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Promoter Selectivity of the *Bradyrhizobium japonicum* RpoH Transcription Factors In Vivo and In Vitro

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Expression of the *dnaKJ* and *groESL*$_1$ heat shock operons of *Bradyrhizobium japonicum* depends on a σ^{32}-like transcription factor. Three such factors (RpoH$_1$, RpoH$_2$, and RpoH$_3$) have previously been identified in this organism. We report here that they direct transcription from some but not all σ^{32}-type promoters when the respective *rpoH* genes are expressed in *Escherichia coli*. All three RpoH factors were purified as soluble C-terminally histidine-tagged proteins, although the bulk of overproduced RpoH$_3$ was insoluble. The purified proteins were recognized by an anti-*E. coli* σ^{32} serum. While RpoH$_1$ and RpoH$_2$ productively interacted with *E. coli* core RNA polymerase and produced *E. coli groE* transcript in vitro, RpoH$_3$ was unable to do so. *B. japonicum* core RNA polymerase was prepared and reconstituted with the RpoH proteins. Again, RpoH$_1$ and RpoH$_2$ were active, and they initiated transcription at the *B. japonicum groESL*$_1$ and *dnaKJ* promoters. In all cases, the in vitro start site was shown to be identical to the start site determined in vivo. Promoter competition experiments revealed that the *B. japonicum dnaKJ* and *groESL*$_1$ promoters were suboptimal for transcription by RpoH$_1$- or RpoH$_2$-containing RNA polymerase from *B. japonicum*. In a mixture of different templates, the *E. coli groESL* promoter was preferred over any other promoter. Differences were observed in the specificities of both sigma factors toward *B. japonicum rpoH*-dependent promoters. We conclude that the primary function of RpoH$_2$ is to supply the cell with DnaKJ under normal growth conditions whereas RpoH$_1$ is responsible mainly for increasing the level of GroESL$_1$ after a heat shock.

The σ subunit confers promoter recognition ability upon bacterial RNA polymerases. Sigma factors specifically interact with the catalytic, four-subunit core RNA polymerase ($\alpha_2\beta\beta'$). Only after assembly with σ to form the so-called holo-RNA polymerase can the enzyme recognize promoter sequences and initiate transcription accurately. Upon initiation, the σ factor is released and the core enzyme elongates the RNA chain (reviewed in reference 9).

Bacteria often contain multiple sigma factors (for reviews, see references 8, 10, and 11). Under normal growth conditions, transcription of most genes in *Escherichia coli* is initiated by RNA polymerase containing the major sigma factor σ^{70} (also called σ^{D}). Alternative sigma factors coordinate transcription of distinct subsets of coregulated genes which, for example, are required for nitrogen assimilation (σ^{54} [σ^{N}]), for flagellum gene expression (σ^{28} [σ^{F}]), during stationary phase (σ^{38} [σ^{S}]), or under conditions of heat stress (either σ^{32} [σ^{H}] or σ^{24} [σ^{E}], depending on whether the stress signal is elicited in the cytoplasm or the periplasm) (17).

We are studying the regulation of heat shock genes in *Bradyrhizobium japonicum*, the nitrogen-fixing root nodule symbiont of the soybean plant. This organism uses three different strategies to control the expression of heat shock proteins (Hsps) at elevated temperatures. Two mechanisms rely on conserved DNA elements (CIRCE and ROSE) which are located immediately downstream of the transcription start sites of some heat shock operons (2, 18, 20). In both cases, it is likely that a putative repressor protein binds to these sites and prevents transcription under normal growth conditions. Regulation of a third class of heat shock genes is mediated by σ^{32}-like factors (2, 16). *B. japonicum* is the first known organism which contains an *rpoH* gene family coding for three different σ^{32}-like factors (RpoH$_1$, RpoH$_2$, and RpoH$_3$) (18, 19). Notably, these proteins differ not only in their primary sequence (the number of identical amino acids varies between 49 and 66%) but also in their significance to survival and in the way they are regulated. RpoH$_2$ appeared to be crucial for survival under all conditions because several attempts to disrupt the corresponding gene failed. By contrast, RpoH$_1$ and RpoH$_3$ could be deleted individually. Even the *rpoH*$_1$-*rpoH*$_3$ double mutant had no obvious growth defect under various conditions.

All three RpoH proteins were capable of producing high levels of β-galactosidase from a *groE-lacZ* fusion in a σ^{32}-deficient *E. coli* strain (19). In fact, *rpoH*$_1$ had originally been discovered by searching for this phenotype in a complementation approach (18). A puzzling observation was made when the abilities of the RpoH factors to complement the temperature-sensitive phenotype of the *rpoH* mutant were compared. Only RpoH$_2$ reached a similar complementation capacity to *E. coli* σ^{32} in allowing the strain to grow at otherwise restrictive temperatures (19). While RpoH$_1$ still permitted growth at intermediate temperatures, RpoH$_3$ almost completely failed. Taken together, these results could be taken as a hint of a somewhat overlapping but also divergent promoter specificity of the three RpoH factors of *B. japonicum*.

In the present study, we set out to systematically test the promoter specificities of the RpoH factors. To complete our previous studies, we checked which Hsps were produced in each complemented *E. coli* strain. We were particularly interested in establishing an in vitro transcription system to approach this question. By the successful use of purified components, we show that RpoH$_1$ and RpoH$_2$ indeed favor different promoters.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *E. coli* cells were grown in Luria-Bertani (LB) medium (15) supplemented with ampicillin (200 μg/ml) or

kanamycin (30 μg/ml) if required. *E. coli* A7448 (4, 30) was grown at 28°C; the routine growth temperature for other *E. coli* strains was 37°C. *B. japonicum* was grown aerobically at 28°C in PSY medium (22) supplemented with 0.1% (wt/vol) arabinose and 100 μg of spectinomycin per ml.

