from Bio-Rad), and the β, β', σ70, and α subunits are labeled on the right. Fractions are from either *E. coli* (lanes 1–3) or *R. capsulatus* (lanes 4–6). Lanes 1 and 4, PEG precipitation fraction; lanes 2 and 5, heparin-agarose fraction; lanes 3 and 6, DEAE-Sepharose fraction; lane 7, 1 μg of HPLC-purified *E. coli* RNAP holoenzyme (provided by Robert Landick, University of Wisconsin, Madison).

**Table III**

Summary of RNA polymerase purification from *R. capsulatus* and *E. coli*

| Strain             | Fraction     | Volume | Total protein | Total activity a | Specific activity | Yield | Fold purification |
|--------------------|--------------|--------|---------------|-----------------|------------------|-------|------------------|
| *E. coli* TB1      | PEG supernatant | 6.5    | 679           | 11.6            |                  |       |                  |
| *E. coli* TB1      | Heparin-agarose | 1.4    | 2,314         | 551             |                  | 100   | 36               |
| *E. coli* TB1      | DEAE-Sepharose | 0.26   | 725           | 2,231           |                  | 75    | 150              |
| *R. capsulatus* SB1003 | PEG supernatant | 4.6    | 4,885         | 17.7            |                  |       |                  |
| *R. capsulatus* SB1003 | Heparin-agarose | 1.1    | 8,646         | 524             |                  | 100   | 35               |
| *R. capsulatus* SB1003 | DEAE-Sepharose | 0.2   | 3,469         | 1,927           |                  | 40    | 140              |

*a* For nonspecific assays, 1 unit represents incorporation of 1 nmol of [3H]UTP in 15 min at 30 °C.

The starting material was 200 g of cells (wet weight) for *R. capsulatus* SB1003 and 10 g of cells (wet weight) for *E. coli* TB1. Each assay was repeated at least twice.
the presence of rifampicin (Fig. 2A). Monoclonal antibody 2G10 has been shown to inhibit transcription of the E. coli RNAP/σ^70 holoenzyme in vitro (34). The 2G10 antibody also inhibited R. capsulatus RNAP production of the 141-nucleotide product although a 230-nucleotide readthrough transcript was unaffected by the antibodies (e.g. Fig. 2B, lane 5; see below).

Occasionally, larger transcripts of varying intensity were observed using the T7A1 linear template and other templates (Fig. 2B). In particular, a 230-nucleotide transcript, of approximately the same length as the template, appeared to be relatively more intense when using R. capsulatus RNAP preparations that have a higher core/σ^70 ratio. ("Experimental Procedures" describes the purification of such core-enriched preparations from R. capsulatus). To understand more fully the basis for these products we investigated their synthesis. Synthesis of the 230-nucleotide transcript (designated readthrough in Fig. 2B) was sensitive to rifampicin and RNase (data not shown) but not to the monoclonal antibody 2G10 (Fig. 2B, lane 5) or low levels of potassium acetate (Fig. 2B, lane 3). We conclude that this transcript is the product of end-to-end readthrough of the T7A1 230-nucleotide template by the core R. capsulatus RNAP (which was also observed for E. coli RNAP; Fig. 2B, lanes 7–9). The 371-, 601-, and 831-nucleotide transcripts (designated transposition in Fig. 2B) were sensitive to low levels of potassium acetate, rifampicin, RNase, and to the monoclonal antibody 2G10 (Fig. 2B, lanes 3 and 5, respectively) and thus are probably the result of initiation from the T7A1 promoter and transposition to a neighboring template, as has been characterized with the T7 RNAP (35) and E. coli RNAP (e.g. Ref. 36).

