Activity of *Rhodobacter sphaeroides* RpoH_{II}, a Second Member of the Heat Shock Sigma Factor Family

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Received 22 March 2006/Accepted 31 May 2006

We have identified a second RpoH homolog, RpoH_{II}, in the α -proteobacterium *Rhodobacter sphaeroides*. Primary amino acid sequence comparisons demonstrate that *R. sphaeroides* RpoH_{II} belongs to a phylogenetically distinct group with RpoH orthologs from α -proteobacteria that contain two *rpoH* genes. Like its previously identified paralog, RpoH_I, RpoH_{II} is able to complement the temperature-sensitive phenotype of an *Escherichia coli* σ^{32} (*rpoH*) mutant. In addition, we show that recombinant RpoH_I and RpoH_{II} each transcribe two *E. coli* σ^{32} -dependent promoters (*rpoD* P_{HS} and *dnaK* P1) when reconstituted with *E. coli* core RNA polymerase. We observed differences, however, in the ability of each sigma factor to recognize six *R. sphaeroides* promoters (*cycA* P1, *groESL*₁, *rpoD* P_{HS}, *dnaK* P1, *hslO*, and *ecfE*), all of which resemble the *E. coli* σ^{32} promoter consensus. While RpoH_I reconstituted with *R. sphaeroides* core RNA polymerase transcribed all six promoters, RpoH_{II} produced detectable transcripts from only four promoters (*cycA* P1, *groESL*₁, *hslO*, and *ecfE*). These results, in combination with previous work demonstrating that an RpoH_I mutant mounts a typical heat shock response, suggest that while RpoH_I and RpoH_{II} have redundant roles in response to heat, they may also have roles in response to other environmental stresses.

The ability to regulate gene expression through the use of alternate sigma factors enables bacteria to respond quickly to changes in the environment. These secondary sigma factors guide RNA polymerase to promoter elements whose target sequences differ from those of the primary sigma factors, thereby directing the transcription of a specific set of genes (44). One such rapid response to environmental perturbations, known as the heat shock response, uses an alternate sigma factor to increase the expression of proteins needed to maintain an intracellular milieu conducive for protein folding. While these heat-inducible gene products have traditionally been referred to as the heat shock proteins, increased heat shock protein expression is also induced by other stress conditions (11, 33). We are interested in alternate sigma factors that play a role in the heat shock or other stress responses of the α -proteobacterium *Rhodobacter sphaeroides*.

In the γ -proteobacterium *Escherichia coli*, σ^{32} (encoded by *rpoH*) is the alternate sigma factor responsible for recognizing heat shock gene promoters (12). As a member of the σ^{70} family of eubacterial sigma factors, σ^{32} recognizes unique promoter sequences centered at positions -10 and -35 relative to the transcriptional start site (44). All σ^{32} -like proteins are defined by a conserved region known as the “RpoH box”; they also contain conserved sequences in regions 2.4 and 4.2 that recognize the -10 and -35 elements, respectively, of heat shock promoters (29). While *E. coli*, with its single *rpoH* gene, sets the stage for much of what we know about bacterial heat shock regulation, several α -proteobacteria are known to encode multiple RpoH homologs. Three *rpoH*-like genes in *Bradyrhizobium japonicum* have been reported (31, 32), while *Sinorhizobium meliloti* contains two *rpoH* genes (36, 37). Individual

RpoH family members from these bacteria can completely, or partially, complement the temperature sensitivity of an *E. coli* *rpoH* mutant (31, 36, 37), indicating that they are functionally similar to σ^{32} . In both *Rhizobium* species, however, each of the RpoH homologs appears to have different but overlapping roles in the organism’s response to stress (31, 32, 36, 37).

Past work established that *R. sphaeroides* RpoH_I (previously called σ^{37}) was a member of the σ^{32} family of alternate sigma factors, since the *rpoH*_I gene complemented the inability of an *E. coli* σ^{32} mutant to support phage growth (19). In addition, a ~ 37 -kDa protein isolated from RNA polymerase preparations transcribed several *E. coli* heat shock promoters in vitro when reconstituted with core RNA polymerase. The *R. sphaeroides* RpoH_I-null mutant (Δ RpoH_I), however, mounted a typical heat shock response, implying the existence of a second system by which this bacterium could activate heat shock promoters (19, 24). Further evidence supporting this idea came from in vitro transcription assays that demonstrated that a ~ 38 -kDa protein (previously called σ^{38}) purified from Δ RpoH_I cells recognized both a known *E. coli* σ^{32} promoter (19) and the *R. sphaeroides* *cycA* P1 promoter, which contains sequence elements related to the *E. coli* σ^{32} promoter consensus (24). The identity of this ~ 38 -kDa protein, however, and its possible similarity to other alternate sigma factors were unknown at the time.

In this report, we illustrate that the *rpoH*_{II} gene encodes a second alternate sigma factor of the σ^{32} family in *R. sphaeroides*. We also show that recombinant RpoH_I and RpoH_{II} can each transcribe several heat shock promoters when reconstituted with core RNA polymerase from either *E. coli* or *R. sphaeroides*. While both RpoH_I and RpoH_{II} recognize *R. sphaeroides* promoters that resemble the *E. coli* σ^{32} consensus, there are differences in the ability of each protein to recognize individual promoters in vitro. We discuss the possibility that each *R. sphaeroides* RpoH homolog may regulate different, yet

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overlapping, regulons in response to one or more environmental stress signals.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* DH5 α was used as a plasmid host. Cells were grown in Luria-Bertani (LB) medium (38) supplemented with ampicillin (100 μ g/ml) as necessary. For the complementation assays, we grew *E. coli* CAG9333, an *rpoH* mutant capable of growth at 40°C (22), in LB medium supplemented with ampicillin (25 μ g/ml) when required. All *E. coli* cells were grown at 37°C unless otherwise indicated.