Plasmid constructions. Recombinant DNA techniques were performed by standard methods (23). The individual *rpoH* genes were amplified from plasmids containing these genes (18, 19) by PCR with Vent DNA polymerase as specified by the manufacturer (New England Biolabs, Beverly, Mass.). The oligonucleotides used as primers were designed so that they introduced a *Nde*I recognition site overlapping the start codon of each gene and either a *Xho*I site immediately downstream of the 3′ ends of $rpoH_1$ and $rpoH_2$ or a *Not*I site at the equivalent position of $rpoH_3$. The PCR-generated fragments were cut with *Nde*I-*Xho*I or *Nde*I-*Not*I and ligated into pET24b(+) (Novagen, ams Biotechnology, Lugano, Switzerland) digested with the same enzymes. The resulting plasmids, pRJ5086, pRJ5101, and pRJ5102, encoded C-terminally histidine-tagged $RpoH_1$, $RpoH_2$, and $RpoH_3$ proteins, respectively. The vector-encoded amino acids leucine and glutamic acid were introduced between the carboxy-terminal amino acid of $RpoH_1$ or $RpoH_2$ and the histidine tag. A stretch of five amino acids (AAALE) was introduced at the corresponding position of $RpoH_3$-His_6.

Plasmids used as transcription templates were based on pRJ9519, which contains the *B. japonicum rrn* terminator. It derives from pRJ9601 (3) from which the *rrn* promoter and an internal *Eco*RI restriction site upstream of the terminator have been deleted. Plasmid pRJ5099 carries a 0.9-kb *Eco*RI-*Bgl*II fragment containing the *B. japonicum dnaKJ* promoter region (16). The *B. japonicum* $groESL_1$ template pRJ5134 contains a *Bgl*II-*Eco*RV-digested 0.4-kb PCR fragment originating from pRJ8067 (2). While the *Bgl*II site was present in the sequence upstream of $groESL_1$, the *Eco*RV site was introduced into the $groES_1$ gene by the PCR primer (Sig106, 5′-GGCTTGATATCGATCGGGATC-3′ [recognition site underlined]). Plasmid pEC5162 bears a 0.4-kb *E. coli groESL* promoter fragment that was PCR amplified from pEC8182 (kindly provided by H.-M. Fischer, Zürich, Switzerland), a subclone of pND5 (12). *Eco*RI restriction sites at either end of the fragment were created by the PCR primers (Sig122, 5′-GCGAATTCCCTGGGCCAGCCC-3′; Sig123, TCGAATTCTTTACGCTTGACG-3′ [*Eco*RI sites underlined]).

The correct nucleotide sequences of all PCR-amplified fragments introduced into pET24b(+) or the transcription vector pRJ9519 were confirmed by sequencing. It revealed for pEC5162 that in this particular case the *Eco*RI site in pRJ9519, which was assumed to be destroyed (see above), was present and had been cut by *Eco*RI, resulting in a product that was approximately 100 bp shorter than expected.

Overproduction and purification of RpoH-His_6 proteins. Freshly transformed *E. coli* BL21/pLys cells (26) carrying pRJ5086, pRJ5101, or pRJ5102 were used for overproduction of RpoH proteins. When the cells had reached an optical density at 600 nm of 0.7 at 28°C, production of the recombinant proteins was induced by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After 2 h, the cells were harvested and disrupted in a French pressure cell, and proteins were purified from the soluble fraction of a 500-ml cell culture ($RpoH_1$ and $RpoH_2$) or 2,000-ml culture ($RpoH_3$). The extracts were loaded onto a 1.5-ml Ni-nitrilotriacetic acid-agarose column (Qiagen, Hilden, Germany), and chromatography was performed as specified in the pET System Manual (Novagen). Protein fractions were dialyzed against storage buffer (20 mM Tris-HCl [pH 8.0], 200 mM NaCl, 50% glycerol) and then stored at −20°C.

Purification of *B. japonicum* RNA polymerase core enzyme. RNA polymerase was prepared as described previously (3). Core RNA polymerase was separated from holoenzyme on a Resource Q column (6 ml; Pharmacia, Uppsala, Sweden). Up to 1.4 mg of RNA polymerase was loaded onto the column equilibrated with T_{50}GED (50 mM Tris-HCl [pH 8.0], 10% glycerol, 1 mM EDTA, 0.1 mM dithiothreitol) containing 0.1 M NaCl. After the column was washed with the same buffer, bound protein was eluted with 170 ml of a linear 0.1 to 0.325 M NaCl gradient. Peak fractions which were devoid of sigma factor (judged by sodium dodecyl sulfate [SDS]-polyacrylamide gel electrophoresis [12% polyacrylamide]) were pooled, dialyzed against T_{10}GED buffer containing 0.02 M NaCl and 50% (vol/vol) glycerol, and concentrated by ultrafiltration.

Western blot (immunoblot) analysis. Crude extracts of *E. coli* cells were prepared, separated on SDS–12% polyacrylamide gels, and transferred to nitrocellulose membranes as described previously (18). The following antisera were used: anti-*E. coli* σ^{32}, DnaK, and GrpE sera (B. Bukau, Freiburg, Germany; 3,000-fold dilutions [7]); anti-*E. coli* GroEL serum (Epicentre Technologies, Madison, Wis.; 20,000-fold dilution); and anti-*E. coli* IbpA serum, which recognizes the small Hsps IbpA and IbpB (A. Easton, St. Louis, Mo.; 1,500-fold dilution [1]). The primary rabbit antibodies were detected with a chemiluminescence Western blotting kit (Boehringer GmbH, Mannheim, Germany).