The R. capsulatus RNAP/σ^70 holoenzyme was characterized using a supercoiled template that contained a σ^70 promoter. The RNA I promoter (37) from the ColEI plasmids pBR322 and pUC derivatives, also called the replication inhibitor promoter, is a strong σ^70-dependent promoter that utilizes the E. coli RNAP/σ^70 in vitro and in vivo, producing a characteristic 108-nucleotide transcript that inhibits plasmid replication in vivo (38). The R. capsulatus RNAP/σ^70 holoenzyme also produces a 108-nucleotide product in vitro from pUC118 and other pBR322-based plasmids (e.g. Fig. 3A). This transcription is sensitive to the σ^70 monoclonal antibodies 2G10, rifampicin, and is optimal at 100 mM potassium acetate, although the salt requirement was not as stringent as for the T7A1 promoter (not shown). This promoter serves as an internal control for all of the R. capsulatus promoter regions that have been engineered into pUC118.

R. capsulatus RNAP/σ^70 Utilizes R. capsulatus Promoters in Vitro—A number of regulated promoters in R. capsulatus have been analyzed with respect to their in vivo transcription start sites. The fruB gene has been shown by lacZ fusion studies to be induced by the addition of fructose in R. capsulatus (14). Unfortunately, delineation of the in vivo transcription start site for fruB has not been reported. We have attempted to determine the fruB start site by primer extension analysis, but at least five products of similar intensity result. Thus, the fruB start site and promoter shown in Fig. 4 are based on the in vitro site determined in the present study (see below) and must be considered tentative. All other promoters shown in Fig. 4, including that for cycA, are based on the in vivo transcription start sites and correlate with the transcripts observed in vitro, as shown below. (We have determined the in vivo cycA transcription start site, as depicted in Fig. 4.)

To characterize R. capsulatus promoters using the R. capsulatus RNAP/σ^70 holoenzyme we initially used PCR to create linear templates that contain various R. capsulatus promoters predicted either to use σ^70 or unknown factors. Using the purified R. capsulatus RNAP and linear templates that contained the photosynthetic promoter, pucC (Fig. 3B, lane 1) and the cytochrome _c_2 cycA (lane 3) promoter, runoff transcripts were produced in vitro. A 102-nucleotide product was observed in reactions that contained the pucC promoter template, in addition to a probable readthrough transcript at 180 nucleotides (Fig. 3B, lane 1). A 165-nucleotide band was observed in the presence of the cycA promoter template (lane 3). Interestingly, neither the pucC (Fig. 3B, lane 2) nor the cycA promoter (lane 4) was transcribed by E. coli RNAP/σ^70, although a readthrough transcript from the pucC template was observed.

Specific transcription products from templates containing other promoters (fruB, bchC, and pufK) were not detected using linear templates when analyzed with E. coli RNAP or R. capsulatus RNAP. Thus, most promoters were analyzed using supercoiled templates that contained the T4 bacteriophage gene 32 transcriptional terminator engineered downstream. The exact site of termination in vitro from the gene 32 terminator is known for E. coli RNAP, and thus the sizes of RNA

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3 P. J. Cullen, C. K. Kaufman, W. C. Bowman, and R. G. Kranz, unpublished data.
products initiating from a predicted promoter could be calculated. Templates that contained the pufQ, fruB, and bchC promoters gave major specific transcripts of approximately 290, 280, and 145 nucleotides, respectively (Fig. 3A). Each of the transcripts was similar to the level of intensity of the RNA I transcript (108 nucleotides) and was not present in control plasmids that did not contain the promoter insertions (Fig. 3A).

The E. coli RNAP also produced a transcript of the same size as R. capsulatus RNAP from each of the supercoiled templates in vitro (data not shown). Synthesis of the pufQ, fruB, and bchC products was sensitive to rifampicin and to the $\sigma^{290}$ mononuclease antibodies 2G10 (e.g. Fig. 3C for fruB promoter). However, variability for each of these promoters was observed in the potassium acetate requirement. Lower levels of potassium acetate (10 mM) resulted in increased levels of the pufQ product, did not affect the bchC promoter, and abolished transcription from the fruB promoter (e.g. Fig. 3C, lane 7). Minimal transcription was observed when potassium acetate was replaced with KCl (at 100 mM, see Fig. 3C, lane 8).