Comparison of amino acid sequences of RpoH-like proteins. The amino acid alignments and the phylogenetic relationships of RpoH_I and RpoH_{II} were determined by comparing their amino acid sequences with those of other RpoH homologs (DDBJ/EMBL/GenBank accession numbers are in parentheses): *Agrobacterium tumefaciens* (accession number D50828), *Alcaligenes xylosoxydans* (accession number AB009990), *Bartonella quintana* (accession numbers BQ12120 [*rpoH2*] and BQ02820 [*rpoH1*]), *Bradyrhizobium japonicum* (accession numbers U55047 [*rpoH_I*], Y09502 [*rpoH₂*], and Y09666 [*rpoH₃*]), *Brucella melitensis* (accession numbers AB3287 [gene BMEI0280] and AD3299 [gene BMEI0378]), *Buchnera aphidicola* (accession number BAU35400), *Caulobacter crescentus* (accession number U39791), *Citrobacter freundii* (accession number X14960), *Coxiella burnetii* (accession number AF120928), *Enterobacter cloacae* (accession number D50829), *Escherichia coli* K-12 (accession number U00096), *Haemophilus influenzae* (accession number U32713), *Hydrogenophilus thermolutesolus* (accession number AB009991), *Jannaschia* sp. strain CCS1 (genome draft in progress for gene 3800 and gene 400 [JGI]), *Mesorhizobium loti* (accession numbers MLR3862 [RpoH-like sigma factor C] and MLR3741 [sigma factor]), *Methylovorus* sp. strain SS1 (accession number AF177466), *Myxococcus xanthus* (accession numbers X55500 [*sigB*], L12992 [*sigC*], and AF023661 [*sigE*]), *Proteus mirabilis* (accession number D50830), *Pseudomonas aeruginosa* (accession number S77322), *Pseudomonas putida* (accession number AB025418), *Ralstonia* sp. strain CH34 (accession number J05278), *Rhodobacter capsulatus* (accession number AF017436), *Rhodobacter sphaeroides* (accession numbers U82397 [*rpoH_I*] and CP000143 [*rpoH_{II}*]), *Rickettsia prowazekii* strain Madrid E (accession number AJ235271), *Serratia marcescens* (accession number D50831), *Silicibacter pomeroyi* (accession numbers SPO0406 [*rpoH-1*] and SPO1409 [*rpoH-2*]), *Sinorhizobium meliloti* (accession numbers AF128845 [*rpoH_I*] and AF149031 [*rpoH₂*]), *Stigmatella aurantiaca* (accession numbers U27311 [*sigC*] and U214970 [*sigB*]), *Vibrio cholerae* (accession number U44432), *Xanthomonas campestris* pv. *campestris* (accession number AF042156), and *Zymomonas mobilis* (accession number D50832).

Amino acid alignments were generated using ClustalW and BoxShade 3.21. The phylogenetic tree was created using PAUP* version 4.0beta (Sinauer Associates, Sunderland, Mass.).

Plasmid constructions. The *rpoH_I* and *rpoH_{II}* genes were amplified from an *R. sphaeroides* 2.4.1 cosmid (pU18106) and genomic DNA, respectively, with 2.5 units of *Pfu* polymerase (Stratagene, La Jolla, CA) using primers specific for each gene. The PCR products were cloned into pUC18 and pUC19 in each orientation relative to the *lac* promoter. N-terminal hexahistidine-tagged RpoH_I and RpoH_{II} were obtained by cloning either *rpoH_I* or *rpoH_{II}* into pET-15b at the NdeI and NdeI-BamHI sites, respectively (Novagen, Madison, WI). The cloned portions of the resulting plasmids, pHAG7 and pHAG16, were sequenced to ensure that they encoded the desired His₆-tagged versions of RpoH_I and RpoH_{II}, respectively.

Plasmids used as in vitro transcription templates were derived from either pRKK96 (34) or pRKK137 (24), which both carry the spot 42 transcription terminator (1). Transcription templates containing *R. sphaeroides* *cycA* P1 as well as the *E. coli* *dnaK* P1 and *rpoD* P_{HS} promoters have been described previously (19, 24). Candidate *R. sphaeroides* promoters (*groESL_I*, *rpoD* P_{HS}, *dnaK*, *hslO*, and *ecfE*) were PCR amplified from 2.4.1 genomic DNA as described above and sequenced with vector-specific primers to guarantee that the desired region was cloned in the proper orientation. All potential promoter regions lie within 150 bp of the predicted start of translation, except *rpoD* P_{HS}, which is positioned ~400 bp upstream of the open reading frame start. Sequences of primers used in this study are available upon request.

Proteins used for in vitro transcription assays. Conditions for expression and purification of His₆-RpoH_I, His₆-RpoH_{II}, and *R. sphaeroides* core RNA polymerase were described previously (1), with the following exceptions. Core RNA polymerase was obtained from an Δ RpoH_I null strain (19) by affinity chromatography (~40 g of cells) on a ~2-ml resin bed containing the 4RA2 monoclonal antibody against the α subunit of *E. coli* RNA polymerase (Richard Burgess, University of Wisconsin—Madison). Proteins bound to the column were eluted

in seven 1-ml fractions with Tris-EDTA buffer (10 mM Tris-HCl [pH 8.0] and 0.1 mM EDTA) supplemented with 0.75 M NaCl and 40% propylene glycol. Fractions containing core RNA polymerase subunits, as visualized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), were combined and dialyzed against 1 liter of TGED medium (10 mM Tris-HCl [pH 7.9], 10% glycerol, 0.1 mM EDTA, and fresh 0.1 mM dithiothreitol) containing 47.5% glycerol and 100 mM NaCl. *E. coli* core RNA polymerase was purchased from Epicenter Technologies, Inc. (Madison, WI). All protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). The proteins were stored at -20°C prior to use.

