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Dissection of the Transcription Machinery for Housekeeping Genes of *Bradyrhizobium japonicum*

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By using a PCR approach, the *Bradyrhizobium japonicum sigA* gene, which encodes the primary RNA polymerase sigma factor, σ^{80}, was cloned and its nucleotide sequence was established. The deduced protein is highly homologous to the SigA protein of *Rhizobium meliloti* (72% amino acid sequence identity) but less so to RpoD of *Escherichia coli* (51% identity). Well conserved is the C-terminal end of the protein, which is probably involved in promoter recognition and binding of the RNA polymerase core enzyme. A remarkable feature of the primary sequence is an alanine- and proline-rich segment of 24 amino acids between conserved regions 1 and 2, which might function as an interdomain linker. We purified the *B. japonicum* RNA polymerase holoenzyme. One of the subunits had an apparent molecular mass of 90 kDa and corresponded to the *sigA* gene product, as judged by N-terminal amino acid sequencing. The purified RNA polymerase was used in an in vitro transcription system to determine the transcription start sites of the *rrn* and *groESL*$_4$ operons. They were identical to those previously identified in vivo. The *rrn* promoter was cloned upstream of a rho-independent terminator, yielding a transcript of about 240 bases. This served as a suitable template to analyze promoter activity. Then mutant derivatives of the *rrn* promoter were constructed and tested in in vitro transcription experiments. Several base pairs essential for promoter activity were thus identified. The results suggest that the well-characterized −35/−10 promoter class is predominantly used in *B. japonicum* for the expression of "housekeeping" genes.

The interactions of *Bradyrhizobium*, *Azorhizobium*, and *Rhizobium* strains with their legume host plants are controlled mainly by a set of nodulation (*nod*) and nitrogen fixation (*nif* and *fix*) genes (11, 14). To ensure proper timing of gene expression, many of these genes are subject to positive regulation. The promoter sequences of the symbiotic genes have no similarities to the −35/−10 promoter class first characterized in *Escherichia coli*. The promoters of the *nif* genes belong to the −24/−12 class (11), and recognition by the RNA polymerase requires the presence of an alternative sigma factor, σ^{54}. The expression of *nod* genes, however, whose promoter regions contain a highly conserved sequence, the *nod* box, may depend on the activity of the primary sigma factor (13, 28). For *Rhizobium meliloti*, in vitro transcription of some symbiotic genes has previously been reported (1, 9, 12, 13). In contrast, the promoter structure of "housekeeping" genes has not been studied systematically in rhizobia. Based simply on a sequence alignment, the putative recognition site for the primary sigma factor of *Bradyrhizobium japonicum*, which bore no similarity to known promoter structures (4), has been proposed.

Recently, we cloned and characterized the single *rrn* operon of *B. japonicum*, coding for three rRNA and two tRNA species. The in vivo transcription start site of *rrn* was determined by primer extension experiments (17). Because the rRNAs are essential under all growth conditions, we expected the corresponding operon to be preceded by a promoter that is typical for housekeeping genes. As a consequence, this promoter should be recognized by the primary sigma factor. The purification of the RNA polymerase holoenzyme (RNAP) has been previously reported, and it was suggested that a prominent protein of around 90 kDa corresponds to the primary sigma factor (26, 33). In vitro transcription of *B. japonicum* genes starting from specific promoters has so far not been achieved with these preparations.

Here we describe the characterization of the *rrn* promoter as a typical −35/−10 promoter and the identification of the primary RNAP sigma factor, which is responsible for recognition of this promoter.

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MATERIALS AND METHODS

Purification of RNA polymerase. *B. japonicum* 110*spc*4 (26) was grown aerobically at 30°C in peptone-salts-yeast extract medium (26) supplemented with 0.2% arabinose. Cells were harvested at late exponential phase. All purification steps were performed at 4°C. Cells (25 g [wet weight]) were resuspended in 70 ml of TGED buffer (10 mM Tris-HCl [pH 8.0], 10% glycerol, 1 mM EDTA, 0.1 mM dithiothreitol [DTT]) containing 0.02 M NaCl and disrupted in a French pressure cell (three passes at 11,000 lb/in^2). The crude extract was treated with polyethyleneimine to a final concentration of 0.3%. The pellet obtained after centrifugation (15 min; 27,000 × *g*) was washed with TGED buffer (0.2 M NaCl), and protein containing RNAP was dissolved in TGED buffer (0.8 M NaCl) and precipitated again by adding solid $(NH_4)_2SO_4$ to 65% final saturation (43 g per 100 ml). The precipitate was dissolved in 30 ml of TGED buffer (0.02 M NaCl), dialyzed against 1 liter of TGED buffer (0.02 M NaCl), and loaded onto an EMD-DEAE column (Merck, Darmstadt, Germany), from which it was eluted by a linear 0.02 to 1.2 M NaCl gradient. Fractions containing RNAP (as judged by standard transcription assays) were pooled and loaded onto a heparin agarose column (Bio-Rad Laboratories, Richmond, Calif.). Equilibration and elution buffers were similar to those used in the EMD-DEAE chromatography. Peak fractions containing the enzyme were diluted with a sixfold excess of TGED buffer (0.02 M NaCl) and fractionated on DNA-cellulose (Sigma Chemical Company, St. Louis, Mo.) by a linear 0.15 to 1.2 M NaCl gradient. Peak fractions were pooled, concentrated by ultrafiltration, and loaded onto a Sephacryl-S300 column (Pharmacia LKB Biotechnology, Uppsala, Sweden) equilibrated with TGED buffer containing 0.5 M NaCl. Active fractions were tested for purity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and stored in TGED buffer (0.02 M NaCl) containing 50% glycerol. Protein concentrations were determined with Bio-Rad assay solution, with bovine gamma globulin as the standard.