In vitro transcription. Multiple-round transcription assays with RNA polymerase holoenzyme were carried out in a volume of 20 μl under standard conditions as described previously (3). RNA polymerase core enzyme (1.15 μg of *B. japonicum* enzyme or 1 U of *E. coli* enzyme [Boehringer Mannheim] per assay) was reconstituted with RpoH factors (0.1 μg per assay; molar ratio of core RNA polymerase and RpoH protein, 1:1) for 20 min at 4°C in a volume of 10 μl containing 40 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol, 150 mM KCl, 0.4 mM K_3PO_4, and 0.2 mg of bovine serum albumin per ml. The reaction was started by adding the reconstituted enzyme to a 10-μl sample containing the same components as the reconstitution buffer plus 1 mM of each nucleoside triphosphate, 1 μCi of [α-^{32}P]UTP (800 Ci/mmol; DuPont, Bad Homburg, Germany), 1 U of RNase inhibitor per μl, and 20 nM DNA template (final concentrations).

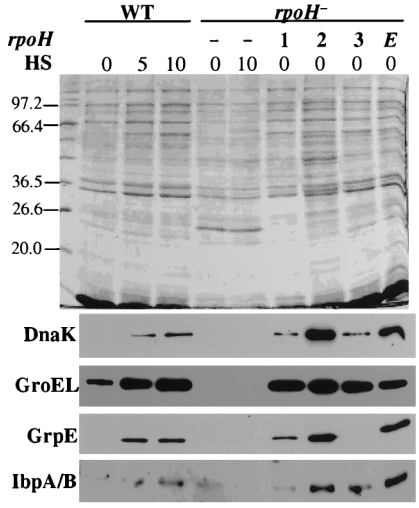

FIG. 1. Detection of heat shock proteins produced by *E. coli* MC4100 (WT) and *E. coli* A7448 ($rpoH^-$). *E. coli* A7448 transformed with pUC18 (−), pRJ5000 ($rpoH_1$, indicated as 1), pRJ5002 ($rpoH_2$, indicated as 2), pRJ5040 ($rpoH_3$, indicated as 3), or pEC5007 (*E. coli rpoH*, indicated as *E*) was grown in the presence of 0.5 mM IPTG. Crude cell extracts were prepared from cells grown at 28°C (0) or heat-shocked cells (heat shock [HS] from 28 to 43°C for 5 or 10 min as indicated) and separated on a SDS–12% polyacrylamide gel. A Coomassie blue-stained gel is shown at the top. The apparent molecular masses (in kilodaltons) of relevant marker proteins are indicated to the left of the gel. Similar gels with suitably diluted samples (1/5, 1/10, 1/3, and 1/15 of the amounts loaded onto the stained gel) were used for immunodetection of the Hsps DnaK, GroEL, GrpE, and IbpA plus IbpB, respectively.

For single-round transcription experiments, the DNA template was added to the reconstitution sample before the remaining components together with 100 ng of heparin per ml (final concentration) were added. The template concentration in promoter competition experiments was 10 nM for each plasmid.

Suitable RNA size markers were synthesized in vitro with T7 or T3 RNA polymerase with linearized pBluescript-based plasmids as templates.

Transcript mapping. RNA isolation and primer extension analysis was performed as described elsewhere (2). The start site of transcripts synthesized in vitro was determined as described previously (3). The oligonucleotides 702 (2) and DnaK12 (16) were used to determine the start sites of in vitro-synthesized *B. japonicum* $groESL_1$ or *dnaK* transcripts. Oligonucleotide Sig123, which was used to amplify the promoter region, was also used to determine the in vivo and in vitro start site of the *E. coli groESL* operon.

RESULTS

Plasmid-encoded RpoH proteins of *B. japonicum* selectively transcribe heat shock promoters in *E. coli*. We have previously described that all three RpoH proteins of *B. japonicum* were functionally active in a σ^{32}-negative *E. coli* reporter strain which contains a chromosomally integrated *E. coli groE-lacZ* fusion (19). This strain produced high β-galactosidase activity when it was complemented with any of the three plasmids harboring an *rpoH* gene under the control of the *lac* promoter. We now monitored the ability of these complemented strains to produce heat shock proteins in *E. coli*. To this end, we performed immunoblot analyses with antibodies raised against different Hsps. Extracts from normally grown and heat-shocked cells of *E. coli* MC4100 and the σ^{32}-negative strain containing pUC18 without insert served as controls. The typical heat-inducible synthesis of the Hsps GroEL, DnaK, GrpE, and IbpA plus IbpB was observed in the wild type but not in the mutant (Fig. 1). As expected from the activation of the *groE-lacZ* fusion, all strains complemented by *rpoH* produced high levels of GroEL. However, the amounts of other Hsps in