In vitro transcription assays. Core RNA polymerase, whether from *E. coli* or *R. sphaeroides*, was reconstituted with either His₆-RpoH_I or His₆-RpoH_{II} by incubating the proteins for 1 h at 32°C in transcription buffer (40 mM Tris-HCl [pH 7.9], 2 mM EDTA, 5 mM MgCl₂, 100 mM KCl, 1 mM dithiothreitol, 100 μ g/ml acetylated bovine serum albumin). The mixture was added to a 20- μ l reaction mixture containing 20 nM supercoiled plasmid template in transcription buffer and incubated for 25 min at 32°C. Transcription assays were initiated with the addition of a mixture of ribonucleotide triphosphates at final concentrations of 250 μ M GTP, CTP, and ATP; 25 μ M UTP; and 1 μ Ci [α -³²P]UTP (3,000 Ci/mmol). Reaction mixtures were incubated at 32°C for 25 min and terminated with 6 μ l of 95% (wt/vol) formamide loading buffer (6). Samples were heated to 95°C and resolved on 6% polyacrylamide-7 M urea gels (19). The transcripts were visualized and quantified using the Molecular Dynamics (Sunnyvale, CA) PhosphorImaging system and ImageQuant software.

Identification of candidate RpoH-dependent promoters from *R. sphaeroides*. The *R. sphaeroides* genome (GenBank accession numbers CP000143 to CP000147) was scanned using the program PromScan (<http://www.promscan.uklinux.net>) to search for candidate σ^{32} -like promoter sequences. This query used DNA sequences upstream of four *R. sphaeroides* promoters whose in vivo transcript levels increased upon an increase in temperature (*cycA* P1, *groESL_I*, *rpoD*, P_{HS}, and *rmB*) (19). We chose to analyze candidate promoters positioned upstream of genes encoding known proteins in other bacteria.

RESULTS

Primary sequence similarity of *R. sphaeroides* RpoH homologs. The *R. sphaeroides* genome sequence predicts that this α -proteobacterium encodes two members of the heat shock sigma factor family, RpoH_I (19) and RpoH_{II} (see below). *R. sphaeroides* RpoH_{II} shares 46% amino acid identity (64% similarity) with its paralog, *R. sphaeroides* RpoH_I (19), and 36% amino acid identity with *E. coli* σ^{32} (23) (Fig. 1A). RpoH_{II} shares the greatest degree of amino acid identity with RpoH proteins from α -proteobacteria known to contain two RpoH factors. It displays 50% amino acid identity to *Mesorhizobium loti* RpoH-like sigma factor C, 47% identity to *Bartonella quintana* RpoH2, 46% identity to a *Brucella melitensis* σ^{32} factor, and 42% identity to *Sinorhizobium meliloti* RpoH₂ (36, 37). Together, *R. sphaeroides* RpoH_{II} and RpoH_I are most similar, displaying ~81% and ~84% amino acid identities, respectively, to the cognate RpoH proteins of two marine heterotrophs of the *Roseobacter* clade, *Jannaschia helgolandensis* (42) and *Silicibacter pomeroyi* (27). These values are consistent with a phylogenetic tree of σ^{32} homologs (Fig. 2), which predicts that six of these proteins form a distinct cluster with *R. sphaeroides* RpoH_{II}. In contrast, *R. sphaeroides* RpoH_I falls into a larger cluster with proteins from α -proteobacterial species that contain either one or more *rpoH* genes.

Figure 1B and C shows amino acid alignments of RpoH proteins from α -proteobacteria known to contain multiple *rpoH* genes and *E. coli* σ^{32} . All proteins in the heat shock family of alternate sigma factors contain the RpoH box, a conserved stretch of nine amino acids in region C (29) that has been implicated in the regulation of *E. coli* σ^{32} activity (3, 18, 28). The second amino acid residue in the RpoH box is characteristically a lysine (K) in proteins from the α -proteobacteria

FIG. 1. Alignments of proteobacterial RpoH proteins. (A) Alignment of *R. sphaeroides* RpoH_I and RpoH_{II} and *E. coli* σ^{32} . Identical/similar amino acid residues are shaded. Numbers on the left indicate the amino acid position relative to the start of each protein. Regions that are conserved among eubacterial sigma factors are denoted by the numbers below the sequences. Region C and the RpoH box are also shown. Gaps introduced to maximize the alignment are indicated by dashes. (B) Partial alignment of RpoH homologs from eight α -proteobacteria containing multiple *rpoH* genes and *E. coli* σ^{32} (γ subgroup) showing the amino acid sequence from region 2.1 to the end of region C. (C) Region 4.2 of the proteins listed in B showing the predicted helix-turn-helix (H-T-H) DNA-binding motif. For simplicity, the "... " indicates an intervening sequence between region C and region 4.2. Abbreviations: Bqui, *Bartonella quintana*; Bjap, *Bradyrhizobium japonicum*; BmelAB3287, *Brucella meli*ensis RpoH gene BMEI0280; BmelAD3299, *B. meli*ensis RpoH gene BMEI0378; Ecoli, *Escherichia coli*; Jann3800, *Jannaschia* sp. strain CCS1-3800/CDS; Jann0400, *Jannaschia* sp. strain CCS1-0400/CDS; MlotRpoHC, *Mesorhizobium loti* RpoH-like sigma factor C; MlotMLR3741, *M. loti* sigma factor; RspH1, *R. sphaeroides* RpoH_I; RspH2, *R. sphaeroides* RpoH_{II}; Spom, *Silicibacter pomeroyi*; Smel, *Sinorhizobium meliloti*. See Materials and Methods for accession numbers.

and an arginine (R) in those from the γ -proteobacteria (19). Not surprisingly, both RpoH paralogs from *R. sphaeroides* contain a lysine at this position. While the third amino acid residue in the RpoH box from γ -proteobacteria has invariably been a lysine, this residue is either an arginine or a lysine in proteins related to RpoH_I from the α subdivision (45). In contrast, proteins that cluster phylogenetically with *R. sphaeroides* RpoH_{II} contain an uncharged amino acid, either alanine (A), serine (S), or valine (V), in the analogous position. This alignment also illustrates that *R. sphaeroides* RpoH_{II} and its most closely related homologs have considerably less amino acid conservation than the RpoH_I orthologs (Fig. 1B) in regions 2.1

and 2.2, which comprise a domain implicated in the binding of core RNA polymerase (7, 13).