Specific in vitro transcription assay. RNA was synthesized in a 20-μl reaction volume containing 40 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM DTT, 150 mM KCl, 0.4 mM K_3PO_4, 0.1 mg of bovine serum albumin per ml, 20 nM plasmid DNA as template, 1 mM (each) nucleoside triphosphate, and 1 μCi of [α-^{32}P]UTP (800 Ci/mmol; DuPont NEN, Boston, Mass.). The reaction was started by adding 1.4 μg of *B. japonicum* RNAP or 1 U of *E. coli* RNAP (Boehringer, Mannheim, Germany) and incubated for 20 min at 37°C. As shown previously (26), *B. japonicum* RNAP has optimal activity between 35 and 40°C. The RNAP is limiting in this assay. The reaction was terminated by placing the reaction tubes on ice. The RNA was purified by extraction with phenol-methylene chloride (1:1) and subsequent ethanol precipitation. The RNA was dissolved in 6 μl of loading dye (38% formamide, 8 mM EDTA [pH 8.0], 0.02% bromophenol blue, and 0.02% xylene cyanol FF), heated to 85°C for 2 min, and loaded onto a 6% polyacrylamide–7 M urea gel. The transcripts obtained were analyzed by using a PhosphorImager SF (Molecular Dynamics, Sunnyvale, Calif.).

General in vitro transcription assay. The assay conditions were essentially identical to those described above for the specific in vitro transcription assay, except that [5,6-^{3}H]UTP (DuPont NEN) was used as the label. The reaction was terminated by spotting the solution on DE81 DEAE filters (Whatman Scientific Limited, Maidstone, United Kingdom). Unincorporated nucleotides were removed by washing the filters with 0.5 M sodium phosphate buffer (pH 6.5), water, and ethanol. Radioactivity was determined by liquid scintillation counting.

RNA size markers. RNA fragments of suitable sizes were produced with T7 RNA polymerase as previously described (18). The template, pRJ9601, was cut with *Eco*RI and *Sma*I to yield runoff transcripts of 231 and 118 nucleotides, respectively.

Primer extension experiments. Plasmids pRJ9503 and pRJ8516 were used for the determination of the in vitro transcription start sites of the *rrn* and *groESL*$_4$ genes. RNA subjected to primer extension was synthesized as described above for the specific transcription assay with the exception that no radiolabeled nucleoside triphosphate was used. Plasmid pRJ9503 consists of the vector pBluescript SK+ (Stratagene, La Jolla, Calif.) containing a 420-bp *Sac*I-*Hin*dIII fragment with the *rrn* promoter. Plasmid pRJ8516 is a pUC18 derivative that contains the *groESL*$_4$ genes on a 6.9-kb *Eco*RI-*Bam*HI fragment (3). The primer extension experiments were done with the oligonucleotides 16S6, 16S8, and ES4UP (2, 17), which had been used for the determination of the in vivo transcription start site. They were labeled with [γ-^{32}P]ATP by using T4 polynucleotide kinase. Free nucleotides were removed by gel filtration. RNA obtained by in vitro transcription was dissolved in 3 μl of hybridization buffer (100 mM Tris-HCl [pH 8.3], 20 mM $MgCl_2$, 160 mM KCl). Two microliters of the labeled oligonucleotides (approximately 10^5 cpm) was annealed to the RNA by incubation at 85°C for 3 min and subsequent rapid cooling at −20°C. For the elongation reaction, 5 μl of a 2 mM deoxynucleoside triphosphate solution, 0.5 μl of 100 mM DTT, 0.5 μl of RNasin (10 U), and 10 U of SuperScript RNaseH$^-$ reverse transcriptase (GIBCO BRL, Gaithersburg, Md.) were added. The reaction was incubated at 42°C for 30 min and terminated with 10 μl of stop solution (95% formamide, 20 mM EDTA [pH 8.0], 0.05% bromophenol blue, and 0.05% xylene cyanol FF). The primer extension products were separated on a 6% polyacrylamide–7 M urea gel. The gel run was analyzed by using a PhosphorImager SF.