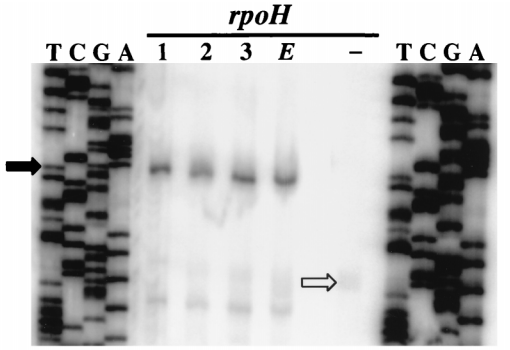

FIG. 2. Primer extension analysis of *groESL* transcripts synthesized in *E. coli* A7448. Reverse transcripts were obtained with 15 μg of total RNA isolated from the strain complemented with *B. japonicum* $rpoH_1$ (lane 1), $rpoH_2$ (lane 2), or $rpoH_3$ (lane 3) or *E. coli rpoH* (lane *E*) and 30 μg of RNA from the same strain transformed with pUC18 (lane −). Oligonucleotide Sig123 was used as the primer for the primer extension and corresponding sequencing reactions (lanes T, C, G, and A). The transcription start site used by the σ^{32}-like proteins is indicated by a solid arrow; the open arrow marks the transcript initiated at the σ^{70}-dependent promoter.

these strains were quite different. While *E. coli* σ^{32} and *B. japonicum* $RpoH_2$ produced comparable, high levels of DnaK, GrpE, and small Hsps, $RpoH_1$ and in particular $RpoH_3$ produced only small or undetectable amounts of DnaK and GrpE. This result indicated that the sigma factors conferred different promoter specificities upon *E. coli* core RNA polymerase.

The *B. japonicum* RpoH factors use the same in vivo start site as *E. coli* σ^{32}. Since all four RpoH factors synthesized GroEL protein efficiently, we analyzed whether the heterologous proteins initiated transcription at the same position as *E. coli* σ^{32}. Total RNA was isolated from each complemented strain, and the 5′ end of the *groESL* transcript was determined by primer extension. This strain contains two *groESL* promoter regions: the original operon and a chromosomally integrated *groE-lacZ* fusion. Transcripts of both operons were detected by the primer used in these experiments. The transcripts formed by all four RpoH proteins start at the identical position, which

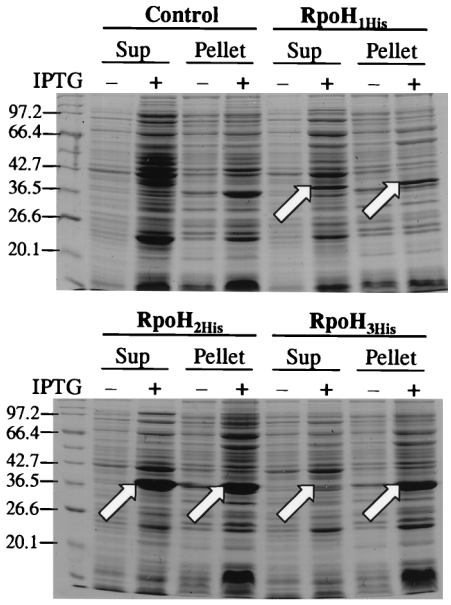

FIG. 3. Solubility of overproduced *B. japonicum* RpoH proteins. The supernatant (Sup) and pellet (Pellet) fraction of crude cell extracts prepared before (−) or after (+) IPTG induction were separated by centrifugation at 15,000 rpm (SS-34 rotor) at 4°C for 30 min. Control cells contain the vector pET24b(+) without insert. $RpoH_{1His}$, $RpoH_{2His}$, and $RpoH_{3His}$ were produced from plasmids pRJ5086, pRJ5101, and pRJ5102, respectively. After electrophoresis in an SDS–12% polyacrylamide gel, the proteins were stained with Coomassie blue. The apparent molecular masses (in kilodaltons) of marker proteins are indicated to the left of the gel. Arrows point to the overproduced $RpoH_{His}$ protein.

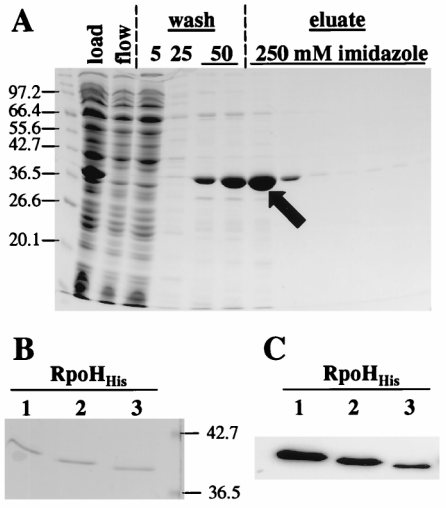

FIG. 4. Purification of $RpoH_{2His}$ and immunodetection of *B. japonicum* $RpoH_{His}$ proteins. (A) Aliquots of the $RpoH_{2His}$ supernatant fraction which was loaded onto the column (load), the flowthrough (flow), the different wash (wash), and the elution (eluate) fractions were separated on an SDS–12% polyacrylamide gel, and the proteins were stained with Coomassie blue. The imidazole concentration in the wash and elution buffers is indicated. The apparent molecular masses (in kilodaltons) of the marker proteins are indicated to the left of the gel. The arrow points to the protein fraction which was subsequently used for in vitro experiments. (B) Coomassie blue-stained SDS–12% polyacrylamide gel of 100 ng of each purified *B. japonicum* $RpoH_{His}$ protein. The apparent molecular masses of two marker proteins are indicated to the right of the gel. (C) Immunodetection of purified *B. japonicum* RpoH proteins with anti-*E. coli* σ^{32} serum. For each protein, a 5-ng sample was subjected to Western blot analysis.