Function of *R. sphaeroides* *rpoH* genes in an *E. coli* *rpoH* mutant. Since *R. sphaeroides* RpoH_{II} has significant amino acid conservation in regions 2.4 (−10 promoter recognition) and 4.2 (−35 promoter recognition) (Fig. 1B and C), we predicted that it should recognize heat shock promoters. To test this hypothesis, we asked whether the *R. sphaeroides* *rpoH*_{II} gene could complement the temperature-sensitive phenotype of the *E. coli* σ^{32} -null strain CAG9333. While this tester strain lacks a functional copy of *rpoH*, a compensatory R40 mutation that results in enhanced GroES and GroEL synthesis allows for

FIG. 2. Phylogenetic tree of σ^{32} homologs from 31 different proteobacterial species analyzed by the neighbor-joining method using PAUP* 4.0beta (Sinauer Associates, Sunderland, Mass.). Sigma factor names other than RpoH are shown in parentheses. The subdivisions (α , β , γ , and δ) of the proteobacteria are shown to the right of the tree. As indicated by the numbers to the right of each branch point, 100 pseudoreplicates were used in this bootstrap analysis. Only values greater than 70% are shown. See Materials and Methods for accession numbers.

growth at up to 40°C (22). When we introduced a plasmid containing *rpoH_{II}* downstream of the *lac* promoter into CAG9333 and tested for growth at 37°C (permissive temperature) and 44°C (restrictive temperature), we found that *rpoH_{II}* was sufficient to restore growth at 44°C. In contrast, CAG9333, containing a control plasmid (pUC18), did not grow at 44°C (Table 1). We had previously shown that *R. sphaeroides* *rpoH_I*

supported phage growth of an *E. coli* σ^{32} mutant (19), so we expected a copy of this gene to allow for growth at 44°C. CAG9333 also carries a chromosomal *rpoD* P_{HS}::*lacZ* fusion that can be used to score for transcription from this known heat shock promoter (22). We demonstrated in previous work that RNA polymerase holoenzymes purified from either *R. sphaeroides* wild-type or Δ RpoH_I cells were able to transcribe the *E. coli* *rpoD* P_{HS} promoter in vitro (19). As expected, strains carrying either *rpoH_I* or *rpoH_{II}* formed blue colonies on plates containing X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) and IPTG (isopropyl- β -D-thiogalactopyranoside) (Table 1). These results indicate that the *rpoH_{II}* gene product as well as the *rpoH_I* gene product can functionally replace σ^{32} , presumably because they are capable of binding *E. coli* core RNA polymerase and transcribing one or more heat shock promoters. **Recombinant RpoH_I and RpoH_{II} can direct transcription from *E. coli* heat shock promoters in vitro.** To test the hypothesis that recombinant RpoH_I and RpoH_{II} can direct transcription from *E. coli* heat shock promoters in vitro, we reconsti-

TABLE 1. Complementation of an *E. coli* Δ RpoH mutant (CAG 9333)^a

Complementing gene	37°C	44°C	<i>rpoD</i> P _{HS} :: <i>lacZ</i> expression ^b
<i>rpoH_I</i>	+	+	+
<i>rpoH_{II}</i>	+	+	+
None ^c	+	—	—

^a All phenotypes were scored on solid medium.
^b Scored by monitoring a LacZ phenotype (see the text); “+,” blue; “—,” white.
^c Cells containing pUC18 control plasmid.

TABLE 2. Comparison of promoters from *R. sphaeroides* and *E. coli* recognized by RpoH_I and RpoH_{II}

Promoter	-35 region ^a	Spacer length (bp)	-10 region ^a
<i>R. sphaeroides</i>			
<i>cycA</i> P1	GTATTGATT	13	ACCTATAT
<i>groESL</i> ₁	CCGTTGACA	14	TCATATCT
<i>rpoD</i> P _{HS}	CCCTTGAAA	14	ACCCATGT
<i>dnaK</i>	CGCTTGCAG	14	CCCTATAT
<i>hslO</i>	CCCTTGGTC	13	GCCTATAT
<i>ecfE</i>	CCCTTGAGG	14	GACCATCT
<i>E. coli</i>			
<i>rpoD</i> P _{HS}	CCCTTGAAA	14	GACGATAT
<i>dnaK</i> P1	CCCTTGATG	13	CCCCATTT
σ^{32} consensus	CNCTTGAAA	13/14	CCCCATNT

^a Those bases matching the *E. coli* σ^{32} consensus sequence (11) are shown in boldface type. The N indicates no base preference.

tuted *E. coli* core RNA polymerase with histidine-tagged versions of either RpoH_I or RpoH_{II} and assayed transcription from two known *E. coli* heat shock promoters, *rpoD* P_{HS} and *dnaK* P1 (Table 2). In order to determine the amount of sigma factor required to produce optimal transcription from each promoter, we performed multiple-round transcription using a fixed amount of *E. coli* core RNA polymerase (37.5 nM) and increasing amounts of either RpoH_I (5.75 nM to 115 nM) or RpoH_{II} (2 nM to 40 nM). As expected, transcript abundance from *rpoD* P_{HS} and *dnaK* P1 increased with increasing concentrations of either RpoH_I or RpoH_{II} (titration curves represent-

ing an average of several assays are shown in Fig. 3C and D). In the presence of RpoH_I, we reproducibly obtained optimum transcript levels from both promoters when the sigma factor was in an approximately twofold molar excess over core RNA polymerase (Fig. 3C). In comparison, adequate transcription from reaction mixtures containing RpoH_{II} required an approximately equimolar ratio of sigma to core RNA polymerase (Fig. 3D) over the course of several different assays. The transcripts derived from each of these promoters (Fig. 3A and B) are identical in length to those synthesized by *E. coli* E σ^{32} (20), indicating that pure RpoH_I and RpoH_{II} each recognize the established heat shock promoters.