Generation of hybridization probes. Amplification and labeling of DNA fragments with digoxigenin-11-dUTP (Boehringer) by using *Taq* DNA polymerase (Boehringer) were done according to the manufacturer's instructions. Oligonucleotides sig1 (5′-CGGGGTACCAGGAGGGIAACATCGGCCTGATG-3′) and sig2 (5′-GATCGAGCTCTAGATGGCCTGGCGGATCCACCA-3′) were designed according to the amino acid sequence in the highly conserved region 2 of the primary sigma factors. At the 5′ end, recognition sites for restriction enzymes were introduced to facilitate subsequent cloning of the amplified fragment.

Site-specific mutagenesis. Site-specific mutagenesis was performed by the method of Merino et al. (22). A 340-bp *Sac*I-*Sma*I fragment containing the promoter region of the *rrn* operon and a 220-bp *Stu*I-*Xho*I fragment containing the terminator of the *rrn* operon were cloned into vector pBluescript SK+, yielding plasmid pRJ9601. The 560-bp *Sac*I-*Xho*I insert fragment of plasmid pRJ9601 was cloned into M13mp18, and single-stranded DNA was isolated. For mutagenesis, the oligonucleotides listed in Fig. 1 were designed. The oligonucleotides and reverse M13 sequencing primer were used to amplify a fragment containing the mutated promoter. The reaction mixture (100-μl final volume) consisted of 25 pmol of each oligonucleotide, 400 ng of single-stranded DNA, 4 μl of 5 mM deoxynucleoside triphosphate, 10 μl of 10× reaction buffer, and 2 U of $Vent_R$ DNA polymerase (New England Biolabs, Beverly, Mass.). Twenty-five cycles were performed under the following conditions: 1 min at 94°C, 30 s at 55°C, and 10 s at 72°C. The mutated fragment was isolated from an agarose gel and used as a primer in a second PCR. In this reaction, the universal M13 sequencing primer was used as the second primer. The 25 cycles were performed under the following conditions: 1 min at 94°C, 30 s at 60°C, and 30 s at 72°C. Amplified fragments containing the mutated promoter and terminator regions were cloned into pBluescript SK+, resulting in plasmids pRJ9621 to pRJ9625 and pRJ9628. Plasmid pRJ9627 consists of a pBluescript SK+ vector containing unmodified promoter and terminator regions. All final constructs were confirmed by sequencing.

DNA manipulations and sequencing. For routine work with recombinant DNA, established protocols were used (29). DNA sequencing was done with a DNA sequencer (model 373A; Applied Biosystems, Foster City, Calif.). The UWGCG (Genetics Computer Group of the University of Wisconsin, Madison) software package was used for DNA sequence analysis.

N-terminal protein sequencing. N-terminal sequencing of *B. japonicum* RNAP σ^{80} was done as described previously (31).

Nucleotide sequence accession number. The *sigA* nucleotide sequence has been assigned GenBank accession number X99588.

Oligonucleotide	Mutation
5′-pGGTTGGGGTTATAG**CA**CCCACCTTCCGGGC-3′	mut2
5′-pGTTATAGGCCCCACCCCGGGCTGTCAACGC-3′	mut3
AA Δ 2 nt	
5′-pTATAGGCCCCACC**GG**TTCCGGGCTGTCAAC-3′	mut4
5′-pTCAGTGGTTGGGGT**GG**TAGGCCCCACCTTC-3′	mut5
5′-pCACCTTCCGGGCTG**GG**AACGCCAATCGTCA-3′	mut6
5′-pTCGTCGCACAGTGGAT**CC**TTGAAAAAAATC-3′	mut7

FIG. 1. Oligonucleotides used for site-directed mutagenesis of the *rrn* promoter region. Nucleotides that differ from those in the wild-type sequence are printed in bold. The positions where two adenines were deleted (Δ 2 nt) in the oligonucleotide, leading to mut3, are shown below its DNA sequence.

RESULTS

Determination of the transcription start sites of the *rrn* and *groESL*$_4$ genes used in vitro. In a previous study, the 5′ end of the *B. japonicum rrn* transcript generated in vivo was identified by primer extension experiments (17). In order to confirm that the obtained signal was not the result of processing of the RNA, the in vitro transcription start site was determined. For this purpose, purified RNA polymerase of *B. japonicum* and plasmid pRJ9503, which carries the *rrn* promoter, were used to synthesize RNA. The RNA was subsequently used in primer extension experiments (Fig. 2A); its 5′ end was found to be identical to that reported for RNA isolated from bacterial cells (17). As a second test system, we chose the *groESL*$_4$ operon that encodes chaperonin-like proteins (10). Expression of this operon is already strong under normal growth conditions and is further induced after heat shock (2). Primer extension of RNA synthesized in vitro from the *groESL*$_4$ promoter (Fig. 2B) gave a prominent signal that corresponded to one of the two in vivo transcription start sites (P1 [2]).