The promoters activated by R. capsulatus RNAP/$\sigma^{35}$ in vitro were aligned and compared with the consensus $\sigma^{35}$ recognition sequence of E. coli (Fig. 4). With one significant exception (i.e. pufQ) the comparison suggests that homology in the −35 region (specifically TTGAC) is important for optimal recognition of the promoter by the R. capsulatus $\sigma^{35}$ subunit. The region 4.2 of E. coli $\sigma^{35}$ is responsible for recognition of the −35 hexamer, and specific amino acids Arg-584 and Arg-588 make base-specific contacts with the nucleotides within the −35 region (39, 40). The rpoD gene encoding the R. capsulatus $\sigma^{35}$ protein has recently been sequenced by Haselkorn and colleagues (GenBank accession number U28162). Sequence comparison between the E. coli and R. capsulatus $\sigma^{35}$ shows that Arg-584 and Arg-588 residues are conserved as are the 5 upstream and 10 downstream amino acids. Nucleotides within the conserved −10 hexamer show less homology among strong R. capsulatus $\sigma^{35}$ promoters (Fig. 4A). The region 2.4 of E. coli $\sigma^{35}$ makes base-specific contacts at Thr-440 and Gln-437 with the −10 hexamer (41), and this region is also completely conserved with the R. capsulatus $\sigma^{35}$. The $\sigma^{35}$-rich properties of the −10 region probably aid in open complex formation at this region, but conclusions about exact contacts with $\sigma^{35}$ cannot be made.

It is surprising that the pufQ promoter appears to be utilized by the $\sigma^{35}$ holoenzyme in vitro since this promoter is atypical in the −35 region. Based on this poor −35 homology it has been proposed that a $\sigma$ factor other than the housekeeping subunit may be required for transcription of pufQ (42). However, no genetic evidence has yet been generated to suggest such an alternative $\sigma$ factor, as discussed previously (42). We could not find other sequences near the puf promoter which are similar to the −35 consensus which might yield this in vitro transcript, suggesting that an aberrant start site is not the reason for the observed product. It may be important that all other promoters were optimal at 100 mM potassium acetate, whereas preliminary studies indicate that the puf promoter appears to function at lower salt concentrations. Nevertheless, we cannot rule out the possibility that another promoter in the pufQ fragment is responsible for this transcription product. On the other hand, the consensus −35 hexamer may not always reflect the most important determinant for recognition/transcription.

Creation of $\sigma^{35}$ Type Promoters by Site-directed Mutagenesis: Elements Important in the −35 Recognition Sequence—The

contains R. capsulatus RNAP and pUC:fruB unless otherwise noted: lanes 1, 4, and 5, pUC:fruB only; lane 2, 10 mM EDTA; lane 3, 2G10 antibodies; lane 6, B10RNAP and 1 μg/ml rifampicin; lane 7, 10 mM potassium acetate; lane 8, 100 mM KCl, no potassium acetate.