The results of these in vitro transcription assays also revealed that different levels of transcript were produced from *rpoD* P_{HS} compared to *dnaK* P1 when the *E. coli* core was reconstituted with either RpoH_I or RpoH_{II}. At the concentration of purified RpoH_I required for optimal transcription, the RNA product was twofold more abundant from the *dnaK* P1 promoter than from the *rpoD* P_{HS} promoter (Fig. 3C). Similarly, greater than twofold more transcript resulted from the *dnaK* P1 promoter than from the *rpoD* P_{HS} promoter in the presence of a 1:1 molar ratio of RpoH_{II} to core (Fig. 3D).

Recombinant RpoH_I and RpoH_{II} can direct transcription from *R. sphaeroides* heat shock promoters in vitro. To provide further evidence that RpoH_I and RpoH_{II} are members of the σ^{32} family, we tested their ability to transcribe the *R. sphaeroides* *cycA* P1 and *groESL*₁ promoters (Table 2). Primer extension assays indicate that the abundance of *cycA* P1 and *groESL*₁-specific transcripts increases when either *R. sphaeroides* wild-type or Δ RpoH_I cells mount a heat shock response

FIG. 3. In vitro transcription of *E. coli* *rpoD* P_{HS} and *dnaK* P1 promoters by *E. coli* core RNA polymerase reconstituted with either purified *R. sphaeroides* RpoH_I (A) or RpoH_{II} (B). Shown are products from multiple-round transcription assays performed in the presence of increasing amounts of either RpoH_I (A) (5.75, 11.5, 34.5, 57.5, and 115 nM) or RpoH_{II} (B) (2, 4, 12, 20, and 40 nM) with a constant amount of core RNA polymerase (37.5 nM). Lanes marked with an asterisk contain products from assays using a control template in the presence of either a 3:1 (A) or a 1:1 (B) molar ratio of the indicated RpoH sigma factor to core RNA polymerase. Panels C and D represent the quantification and summary of transcription results from A and B as well as additional assays. The relative transcript abundance (pixel intensity) from each promoter was measured by pixel intensity on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and adjusted for background.

FIG. 4. In vitro transcription of *R. sphaeroides* *cycA* P1 and *groESL*₁ promoters after adding either RpoH_I (A) or RpoH_{II} (B) to *R. sphaeroides* core RNA polymerase. Shown are products from multiple-round transcription assays performed in the presence of increasing amounts of either RpoH_I (A) (20, 40, 80, 160, 480, and 800 nM) or RpoH_{II} (B) (5, 10, 20, 40, 80, and 160 nM) with a constant amount of core RNA polymerase (160 nM). Panels C and D represent the quantification and summary of transcription results from A and B and depict typical titration curves for these two assays. The products marked with an asterisk in B are of unknown origin, but control experiments indicate that they are derived from an RpoH_{II}-dependent promoter on the vector (pRKK96) used to generate these transcription templates (data not shown). The relative transcript abundance (pixel intensity) from each promoter was measured by pixel intensity on a PhosphorImager (Molecular Dynamics, Sunnyvale, CA) and adjusted for background.

(19); hence, we predicted that these promoters would be recognized by RNA polymerase containing either RpoH_I or RpoH_{II} in vitro. We also tested the more physiologically relevant holo-RNA polymerase in these reactions by reconstituting the individual RpoH proteins with core enzyme isolated from *R. sphaeroides*. To eliminate RpoH_I activity from our core RNA polymerase preparations, we purified this enzyme from an RpoH_I mutant (19). The in vivo levels of RpoH_I and RpoH_{II} differ vastly. Protein gels of purified holo-RNA polymerase (24), as well as Western blot analyses of RpoH_I and RpoH_{II} levels in crude cell extracts (data not shown), indicate that RpoH_I is considerably more abundant than RpoH_{II} under aerobic growth conditions. This *R. sphaeroides* core RNA polymerase does not contain any detectable RpoH_{II} activity with these promoters by multiple-round transcription (data not shown).

When we performed multiple-round transcription using a fixed amount of *R. sphaeroides* core RNA polymerase (160 nM) and increasing amounts of RpoH_I (20 nM to 800 nM), we found that increasing the concentration of RpoH_I stimulated transcription from both *cycA* P1 and *groESL*₁ (results from a representative assay are shown in Fig. 4A). While maximal levels of the *groESL*₁ transcript appeared to require a sigma-to-core ratio of only 0.5 in the presence of RpoH_I, we chose to focus on a ratio of sigma to core that was optimal for both promoters tested. Thus, with this reconstituted RNA polymerase holoenzyme, transcript abundance from *cycA* P1 was ap-

proximately twofold greater than that from *groESL*₁ when the molar ratio of sigma to core was 1:1 (Fig. 4C). Similarly, when we increased the molar concentration of pure RpoH_{II} (5 nM to 160 nM) in the presence of a constant concentration of core RNA polymerase (160 nM), the transcript abundance from both promoters increased, reaching a maximum at 80 to 160 nM RpoH_{II} (Fig. 4B). At all levels of RpoH_{II} tested, however, we detected only low levels of transcript produced from *groESL*₁. Consequently, RpoH_{II}-dependent transcription from *cycA* P1 was approximately 16-fold greater than that from *groESL*₁ when the sigma-to-core RNA polymerase molar ratio was approximately 1:1 (Fig. 4D).

If transcript abundance is taken as an estimate of promoter function, *cycA* P1 is transcribed with greater efficiency than *groESL*₁ in vitro when core RNA polymerase is reconstituted with either RpoH_I or RpoH_{II}. On the other hand, if one analyzes the -10 and -35 regions of *cycA* P1 and *groESL*₁, it is the *R. sphaeroides* *groESL*₁ promoter that is most similar in sequence to the *E. coli* σ^{32} consensus (Table 2), intimating that sequence comparisons alone may not be good indicators of promoter recognition by either RpoH_I or RpoH_{II} (see Discussion). The molar concentrations of purified RpoH proteins essential to observe optimal transcription were similar to that seen when we used *E. coli* core RNA polymerase in reactions instead. However, as indicated by control experiments, each of the *E. coli* heat shock promoters that we tested are stronger than the *R. sphaeroides* promoters analyzed (data not shown).