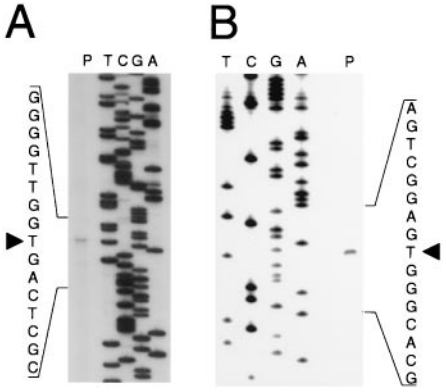

FIG. 2. Primer extension analysis of in vitro-synthesized RNA. The transcription start sites are depicted by arrows. The sequence reactions (T, C, G, and A) were done with the same oligonucleotides. (A) Transcription start site of the *rrn* operon. Lane P, primer extension signal. The signal position is identical to that obtained with RNA isolated from cells (17). (B) Transcription start site of the *groESL*$_4$ operon. Lane P, primer extension signal; it corresponds to the in vivo transcription start site downstream of the P1 promoter (2). The activity of the P2 promoter could not be detected in vitro.

Gene	Nucleotide sequence				
	-40	-30	-20	-10	-1
Ec consensus		TTGACA		TATAAT	
Bj *rrn*	ACTGGCGTTGACAGCCCGGAAGGTGGGGCCTATAAGCCCAACC				
Bj *fbcFH*	AGCGGGCTTGcgcTCGCCCGGCAAGGGTGCcAatAgAACGATT				
Bj *glnA*	TCCGGGCTgGACgCTATCTGAGCCGGGTGCTATccgCATTTT				
Bj *glnB* P1	GAACCGTTTGcCtATTTC.GTAGTCTTCGGTATttTGGGCCGG				
Bj *groESL*$_5$	CCTTTGCTTGACAGGATCGCGGCCAAGCCATATttcGGGCGC				
Bj *groESL*$_2$	CTTTGTCTTGttGCCGA.CCAAGCCGGCTTATttTCGGAGCG				
Bj *groESL*$_4$ P1	GAAGCCCTTGcCcCCGCAAAATTCCTGTCCTATttc.AGCCTC				
Bj *etfSL*	TCGCGCGTTGACgGGGCCGGGTCAGGCCTTAggtTCCGCGCCA(-1)				

FIG. 3. Alignment of *B. japonicum* promoter regions. Several *B. japonicum* (Bj) upstream sequences were aligned with respect to their transcription start points. The −35 and −10 regions are boxed and compared with those of the *E. coli* (Ec) σ^{70} consensus sequence. Nucleotides that differ from those in the *E. coli* consensus sequence are depicted in lowercase letters. Nucleotides matching the extended −10 promoter motif are underlined.

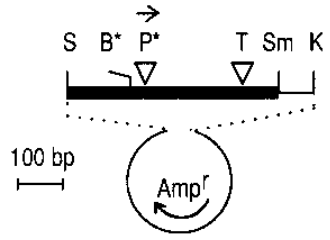

FIG. 4. Plasmid used for in vitro transcription assays. The circle represents the vector pBluescript SK+. The black bar denotes the fragment that contains the mutated promoter derivatives (depicted by P* [see Fig. 5]) and a rho-independent terminator structure (T). This fragment gives rise to a 240-nucleotide transcript. In plasmid pRJ9628, the region between the *Sac*I (S) and the introduced *Bam*HI (B*) recognition sites was deleted. The *Bam*HI site is not present in the other constructs. The horizontal arrow shows the orientation of the promoter region. Other abbreviations: K, *Kpn*I; Sm, *Sma*I.

These results show that the purified *B. japonicum* RNAP is able to initiate transcription at *B. japonicum* promoters in vitro. To obtain more information about the promoter structure, sequences located upstream of previously determined transcription start sites of presumptive *B. japonicum* housekeeping genes were aligned (Fig. 3). The *fbcFH* genes encode the cytochrome bc_1 complex (32). The *glnA* (6) and *glnB* (21) genes are involved in nitrogen assimilation, and *groESL*$_2$, *groESL*$_4$, and *groESL*$_5$ are members of a multigene family encoding chaperonin-like proteins (2, 10). The *etfSL* genes code for subunits of the electron transfer flavoprotein complex (34). All of these genes are supposed to be expressed under standard aerobic growth conditions.

The alignment revealed a substantial degree of similarity between most of the promoters and the consensus of the compiled *E. coli* promoter sequences (19). This is particularly evident in the *rrn* promoter region, which differs only at one position from the *E. coli* −10 consensus sequence. Some promoter regions may contain an extended −10 region, for which a TGN motif located immediately upstream of the −10 box is characteristic. This motif has been identified, e.g., in the *cysG* promoter of *E. coli* (5), and it leads to increased promoter activity. Experiments with *E. coli* promoters demonstrated that the effects of the extended −10 region are additive to the −35/−10 promoter activity (7).