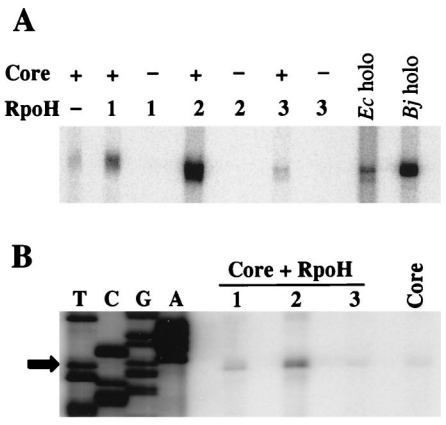

FIG. 5. Transcription of the *E. coli groESL* promoter by *E. coli* RNA polymerase core enzyme and *B. japonicum* RpoH factors. (A) The enzyme combinations tested are indicated. Core, *E. coli* RNA polymerase core enzyme; RpoH, purified $RpoH_{His}$ protein; *Ec* holo, *E. coli* RNA polymerase holoenzyme; *Bj* holo, *B. japonicum* RNA polymerase holoenzyme. (B) Primer extension analysis of in vitro-synthesized RNA. The enzyme with which the transcript was generated is indicated. The primer extension and sequencing reactions (TCGA) were performed with oligonucleotide Sig123. The transcription start site is depicted by an arrow.

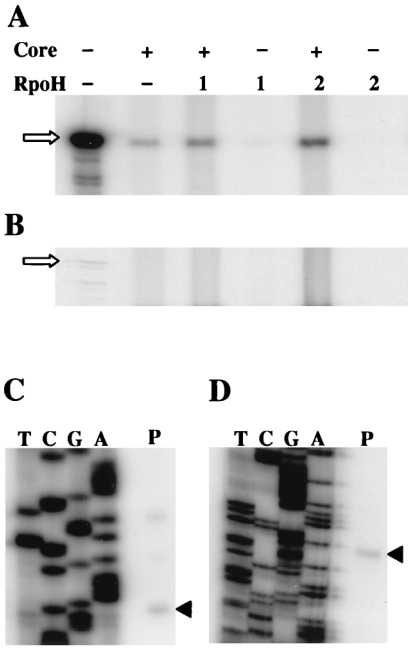

FIG. 6. Transcription of the *B. japonicum groESL*$_1$ promoter and the *dnaKJ* promoter by *B. japonicum* RNA polymerase reconstituted with purified RpoH factors. (A) Transcription of the *B. japonicum groESL*$_1$ promoter. The enzyme combinations tested are indicated. Core, *B. japonicum* RNA polymerase core enzyme; RpoH, purified $RpoH_{His}$ protein. The arrow points to an RNA size marker of 406 nucleotides. (B) Transcription of the *B. japonicum dnaKJ* promoter. The enzyme combinations are as indicated in panel A. (C) Primer extension analysis of in vitro-synthesized *groESL*$_1$ transcript. Oligonucleotide 702 was used for the sequencing (TCGA) and primer extension reaction. The triangle points to the start site, which has been determined in vivo (2). The origin of small amounts of slower-migrating bands is not known because corresponding products were not observed after in vitro transcription. (D) Primer extension analysis of in vitro-synthesized *B. japonicum dnaKJ* transcript. Oligonucleotide DnaK12 was used for the sequencing (TCGA) and primer extension reaction. The 5′ end of the reverse transcript (indicated by a triangle) corresponds to the major start site found in vivo (16).

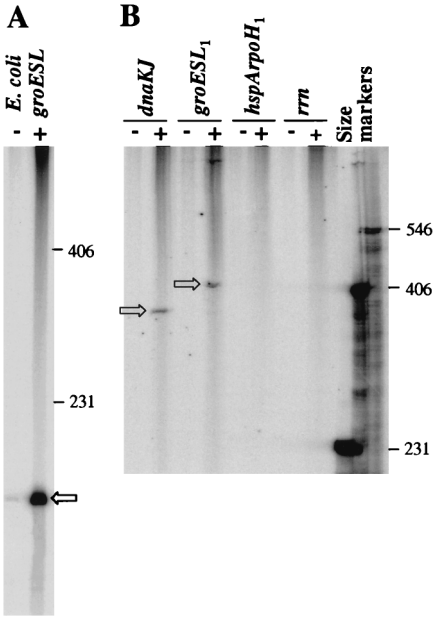

FIG. 7. Single-round transcription of different promoters by *B. japonicum* core RNA polymerase reconstituted with $RpoH_{2His}$. The *E. coli* (A) or *B. japonicum* (B) promoters which were transcribed are indicated. Experiments were performed with core RNA polymerase alone (−) and the same enzyme reconstituted with $RpoH_{2His}$ (+). Arrows point to the transcripts; the position and length (in nucleotides) of RNA size markers are indicated to the right.

corresponds to the previously described σ^{32}-dependent site (Fig. 2) (5). Small amounts of a shorter product probably represent prematurely terminated reverse transcripts. RNA from the mutant containing only the vector resulted in a faint signal that represents transcripts originating from the σ^{70}-dependent promoter (Fig. 2) (30). This transcript was hardly observed with RNA from the *rpoH*$^+$ strains, presumably because the abundant RpoH proteins prevented transcription from the σ^{70} promoter by promoter occlusion. It has been shown that this promoter is very weak and virtually inactive in *rpoH*$^+$ strains (14, 30).