Recognition of additional *R. sphaeroides* promoters by RpoH_I and RpoH_{II}. To further investigate promoters recognized by RpoH_I and RpoH_{II}, we examined the ability of reconstituted RNA polymerase holoenzymes to transcribe other *R. sphaeroides* candidate target genes. Having established the sigma-to-core ratios that are required for efficient transcription from the *cycA* P1 and *groESL*₁ promoters, we chose four additional promoters to assay under these in vitro transcription conditions: *rpoD* P_{HS}, *dnaK* P1, *hslO* (10, 16), and *ecfE* (9). Each of these promoters contains sequences related to the *E. coli* σ^{32} consensus promoter sequence (Table 2). We knew from previous results that *rpoD* P_{HS}-specific transcripts increase in both wild-type and Δ RpoH1 cells upon a temperature increase (19). The remaining three promoters (*dnaK* P1, *hslO*, and *ecfE*) were selected after querying the *R. sphaeroides* genome with a matrix-driven program, PromScan (40), that utilized the -10 and -35 regions of known *R. sphaeroides* heat shock promoters.

RpoH_I reconstituted with *R. sphaeroides* core RNA polymerase stimulated transcription from the two control promoters, *cycA* P1 and *groESL*₁, as well as from each of the four other test promoters, *rpoD* P_{HS}, *dnaK* P1, *hslO*, and *ecfE* (Fig. 5A). When we reconstituted RpoH_{II} with *R. sphaeroides* core enzyme, we detected transcription products from each of the promoters except *rpoD* P_{HS} and *dnaK* P1 (Fig. 5A, lanes 6 and 8). The transcript lengths from each set of promoters transcribed by the two different holoenzymes were identical except for *hslO* (Fig. 5A, lanes 9 and 10), which reproducibly yielded a transcript that was 1 to 2 nucleotides shorter in the presence of RpoH_{II}. One possible explanation for this feature of the *hslO* promoter is that RpoH_{II}-containing RNA polymerase either initiates or terminates transcription at a different position than core enzyme reconstituted with RpoH_I.

The lack of a detectable RpoH_{II}-dependent transcript from the *dnaK* promoter region (Fig. 5A, lane 8) suggests that the *dnaK* operon may not be controlled by this sigma factor (see Discussion). The absence of a detectable transcript from *rpoD* P_{HS} (Fig. 5A, lane 6) using holoenzyme containing RpoH_{II}, however, was surprising, since cells lacking RpoH_I still experience an increase in *rpoD*-specific mRNA from this promoter after a heat shock (19). One feasible explanation for the failure to detect transcription from either *rpoD* P_{HS} or *dnaK* P1 is that a protein(s) present in our *R. sphaeroides* core preparation reduces transcription in the presence of RpoH_{II} (see Discussion).

To test for the presence of such an activity in our *R. sphaeroides* core RNA polymerase, we repeated the above-described transcription reactions using a highly purified preparation of *E. coli* core RNA polymerase. As predicted by the above-described results (Fig. 5A), *E. coli* core RNA polymerase reconstituted with RpoH_I transcribed all six promoters (Fig. 5B). However RNA polymerase holoenzyme formed by adding *E. coli* core enzyme to RpoH_{II} generated detectable transcripts from only five *R. sphaeroides* promoters: *cycA* P1, *groESL*₁, *rpoD* P_{HS}, *hslO*, and *ecfE* (Fig. 5B). The presence of an *rpoD* P_{HS} transcript from assays using RpoH_{II} and *E. coli* core RNA polymerase is consistent with the hypothesis that our *R. sphaeroides* core RNA polymerase may contain a factor(s) capable of inhibiting transcription from this promoter (see Discussion). The inability to detect a *dnaK* P1-specific

FIG. 5. In vitro transcription of *R. sphaeroides* *cycA* P1, *groESL*₁, *rpoD* P_{HS}, *dnaK* P1, *hslO*, and *ecfE* promoters by either RpoH_I (230 nM) or RpoH_{II} (160 nM) reconstituted with either *R. sphaeroides* (160 nM) (A) or *E. coli* (37.5 nM) core RNA polymerase (B). Open arrows denote transcription products of the expected length from each promoter. The product marked with an asterisk in the 4th, 6th, and 12th lanes is of unknown origin, but control experiments indicate that it is derived from an RpoH_{II}-dependent promoter on the vector (pRKK96) used to generate these transcription templates (data not shown).

transcript in assays where RpoH_{II} is added to either *R. sphaeroides* (Fig. 5A, lane 8) or *E. coli* (Fig. 5B, lane 8) core RNA polymerase suggests that this gene either lacks an RpoH_{II}-dependent promoter or contains one whose activity is below the detection level of this assay (see Discussion). Control experiments showed that transcript production from these six promoters is dependent on either RpoH_I or RpoH_{II}, because core RNA polymerase alone, from either *E. coli* or *R. sphaeroides*, does not produce a detectable product from any of the six heat shock promoters (data not shown).

These in vitro transcription assays also revealed that an additional transcript is generated by one of our transcription templates (pRKK96) that is dependent on the presence of RpoH_{II} (see transcription products marked with asterisks in Fig. 4B and 5A and B). The only difference between pRKK96 and pRKK137, the other template used in these assays, is the presence of a ~2-kbp spectinomycin resistance cartridge cloned upstream of the promoter. Thus, it would appear that this RpoH_{II}-dependent transcript is derived from either an uncharacterized promoter within the spectinomycin resistance cartridge or one created by the insertion of this element into

pRKK137. Simple inspection of the relevant sequences did not identify a candidate promoter, so additional experiments are under way to define this proposed RpoH_{II}-dependent promoter.