Mutational and functional analysis of the *rrn* promoter. In order to confirm the involvement of the identified conserved regions in promoter activity, we developed a specific in vitro transcription system. A DNA fragment carrying the *rrn* promoter was cloned upstream of the putative terminator structure of the same operon so that a transcript of 240 nucleotides ought to result (Fig. 4). Subsequently, the putative promoter region was mutagenized with oligonucleotides (Fig. 1 and 5). In the mut2 promoter, two bases were changed to yield an extended −10 region. In mut3 and mut4, the spacing between the −10 and −35 regions was decreased and increased, respectively. The −10 region was mutated in mut5, and the −35 region was mutated in mut6. The mut7 construct had an unchanged promoter sequence, but the upstream region starting at −71 was deleted, thereby removing T-rich DNA segments.

For quantitative analyses of promoter strengths, it is important to have available for comparison a reference transcript that is constitutively produced and of an appropriate size. It turned out that the vector itself produced a transcript of about 80 nucleotides (Fig. 6, lane 3). The promoter activities of the mutated fragments were compared with that of this reference transcript. The activity value of the wild-type 240-nucleotide transcript divided by the activity value of the reference transcript (the analysis was done with a PhosphorImager) was arbitrarily set at 100% activity (Table 1).

Introduction of the TGN motif in front of the −10 box increased the promoter activity sevenfold (Fig. 6, lane 5, and Table 1). Changes in the spacing between the −10 and −35 regions resulted in a significant decrease in promoter activity (Fig. 6, lanes 6 and 7, and Table 1). Similarly, promoter activities dropped drastically when the −10 (Fig. 6, lane 8) and −35 (Fig. 6, lane 9) regions were mutated. In contrast, the deletion of the upstream region beyond position −71 had no significant effect on promoter activity (Fig. 6, lane 10). As a control, wild-type and mutated promoter activities were also tested with *E. coli* RNAP. The results demonstrated that *E. coli* RNAP was able to recognize a *B. japonicum* housekeeping promoter and that the relative promoter activities on the mutated promoter templates were essentially the same (Table 1). Remarkable was the even more increased promoter activity on the fragment with the TGN-extended −10 box with *E. coli* RNAP compared with the data obtained with *B. japonicum* RNAP (Table 1).

Taken together, these results indicate that the basic elements required for promoter recognition by the primary sigma factor of *B. japonicum*, i.e., the −35 and −10 regions, are very similar to those described for σ^{70} of *E. coli* (19).

Cloning and sequencing of *sigA*. The significant similarity between the −35 and −10 regions of *B. japonicum* and *E. coli* suggests that their primary sigma factors are also closely related. The amino acid sequence similarity between members of the σ^{70} family is especially pronounced in so-called region 2 (20). Two oligonucleotides, sig1 and sig2, that fitted to this region were designed by taking into account the codon usage of *B. japonicum* genes (25). The oligonucleotides were used to amplify a fragment from *B. japonicum* total DNA. This fragment was labelled with digoxigenin-11-dUTP (Boehringer) and probed against a *B. japonicum* genomic DNA library of cosmid clones. DNA fragments from hybridizing cosmid 12D7 were subcloned into M13 vectors and sequenced. The nucleotide sequence of a 2.8-kb region revealed one open reading frame (Fig. 7). Because of the similarity of the deduced protein product to members of the σ^{70} family, the gene was named *sigA*. The start codon of *sigA* was preceded by a purine-rich Shine-Dalgarno-like sequence. Downstream of the stop codon a stem-loop structure that could serve as a transcription terminator was identified. The upstream and downstream regions showed no similarity to other known sequences.

The SigA protein. The deduced *sigA* gene product consists of 719 amino acid residues with a predicted molecular weight of 80,652. As shown in Fig. 8, the protein is highly homologous to

Promoter configuration	Plasmid	Promoter sequence
wild type	pRJ9627	-150 -140 -130 -118 TTTTTTTGAGATTTTTTGCGGCGCGGCCGCGGTCGGCGC -110 -100 -90 -79 GCTCATGCCCTCTCTAATCGACGGGAATCACTGGATTTT -70 -60 -50 -40 TTTCAACAATCCACTGTGCGACGATTTGTTGGACGATTG -30 -20 -10 -1 GCGTTGACAGCCCGGAAGGTGGGGCCTATAACCCCAACC
mut2	pRJ9621	GCGTTGACAGCCCGGAAGGTGGG**TG**CTATAACCCCAACC
mut3	pRJ9622	GCGTTGACAGCC.GG..GGTGGGGCCTATAACCCCAACC
mut4	pRJ9623	GTTGACAGCCCGGAA**CC**GGTGGGGCCTATAACCCCAACC
mut5	pRJ9624	GCGTTGACAGCCCGGAAGGTGGGGCC**TACCA**CCCCAACC
mut6	pRJ9625	GCGTT**CCC**AGCCCGGAAGGTGGGGCCTATAACCCCAACC
mut7	pRJ9628	GCGTTGACAGCCCGGAAGGTGGGGCCTATAACCCCAACC