Overexpression and purification of histidine-tagged $RpoH_1$, $RpoH_2$, and $RpoH_3$. Knowing that amino-terminally or carboxy-terminally histidine-tagged σ^{32} of *E. coli* is active in vivo and in vitro (7), we constructed plasmids to produce the *B. japonicum* RpoH proteins with a histidine tag attached to their C termini. Crude cell extracts containing these proteins were prepared and separated into pellet and supernatant fractions (Fig. 3). Surprisingly, the overproduction efficiency and the solubility of the RpoH proteins varied over a wide range. $RpoH_{2His}$ and $RpoH_{3His}$ were overproduced to the highest levels. However, more than 95% of $RpoH_{3His}$ was found in the pellet fraction, indicating that the majority of this protein was insoluble. By contrast, approximately 50% of overproduced $RpoH_{1His}$ and $RpoH_{2His}$ remained in the supernatant. All three proteins were purified from the soluble supernatant fraction by nickel-nitrilotriacetic acid chromatography. The proteins exhibited different binding affinities to the column resin. While some $RpoH_{2His}$ had already eluted during the wash step with an imidazole concentration of 50 mM, $RpoH_{1His}$ and $RpoH_{3His}$ were still retained at this concentration (data not shown). The purification profile for $RpoH_{2His}$ (Fig. 4A) serves as an example to show that the nickel affinity chromatography technique allowed efficient purification of histidine-tagged *B. japonicum* RpoH proteins.

In previous work, we showed that $RpoH_1$ or $RpoH_2$ produced in *E. coli* A7448 was detectable by an anti-*E. coli* σ^{32} serum in an immunoblot (18, 19) but that $RpoH_3$ did not consistently react with the antiserum. By using the purified His-tagged proteins (Fig. 4B), we could now show that this serum also recognizes $RpoH_{3His}$ but clearly does so less well than it recognizes the other proteins (Fig. 4C).

Multiple-round in vitro transcription assays. In a first attempt to test whether the purified RpoH proteins were transcriptionally active in vitro, we simulated the situation in the *E. coli rpoH* mutant in which the proteins had been shown to function in vivo. *E. coli* core RNA polymerase was reconstituted with the individual $RpoH_{His}$ proteins, and the ability to transcribe the *E. coli groESL* promoter was monitored in a multiple-round transcription assay. The core enzyme alone produced small amounts of transcript (Fig. 5A), most probably as a result of some residual σ^{32} protein in the preparation that was detectable by Western blot analysis (data not shown). Transcription was stimulated by the addition of $RpoH_{1His}$ and in particular by $RpoH_{2His}$. $RpoH_{3His}$ appeared to be inactive because the reconstituted enzyme did not produce more transcript than core RNA polymerase alone. Since $RpoH_3$ was active at the *E. coli groESL* promoter in vivo (compare Fig. 1) we conclude that the purified protein was in an inactive state, probably because the small portion of soluble protein was in an inactive conformation. Transcriptional activity of $RpoH_1$ and

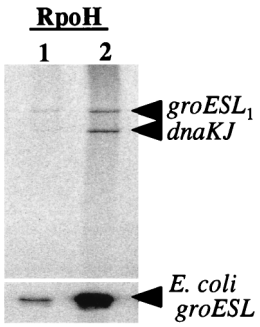

FIG. 8. Single-round promoter competition experiment with *B. japonicum* core RNA polymerase reconstituted with $RpoH_{1His}$ or $RpoH_{2His}$. The *E. coli groESL* promoter and the *B. japonicum dnaKJ*, $groESL_1$, *hspA rpoH*$_1$, and *rrn* promoters were provided at equimolar concentrations (10 nM each; the template concentration is not limiting in this assay). The transcripts obtained are indicated.

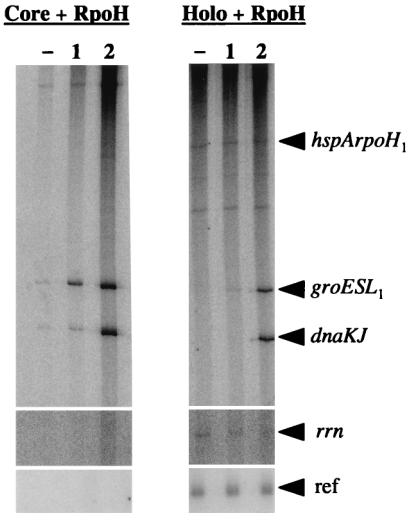

FIG. 9. Single-round promoter competition experiment with *B. japonicum* RNA polymerase core enzyme and holoenzyme reconstituted with purified RpoH proteins. The *B. japonicum dnaKJ*, $groESL_1$, *hspA rpoH*$_1$, and *rrn* promoters were provided at equimolar concentrations (10 nM each). The corresponding transcripts are indicated. The vector-encoded reference transcript (ref) was produced only in the presence of the holoenzyme.

$RpoH_2$ required the presence of RNA polymerase because the sigma factors alone were unable to synthesize transcript. As positive controls, we used *E. coli* and *B. japonicum* holo-RNA polymerases, both of which contain significant amounts of RpoH protein as revealed by Western blot analysis (data not shown). The transcripts produced by these enzymes were indistinguishable from the ones formed by the reconstituted enzymes, indicating that transcription had been initiated at the same position (Fig. 5A).

To confirm that the reconstituted enzymes use the same start site as in vivo, we determined the 5′ end of in vitro-synthesized RNA by primer extension analysis (Fig. 5B). It was found to be identical to the start site of the σ^{32}-dependent *groESL* (5) (compare Fig. 2). The presence of comparable faint signals produced by RNA polymerase core enzyme and $RpoH_3$-containing enzyme again reflected the notion that the latter was not active.