DISCUSSION

Previous studies identified *R. sphaeroides* RpoH_I as a member of the σ^{32} family of alternate sigma factors (19). Several lines of evidence, however, intimated the existence of a second sigma factor that recognized heat-inducible promoters (19, 24). In this work, we provide evidence that *R. sphaeroides* RpoH_{II} is this second sigma factor. Amino acid sequence alignments show that RpoH_{II} is a member of the σ^{32} family. Like its paralog, RpoH_I, expression of RpoH_{II} complements the growth defect of a temperature-sensitive *E. coli* σ^{32} mutant and initiates transcription from the *E. coli* σ^{32} -dependent promoter *rpoD* P_{HS} both in vivo and in vitro. Through the use of purified components, we demonstrate that RpoH_I and RpoH_{II} recognize a number of known and presumed heat shock promoters from *R. sphaeroides*. We also identify differences in the abilities of RpoH_I and RpoH_{II} to recognize individual promoters in vitro.

RpoH_{II} is a second sigma factor that recognizes *R. sphaeroides* heat shock promoters. Previously, two proteins copurifying with *R. sphaeroides* RNA polymerase were eluted from SDS-PAGE gels, individually reconstituted with core enzyme, and shown to transcribe the *E. coli* *dnaK* P1 and *R. sphaeroides* *cycA* P1 promoters (19, 24). One of these proteins, σ^{37} , was tentatively identified as RpoH_I, but the identity of the other protein, known as σ^{38} , was not known. Several lines of evidence link RpoH_{II} to the σ^{38} activity described by previous studies. Both recombinant RpoH_{II} (this work) and gel-purified σ^{38} (19) recognized the heat-inducible promoters *dnaK* P1 and *cycA* P1, and both proteins have similar apparent molecular weights when analyzed by SDS-PAGE (data not shown). Accordingly, antibody raised to His-tagged RpoH_{II} reacts with a protein of similar molecular weight in *R. sphaeroides* RNA polymerase preparations (data not shown). In addition to identifying RpoH_{II} as a second member of the σ^{32} family in *R. sphaeroides*, our experiments provide the first in vitro analysis of promoter recognition by a protein belonging to the clade containing RpoH_{II}.

Comparison of RpoH primary amino acid sequences. There are now seven organisms, all members of the α -proteobacteria, predicted to contain two *rpoH* genes. *R. sphaeroides* RpoH_{II} falls into a distinct phylogenetic clade of alternate sigma factors whose primary amino acid sequences differ somewhat from their respective paralogs, most noticeably in conserved regions 2.1 and 2.2 and the RpoH box. The RpoH box lies in region C, a stretch of 23 amino acids that initially was implicated in the DnaK-mediated degradation of σ^{32} (25, 28). Subsequent studies indicated that instead, region C directly interacts with core RNA polymerase and that the RpoH box may give σ^{32} a competitive advantage over other sigma factors in binding to this enzyme (3, 18). For example, amino acid substitutions in the fifth and sixth residues (F136L and F137E) of the *E. coli* σ^{32} RpoH box (¹³²QRKLFFNLR¹⁴⁰) each decreased the binding of core RNA polymerase (3, 18). These two phenylalanines, together with L135, the fourth residue,

constitute the three most highly conserved RpoH box residues among RpoH homologs (Fig. 1B). Alternatively, the third residue in the RpoH box of proteins that form a distinct cluster with RpoH_{II} is either an alanine, serine, or valine, deviating from the positively charged arginine or lysine otherwise found in this position. To our knowledge, no one has studied the effects that altering this position has on σ^{32} 's, or another RpoH factor's, ability to bind core RNA polymerase. If the RpoH box indeed has a regulatory role, as many lines of evidence suggest, the presence of an uncharged residue at this position may influence the activity of proteins sharing the clade with RpoH_{II}.

Regions 2.1 and 2.2 are conserved among all members of the σ^{70} superfamily. Not surprisingly, amino acid substitutions in these regions alter core RNA polymerase binding (7, 13). In the case of *E. coli* σ^{32} , amino acid substitutions in region 2.2 reduced its affinity for core RNA polymerase (17), while amino acid substitutions in region 2.1 suggested that this region may bind DnaK (15, 35). These observations concur with the view that DnaK and core RNA polymerase compete for binding to specific regions of σ^{32} (45, 46). The proteins most closely related to *R. sphaeroides* RpoH_{II}, however, show less primary amino acid conservation, as a group, in regions 2.1 and 2.2 than their respective paralogs (Fig. 1B). The relative plasticity in the primary amino acid sequences of regions 2.1 and 2.2 among the RpoH_{II} clade may modify their affinities for core RNA polymerase or, possibly, the binding of chaperones that could negatively regulate their activity (14, 41). Thus, the differences in promoter activity that we have observed for RpoH_I and RpoH_{II} may in part be due to their relative efficiencies in binding core RNA polymerase, as reflected by the amino acid sequence variations in regions 2.1 and 2.2 and the RpoH box.

While domains 2 and 4 of the σ^{70} -type sigma factors are structurally conserved, it is the primary amino acid sequence variation in regions 2.4 and 4.2 that accounts for differences in promoter recognition among the diverse members of this superfamily (13). In light of the fact that RpoH_I and RpoH_{II} each recognize several heat shock promoters, it is not surprising that regions 2.4 and 4.2 of these two proteins show little sequence variation from other σ^{32} family members. In addition, both RpoH_I and RpoH_{II} share several amino acid residues with regions 2.4 and 4.2 of *E. coli* σ^{32} that are believed to contact the -10 and -35 elements of the *groE* promoter (21).