FIG. 5. Derivatives of the *rrn* promoter. Sequences of wild-type and mutated *rrn* promoter regions are shown. The −10 and −35 boxes are emphasized in negative print. Mutated nucleotides are indicated in bold; deleted nucleotides are indicated by dots. All promoters contained the same upstream region, except for mut7, which had a deletion starting at position −71; the bases that were mutated to create a *Bam*HI recognition site at −71, which was used for constructing this deletion, are boxed in the wild-type sequence. T-rich sequences are underlined. The mutated fragment of pRJ9622 had suffered a 3-bp deletion, although the oligonucleotide was used with the intention of creating a deletion of two bases.

the SigA protein of *R. meliloti* (72% identity) but less so to σ^{70} of *E. coli* (51% identity). The C-terminal end of the protein known to be involved in promoter recognition and binding of the core enzyme is well conserved (20). Remarkable is an alanine- and proline-rich region (amino acids 243 to 266 [Fig. 8]). This region was not found in other sigma factors described so far.

Purified *B. japonicum* RNA polymerase preparations contain a prominent protein with an apparent molecular mass of 96 kDa that has been suggested to represent the primary sigma factor (26, 33). We purified this protein from an SDS-PAGE gel and determined the N-terminal amino acid sequence. The obtained sequence (XXKAKTLQA, where X is an unidentifiable amino acid) corresponded to the deduced *sigA* gene product (Fig. 9). Because the SigA protein belongs to the σ^{70} family and it is present as a major subunit in a pure RNA polymerase preparation, we believe that the *sigA* gene encodes the primary sigma factor of *B. japonicum* RNAP and that it is responsible for the recognition of promoters of the −35/−10 class.

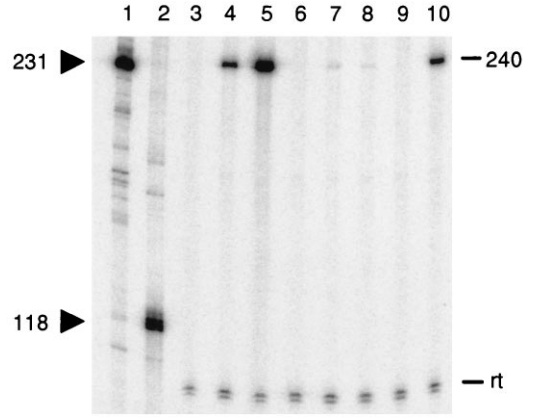

FIG. 6. In vitro transcription of *rrn* promoter derivatives. Lanes 1 and 2 contained RNA size markers. Numbers indicate transcript lengths (in nucleotides). The 240-nucleotide transcripts arise from the tested promoters. The vector-encoded transcript (the reference transcript [rt]) was used as an internal reference for measuring promoter strength. The relative promoter activities are given in Table 1. Lane 3, pBluescript SK+ (vector alone); lane 4, pRJ9627 (wild-type promoter); lane 5, pRJ9621 (extended −10 region); lane 6, pRJ9622 (reduced spacer region); lane 7, pRJ9623 (enlarged spacer region); lane 8, pRJ9624 (mutated −10 box); lane 9, pRJ9625 (mutated −35 box); lane 10, pRJ9628 (deleted T-rich upstream region). All results were confirmed by at least one repetition.

DISCUSSION

The *rrn* promoter and purified RNA polymerase of *B. japonicum* were used to set up a homologous in vitro transcription system. The promoter activity depended on an intact −10 region, an intact −35 region, and correct spacing between the two regions (17 nucleotides). We cannot rule out the possibility that the deletion of several bases in the spacer region also removed a base essential for efficient promoter activity. However, this seems very unlikely because there is no detectable conservation in this region, as judged by alignment of promoter sequences. The nucleotide sequences of the −35 and −10 regions were found to be almost identical to the consensus sequence of the compiled *E. coli* promoters (19). Due to the limited number of known promoter regions, the propagation of a consensus sequence for *B. japonicum* housekeeping genes may be somewhat premature. However, based on mutant phenotype and sequence alignment, the TTG motif in the −35

TABLE 1. In vitro activities of *rrn* promoter derivatives

Plasmid	Promoter configuration	Activity (%) relative to the wild-type promoter with:	
		B. japonicum RNAP[a]	*E. coli* RNAP
pRJ9627	Wild type	100	100
pRJ9621	mut2 (extended −10)	740	1,061
pRJ9622	mut3 (shortened spacer)	2.1	2.3
pRJ9623	mut4 (enlarged spacer)	3.3	0.8
pRJ9624	mut5 (mutated −10)	2.7	1.7
pRJ9625	mut6 (mutated −35)	1.0	0.5
pRJ9628	mut7 (upstream deletion)	114	103

[a] Data are means of two independent experiments.