Next we tested a physiologically more relevant combination by reconstituting *B. japonicum* core RNA polymerase with the purified RpoH factors and transcribing the *B. japonicum* $groESL_1$ and *dnaKJ* promoters, which are dependent on a σ^{32}-type factor in vivo (2, 16). Again, $RpoH_{3His}$ did not stimulate transcription (data not shown), but transcription from both promoters was initiated when RNA polymerase had been reconstituted with $RpoH_{1His}$ or $RpoH_{2His}$ (Fig. 6A and B). The $groESL_1$ and *dnaKJ* transcripts are of the expected lengths (401 and 358 nucleotides, respectively), as judged from the RNA size marker. To demonstrate that these transcripts had been initiated at the same site as in vivo, in vitro-synthesized RNA was subjected to primer extension analysis. The determined 5′ ends of the $groESL_1$ and *dnaKJ* transcripts (Fig. 6C and D, respectively) were mapped to the same positions as described previously (2, 16).

Single-round in vitro transcription assays. To gain more insight into possible promoter specificities of $RpoH_1$ and $RpoH_2$, it was important to establish a single-round transcription system. *B. japonicum* core RNA polymerase was reconstituted with $RpoH_{2His}$, and transcription at five different promoters was assayed under conditions which allowed for the production of only one transcript by each preformed RNA polymerase-template complex. First we tested the *E. coli groESL* promoter, which was transcribed very well by this enzyme combination (Fig. 7A). The *B. japonicum dnaKJ* and $groESL_1$ promoters were also transcribed specifically (Fig. 7B). As a control, we examined the *hspA rpoH*$_1$ and *rrn* promoters, which are expected to be transcribed by *B. japonicum* σ^{80}, the σ^{70} homolog (3, 18). The corresponding products would be 625 and 240 nucleotides long, respectively. Neither promoter was transcribed by the RNA polymerase containing $RpoH_{2His}$, indicating that this sigma factor specifically recognizes σ^{32}-like promoters.

Promoter competition experiments. To further investigate the promoter specificities of $RpoH_1$ and $RpoH_2$, we performed experiments in which an equimolar mix of different templates was provided for transcription. The first mix contained a composite of all five promoters which had been tested individually in the last experiment. *B. japonicum* core RNA polymerase was reconstituted with $RpoH_{1His}$ and $RpoH_{2His}$, and transcription was assayed under single-round conditions (Fig. 8). As suggested by the experiment in Fig. 7, RNA polymerase containing $RpoH_{2His}$ preferentially transcribed from the *E. coli groESL* promoter. (The preference for the *E. coli* promoter may even be underestimated, because the *E. coli groESL* transcript contains 50 uracils that can be radiolabeled whereas the *B. japonicum dnaKJ* and $groESL_1$ transcripts contain 81 and 80 uracils, respectively.) Transcription from the *B. japonicum* σ^{32}-type promoters was much lower with the *dnaKJ* promoter, yielding slightly more transcript than the $groESL_1$ promoter. RNA polymerase containing $RpoH_{1His}$ also transcribed the *E. coli groESL* promoter to the highest level. Small amounts of the $groESL_1$ transcript and very little *dnaKJ* transcript were obtained, which implies that $RpoH_1$ and $RpoH_2$ have different promoter specificities. The *hspA rpoH*$_1$ and *rrn* promoters that were also present in the mixture were not transcribed by the reconstituted enzymes.

After this initial comparative assessment of the promoter specificities conferred by the different RpoH factors, we performed another template competition experiment in which only *B. japonicum* promoters were provided. Not only *B. japonicum* core but also holo-RNA polymerase was mixed with the individual purified RpoH factors. The experiment with holoenzyme showed that the added sigma factors can associate with core enzyme present in this preparation (Fig. 9). As expected, the σ^{80} holoenzyme produced some *hspA rpoH*$_1$ and *rrn* transcript and also an 80-nucleotide reference transcript

that is encoded by each vector carrying one of the promoter fragments (3). These three transcripts were not obtained when core polymerase was reconstituted with RpoH proteins. The experiments with core enzyme and with holoenzyme confirmed the different promoter specificities of $RpoH_1$ and $RpoH_2$. While the latter enzyme transcribed the *groESL*$_1$ and *dnaKJ* promoter well and the *dnaKJ* promoter slightly better than *groESL*$_1$, the $RpoH_1$-containing RNA polymerase produced less transcript and favored the *groESL*$_1$ promoter (Fig. 9; see also Fig. 8).

DISCUSSION

Different promoter specificities of *B. japonicum* RpoH factors. After the previous identification of three disparately regulated *rpoH* genes in *B. japonicum* (18, 19), we were left with the question of why this organism uses a gene family for functions that in other bacteria (e.g., *E. coli*) are accomplished by a single *rpoH* gene. In principle, an organism can use two different strategies to achieve coordinate regulation of a gene under different environmental conditions: (i) The gene can be put under the control of different promoters, or (ii) several copies of this gene may be present, each having a distinctly regulated promoter. A paradigm for the differential regulation of reiterated genes is the *groESL* multigene family of *B. japonicum*. The expression of five *groESL* operons appears to be controlled by four mechanisms, two of which do not contribute to chaperonin production under heat shock conditions (low constitutive expression of *groESL*$_2$ and NifA-dependent expression of *groESL*$_3$) (6). Heat-induced expression of *groESL* genes depends on two separate mechanisms, namely, RpoH factor(s) for *groESL*$_1$ and CIRCE (controlling inverted repeat of chaperone expression) (31) for *groESL*$_4$ and *groESL*$_5$ (2). The *rpoH* genes of *B. japonicum* were also defined as a disparately regulated gene family. Transcription of *rpoH*$_1$, for instance, is heat inducible by a novel mechanism (18, 20), whereas *rpoH*$_2$ is transcribed constitutively from a σ^{70}-type promoter under normal growth conditions and induced from a σ^{E}-type promoter under severe heat shock conditions (19).