Promoter recognition by RpoH_I and RpoH_{II}. Of the six *R. sphaeroides* promoters that we examined, all were transcribed by either *R. sphaeroides* or *E. coli* core RNA polymerase reconstituted with RpoH_I. When recombinant RpoH_{II} was combined with *R. sphaeroides* core enzyme, however, we were unable to detect transcripts from two promoters, *rpoD* P_{HS} and *dnaK* P1. This was surprising, since *rpoD* P_{HS} is one of three promoters we chose to analyze based on the knowledge that their transcript abundance increases in Δ RpoH_I cells after a heat shock (19). On the other hand, when RpoH_{II} was added to *E. coli* core RNA polymerase, we detected a product from *rpoD* P_{HS}. The lack of a product from *rpoD* P_{HS} in the presence of RpoH_{II} and the *R. sphaeroides* core enzyme may have been due to a protein, or proteins, in the core RNA polymerase preparation that reduces in vitro transcription from this particular promoter. Another feasible explanation is that promoter escape from *R. sphaeroides* *rpoD* P_{HS} may be more

efficient when RpoH_{II} is added to *E. coli* core RNA polymerase. For example, Artsimovitch and coworkers reconstituted *E. coli* σ^{32} with either *Bacillus subtilis* or *E. coli* core RNA polymerase and demonstrated that the two holoenzymes differed vastly in their promoter selectivities (4). Their results suggested that while promoter recognition resides mainly in the sigma subunit, promoter utilization depends primarily on core RNA polymerase and that contacts made between the core subunits and promoter DNA may either facilitate or inhibit promoter escape (4). Among the *R. sphaeroides* promoters that we examined, *rpoD* P_{HS} is most similar to the *E. coli* σ^{32} consensus in both the -10 and -35 regions, suggesting that specific contacts made between *E. coli* core RNA polymerase and this promoter may result in more efficient transcription than those made with the *R. sphaeroides* core enzyme.

Why the *R. sphaeroides* *dnaK* P1 promoter was not transcribed by either the *E. coli* or *R. sphaeroides* core enzymes in the presence of RpoH_{II} is unknown at this time. The gene product DnaK is an Hsp70 protein (5, 8), and we saw a diminished increase in the synthesis rate of a ~75-kDa protein in Δ RpoH_I cells after a heat shock relative to wild-type cells (19). If this ~75-kDa protein is DnaK, a second mechanism, independent of RpoH_{II}, may be responsible for the heat induction of this promoter in cells lacking RpoH_I. In contrast, the heat-induced synthesis of a 76-kDa protein in *S. meliloti* appears to be totally dependent on RpoH_I (37). While it is conceivable that *dnaK* P1 is simply not recognized by RpoH_{II}, the -10 region of *dnaK* P1 is almost identical to that of the strongest *R. sphaeroides* promoter for RpoH_{II} that we tested, *cycA* P1. The -35 regions of these two promoters, however, share only the TTG motif, and previous studies have shown that a point mutation in the -35 region of *cycA* P1 (from ⁻³⁴TTGA⁻³¹ to ⁻³⁴TTGC⁻³¹) reduced activity by ~60% (24). Since *dnaK* P1 has a C in this position (TTGC), RpoH_{II} may have a greater requirement for an A in the TTGA motif of the -35 element than RpoH_I.

If we use transcript abundance as an estimate of promoter strength, the strongest *R. sphaeroides* promoter that we tested with either RpoH_I or RpoH_{II} was *cycA* P1. Despite this, the -10 and -35 elements of *cycA* P1 have fewer matches to the *E. coli* σ^{32} consensus sequence than the other five promoters examined (Table 2). Thus, the canonical idea of what makes a strong heat shock promoter may not apply to *R. sphaeroides* RpoH_I and RpoH_{II}. Less-than-optimal heat shock promoter sequences for other α -proteobacteria have been noted (26, 30, 39). Based on the upstream regions of nine *dnaKJ* and *groESL* operons from eight α -proteobacteria, Segal and Ron constructed a putative consensus sequence (CTTG[17 to 18 bp]C YTAT-T-G) that differs from the *E. coli* σ^{32} consensus at several positions (39). Only the *R. sphaeroides* *dnaK* P1 and *hsfO* promoter regions, however, abide by this proposed consensus sequence. The existence of relatively weak promoters that are regulated by two or more sigma factors may represent a mechanism that evolved to more subtly regulate gene expression in the absence or presence of different stress conditions.

Based on transcript abundance alone, both of the *E. coli* promoters that we examined, *dnaK* P1 and *rpoD* P_{HS}, were considerably stronger than any of the six *R. sphaeroides* promoters analyzed. Furthermore, whether a holoenzyme was generated by mixing *E. coli* core RNA polymerase with RpoH_I

or RpoH_{II}, *dnaK* P1 was a stronger promoter than *rpoD* P_{HS}. Among the *E. coli* σ^{32} -dependent promoters that have been studied, those with higher activities tend to more closely mimic the σ^{32} consensus sequence (43). Indeed, the -10 element of *E. coli* *dnaK* P1 is a perfect match to the σ^{32} consensus. In addition, a recent study demonstrated that a tryptophan (W108) in the boundary between regions 2.3 and 2.4 of σ^{32} contacts a conserved -13 C-G base pair (21). The presence of a tryptophan residue at this position in both RpoH_I and RpoH_{II} may explain why these sigma factors transcribe *E. coli* *dnaK* P1, which contains this conserved base pair, with greater efficiency than *rpoD* P_{HS}.

The number of *R. sphaeroides* promoters tested, along with the variation in their sequences, makes it difficult to propose a consensus promoter for either RpoH_I or RpoH_{II}. The fact that these two sigma factors do not recognize an identical repertoire of promoters in vitro makes constructing such a consensus for *R. sphaeroides* even more challenging. A future interest is to determine whether *R. sphaeroides* has separate RpoH_I- and RpoH_{II}-specific target genes that allow this α -proteobacterium to respond to different stress, or other environmental, signals. For example, recent studies have shown that RpoH_{II} is part of the RpoE regulon (2). RpoE is required for *R. sphaeroides* to mount a transcriptional response to singlet oxygen (2). Consequently, RpoH_{II} may directly regulate the expression of genes whose products are required to mitigate the damaging effects of this reactive oxygen species as well as play a role with RpoH_I in responding to stress caused by elevated temperatures.

ACKNOWLEDGMENTS

This study was supported by grants GM37509 and GM75273 (National Institute of General Medical Sciences) and DE-FG02-05ER15653 (Department of Energy) to T.J.D.

We are grateful to Matias Cafaro for constructing the phylogenetic tree and to Yann Dufour for his computer expertise. We also thank Rachelle Stenzel and Archana Bhasin for their discussions and thoughtful reading of the manuscript.

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