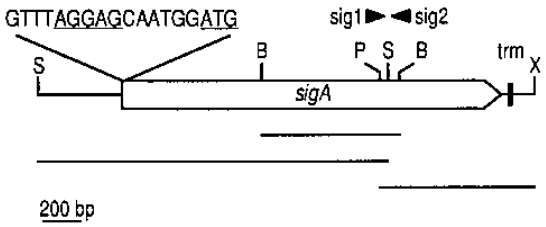

FIG. 7. The *sigA* gene region. The Shine-Dalgarno-like sequence and translation start codon are underlined. Arrowheads denote the annealing sites and orientations of oligonucleotides used for the amplification of a *sigA* fragment for hybridization purposes. Lines show fragments that were cloned and sequenced. The location of a stem-loop structure indicative of a rho-independent terminator (trm) is indicated by a black bar. Abbreviations: B, *Bam*HI; P, *Pst*I; S, *Sal*I; X, *Xho*I.

region and the TAT sequence in the −10 region seem to be crucial for promoter activity. Thus, we propose that the *rrn* promoter of *B. japonicum* belongs to the well-known −35/−10 class and that it is likely that the identified elements suffice for binding of the RNA polymerase and for initiation of transcription. A TGN motif preceding the −10 region in *E. coli* has been described as an additional promoter element that enhances promoter activity (5, 7). The mutational introduction of this motif into the *rrn* promoter led to a sevenfold increase in its in vitro activity, and a promoter alignment revealed that such an extended promoter element might be used in *B. japonicum* as well.

The observed transcript length of 240 nucleotides generated from plasmid templates pRJ9621 to pRJ9625, pRJ9627, and pRJ9628 corresponds to the expected size. This confirms that the integrated terminator structure is functional in vitro and, therefore, probably in vivo. Hence, there appears to be no significant difference in the use of promoter and terminator structures between *B. japonicum* and *E. coli*. In fact, the *B. japonicum rrn* promoter and terminator are recognized by the *E. coli* RNAP as well as by the *B. japonicum* RNAP. The origin of the vector-encoded transcript used as the reference transcript is unknown. With a size of about 80 nucleotides, it is much smaller than the RNA I transcript (108 nucleotides), which is involved in the regulation of plasmid replication at the ColE1 origin (23).

In a previous report, a different base composition of possible housekeeping promoters was suggested (4). This was mainly based on an alignment of the upstream sequences of the *glnA*, *fbcF*, and *fixR* genes. However, *fixR*, whose function is unknown, is aerobically expressed only in the presence of a so-far-unidentified activator (4, 30). Therefore, that gene may not be preceded by a normal housekeeping promoter sequence. The *glnA* gene, which encodes glutamine synthetase I, is expressed under all growth conditions, and its promoter is probably recognized by the primary sigma factor (6). Indeed, a slightly different alignment of this DNA region upstream of the transcription start site results in a reasonably good similarity to the promoter composition suggested here. With regard to a third example, the *fbcF* promoter, it is not known if RNAP is sufficient for expression of the gene; so far, we have been unable to confirm its in vivo transcription start site (32) by in vitro experiments.

The presence of an A- and T-rich upstream promoter (UP) element between −40 and −60 of the *E. coli rrnB* P1 region was shown to increase the promoter activity considerably (27). Such a region was not found at an equivalent position in the *B. japonicum rrn* promoter region. However, there is a T-rich element upstream of position −71. Deletion of this region had no effect on promoter activity in vitro. Nevertheless, this region