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**FIG. 3.** In vitro transcription by R. capsulatus RNAP/$\sigma^{35}$ using various R. capsulatus $\sigma^{35}$ promoters. Panel A, various templates were combined in a standard in vitro transcription reaction with 40 mM R. capsulatus RNAP. Size standards from digestion of pBR322 with HpaII are shown on the left. The DNA templates added to the reactions are as follows: lane 1, T7A1 linear template; lanes 2–7, supercoiled templates; lane 2, pUC: lane 3, pUC:; lane 4, pUC:pufQ, lane 5, pUC:bchC, lane 6, pUC:; lane 7, same as lane 6 but less than 10% template in reaction. Panel B, linear templates in a standard in vitro transcription reaction: lane 1, puc and R. capsulatus RNAP; lane 2, puc and E. coli RNAP; lane 3, cycA and R. capsulatus RNAP; lane 4, cycA and E. coli RNAP. Panel C, analysis of transcripts generated from the fruB promoter in a standard in vitro transcription reaction that
results above suggest that the **Rhodobacter capsulatus** RNAP recognizes -35 elements similar to that of the **E. coli** RNAP. However, the **E. coli** RNAP does not recognize some templates that **Rhodobacter capsulatus** RNAP transcribes (e.g., **cycA** and **pucC**). To determine more carefully the optimal -35 recognition elements for **Rhodobacter capsulatus** RNAP, the **Rhodobacter capsulatus** **nifA1** promoter was used. The natural wild type **nifA1** promoter has only 3 of the TTGAC nucleotides in the -35 region (Fig. 4B). Two distinct mutations were generated to improve this to 4 consensus nucleotide matches, called the **nifA1mut1** and **nifA1mut2** promoters. A third, called the **nifA1mut3** promoter, contained both mutations and has the complete -35 consensus TTGAC sequence. Each of these promoters was cloned in front of the transcription terminator, and supercoiled templates were used for **in vitro** transcription reactions with the **Rhodobacter capsulatus** RNAP/σ^{70} holoenzyme (Fig. 5). Although the pUC118 alone (lane 1) or the wild type **nifA1** (lane 2) template yields no transcripts other than RNA I (108 nucleotides), the **nifA1mut3** template yields an intense 97-nucleotide transcript (lane 5). Levels of this transcript are at least 100-fold higher than could be detected with the other templates, is of the exact size predicted for this promoter and terminator, and it is more intense than the RNA I transcript. We have also observed a modest level of the 97-nucleotide transcript for **mut1** (lane 3) but none for **mut2** (lane 4). These results support the contention that a major recognition determinant for **Rhodobacter capsulatus** RNAP/σ^{70} is TTGAC at the -35 region.

**CONCLUSIONS**

The **Rhodobacter capsulatus** RNA polymerase and associated σ^{70} were purified to homogeneity and used to characterize σ^{70}-dependent promoters. The **Rhodobacter capsulatus** RNAP exhibits structural similarity to the **E. coli** RNAP with regard to subunit sizes and immunological criteria, including the σ^{70} subunit. The molecular basis for rifampicin resistance in **Rhodobacter capsulatus** strain SB1003 was determined, and the **Rhodobacter capsulatus** RNAP enzymes with and without the σ^{70} subunit Rif^R change were characterized. The purified **Rhodobacter capsulatus** RNAP/σ^{70} enzyme transcribed from the T7A1, RNA I, photosynthetic (puc, puf), fructose-inducible (fru), bacteriochlorophyll (behC), and cytochrome c (cycA) promoters. The behC, puc, and fru promoters were only recognized in the context of supercoiled templates, and it is clear that each promoter is affected distinctly by salt concentrations. Interestingly, the pucC and cycA promoters were recognized on linear templates by **Rhodobacter capsulatus** RNAP/σ^{70} but not by the **E. coli** RNAP/σ^{70}. In this context the **Rhodobacter sphaeroides** rrnB promoter was also recognized only by the **Rhodobacter sphaeroides** RRNAP and not **E. coli** RRNAP (4). These results suggest that differences do exist in promoter recognition and/or transcription initiation between photosynthetic and enteric holoenzymes. The molecular basis for these differences, with some possibilities discussed by Karls et al. (4) remains to be determined. It may not be surprising that organisms like **Rhodobacter**, with a genomic composition...
of 65% GC, have evolved some recognition/melting differences from organisms with considerably higher AT content. On the other hand, strong *E. coli* promoters (e.g. T7A1, RNA I) function almost as efficiently using the *R. capsulatus* RNAP, indicating clearly analogous ideal promoter elements. An ideal promoter in the −35 region was determined for the *R. capsulatus* RNApolymerase using site-directed mutagenesis of the *R. capsulatus* bchC promoter (9). In the present study, the −35 region element, TTTGAC, was confirmed by site-directed mutagenesis of the nifA1 promoter; a dramatic increase of *in vitro* transcription was observed with only two changes to the consensus TTGAC pattern. The investigation of activator- and repressor-dependent transcription *in vitro* in *R. capsulatus* will be an important next step to a more complete understanding of signal transduction and gene expression in photosynthetic bacteria.

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