However, the option to differentially regulate each member does not seem to be the only reason for the presence of an *rpoH* multigene family in *B. japonicum*. The situation becomes more complex because the individual *rpoH* genes are not functionally equivalent and have (at least to some extent) different promoter specificities in vivo and in vitro. The finding that all RpoH proteins were capable of synthesizing GroEL in an *E. coli* reporter strain agrees well with their previously shown activation of a *groE-lacZ* fusion. The limited capacity of $RpoH_1$ and $RpoH_3$ to produce other Hsps, especially GrpE, provides a possible answer to why these proteins were insufficient in complementing the temperature-sensitive phenotype of an *E. coli* σ^{32} mutant (19).

The promoter selectivity of $RpoH_1$ and $RpoH_2$ was further corroborated by in vitro studies. Purified $RpoH_{3His}$ protein appeared to be inactive because it showed very little (if any) activity at all promoters tested. $RpoH_{3His}$ also was much less soluble than $RpoH_{1His}$ and $RpoH_{2His}$. Although the small fraction of soluble protein was purified from the crude supernatant, it may still be in an aggregated and transcription-incompetent form. By contrast, $RpoH_{1His}$ and, in particular, $RpoH_{2His}$ were highly active in transcribing σ^{32}-dependent promoters. Much to our surprise, the endogenous *B. japonicum groESL*$_1$ and *dnaKJ* promoters were transcribed less well than the heterologous *E. coli groESL* promoter. This raises the question why the known σ^{32}-type promoters of *B. japonicum* contain a suboptimal promoter sequence. It is particularly puzzling because a heat shock promoter specific for the alpha subdivision of the proteobacteria (to which *B. japonicum* belongs) was proposed on the basis of a comparison of *groESL* and *dnaKJ* promoters from different members of this subdivision (16, 24). The consensus sequence differs from the typical σ^{32}-dependent heat shock promoter of *E. coli* at several positions. However, a comparative functional analysis of heat shock promoters from the alpha and gamma proteobacteria has not been performed yet. Our first attempt in this direction implies that the heat shock promoters of the alpha proteobacteria are not necessarily optimized for recognition by the endogenous sigma factors. In keeping with this finding are the results of a mutational analysis of the σ^{32}-dependent P1 promoter of the *Caulobacter crescentus rpoH* gene, which is autoregulated (29). This promoter also is not optimal, and its activity can be increased substantially by a particular point mutation in the −35 region. Generally, an efficient expression of heat shock genes under normal growth conditions is not required and may even be deleterious. It has been reported for *E. coli* that cells overproducing Hsps are sick (27). Thus, rather weak promoters may have evolved to reduce expression. After a heat shock, the abundant RpoH protein (in *B. japonicum* mainly $RpoH_1$) may override the weak promoter activities.

The purified RpoH factors directed transcription exclusively from σ^{32}-type promoters. A similar strict promoter dependence was also described for the corresponding enzymes of *E. coli* and *C. crescentus* (5, 28) with one notable exception: the P1 promoter of *rrnB*, one of seven *rrn* operons in *E. coli*, can be transcribed from RNA polymerase containing either σ^{70} or σ^{32} (21). This is thought to be important for ribosome synthesis at high temperatures. Neither the in vitro transcription performed in this work nor the earlier characterization of the *rrn* operon of *B. japonicum* (3, 13) gave any indication that a similar situation applies here. This is particularly surprising because *B. japonicum* contains only a single rRNA operon.

Function of $RpoH_1$ and $RpoH_2$ in *B. japonicum*. On the basis of results obtained previously it was proposed that $RpoH_2$ is essential for the synthesis of σ^{32}-dependent proteins under normal growth conditions whereas $RpoH_1$ provides their synthesis under stress conditions (19). The function of $RpoH_3$ remains obscure. Combining all data known at present, we arrive at the following, refined model.

(i) Normal growth conditions. Although the *rpoH*$_2$ gene is transcribed to a considerable extent under these conditions, very little RpoH protein is detectable in cell extracts (18, 19). The corresponding protein is probably unstable during normal growth, as is *E. coli* σ^{32} (25). The observation that neither the *rpoH*$_2$ nor the *dnaK* gene could be deleted had already suggested a direct dependence of the DnaK chaperone, which is required under all conditions, on this sigma factor (16, 19). The in vitro transcription data confirm that $RpoH_2$ directs the transcription of the *dnaKJ* promoter efficiently. The *groESL*$_1$ promoter could also be transcribed in vitro, but this does not seem to play a role in vivo because the *groESL*$_1$ transcript is almost undetectable under normal growth conditions (2).

(ii) Heat shock conditions. A temperature upshift greatly stimulates the production of $RpoH_1$ in *B. japonicum* (18, 19). The parallel induction of the *groESL*$_1$ transcript (2) indicated that it occurred as a result of an increase in $RpoH_1$ production. The in vitro activity of $RpoH_1$ supports this notion. By comparison, the *dnaKJ* promoter was weakly transcribed, which raises the question how heat induction of DnaK is accomplished. Since maximal induction of this protein is already reached a few minutes after a temperature upshift (16), it is highly possible that $RpoH_2$ is responsible for this increase. The $RpoH_2$ level in the cell increases in the initial phase after a

heat shock before it rapidly decreases (19). Under continuous stress conditions, the weak activity of $RpoH_1$ may then suffice to keep the DnaK concentration at an elevated level.

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