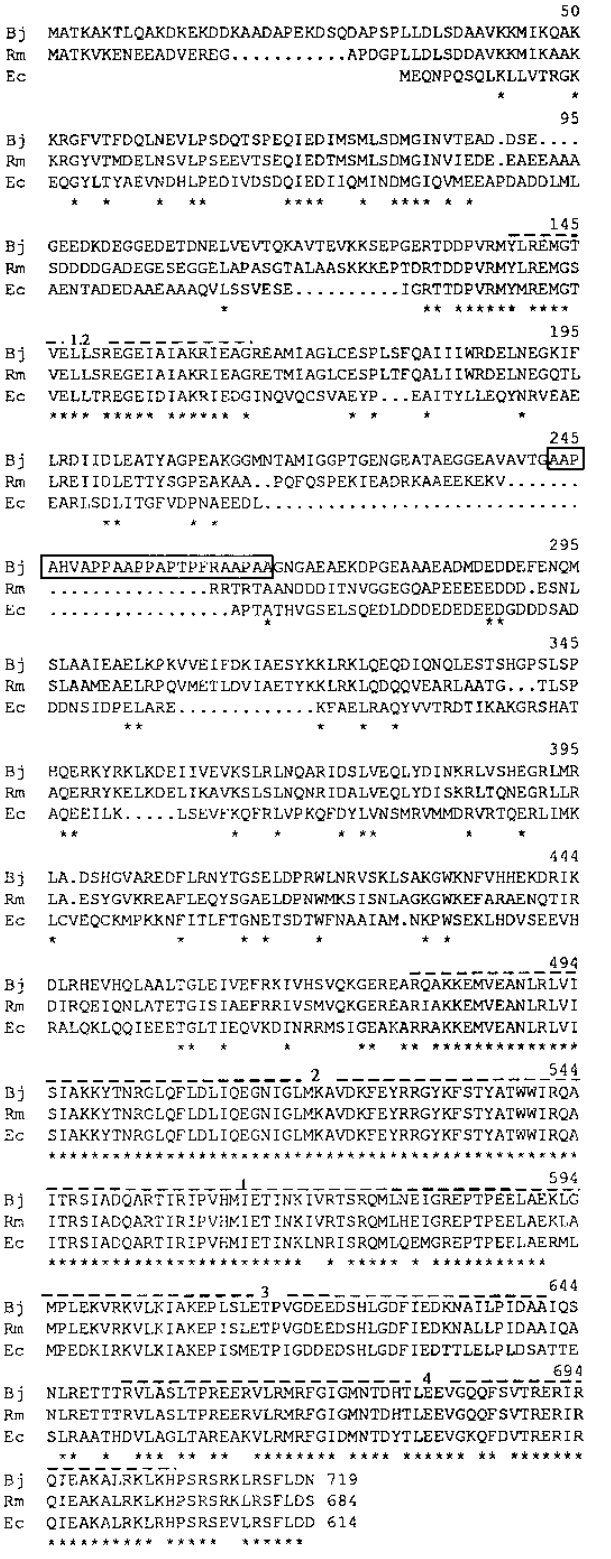

FIG. 8. Amino acid sequence alignment of the primary sigma factors. *B. japonicum* SigA (Bj), *R. meliloti* SigA (Rm; GenBank accession number L47288), and *E. coli* RpoD (Ec; GenBank accession number J01687). The positions of identical amino acid residues are marked by asterisks. Gaps, indicated by dots, were introduced to allow for an optimal alignment. Conserved regions in members of the σ^{70} family are overscored by dashed lines (adapted from reference 20). The alanine- and proline-rich region in the *B. japonicum* sigma factor is boxed (positions 243 to 266).

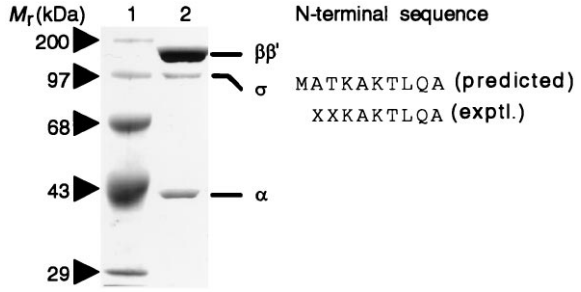

FIG. 9. SDS-PAGE analysis of *B. japonicum* RNAP. Proteins were separated on a 10% polyacrylamide gel and visualized by Coomassie blue staining. Lane 1, molecular mass markers (sizes are shown next to arrowheads on the left); lane 2, *B. japonicum* RNAP (7.5 μg). Subunits are indicated on the right. Predicted and experimentally (exptl.) ascertained N-terminal amino acid sequences of σ^{80} are shown.

might still be important for the regulation of the *rrn* operon in vivo. In *E. coli*, it was shown that AT-rich regions influence promoter strength by bending the DNA (24).

The N-terminal amino acid sequence of a prominent protein in an RNAP preparation revealed that it corresponded to the deduced SigA protein. Therefore, we are sure that the SigA protein with a predicted molecular mass of 80 kDa represents the primary sigma factor of *B. japonicum*. This is also supported by its high degree of sequence similarity to members of the σ^{70} family. The high conservation within the C-terminal end of the proteins from *B. japonicum*, *E. coli*, and *R. meliloti* is a good indication that the sigma factors of the two rhizobial strains recognize in principle the same −35/−10 promoter class as does σ^{70} of *E. coli*. This is in perfect agreement with the in vitro transcription data presented here.

The SigA sequence similarity is less pronounced in the N-terminal region. The function of this region is not fully understood. One function seems to be to prevent the intact sigma factor from binding to DNA (8). An *E. coli* σ^{70} derivative lacking amino acids 130 to 374 showed a functional defect at 42°C, indicating that this region might be critical for thermostability (16). A notable feature in the *B. japonicum* SigA protein is a stretch of 24 amino acids rich in alanine and proline (starting at amino acid position 243). This AP-rich segment is not present in other sigma factors described so far. It may increase the flexibility between region 1 and the rest of the protein. Similar segments have been characterized, for example, in dihydrolipoyl-acetyltransferase, a component of the pyruvate dehydrogenase multienzyme complex of *E. coli* (15). It has been suggested that these peptides form flexible linkers between different domains of the protein.

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