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Rhizobium japonicum Nitrogenase Fe Protein Gene (*nifH*)

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A 12.1-kilobase *Pst*I fragment from *Rhizobium japonicum*, which contains homology to both the *Klebsiella pneumoniae* and the *Rhizobium meliloti nifH* genes, was cloned into vector pHE3. The *nifH*-homologous region was localized on the restriction enzyme cleavage map by Southern blot hybridization experiments. DNA fragments overlapping the *R. japonicum nifH* gene were subcloned into plasmid vectors to allow the expression of this region in *Escherichia coli* minicells. The *nifH* gene product (the polypeptide of the nitrogenase Fe protein) was thus found to have a molecular weight of 33,000. The complete nucleotide sequence of the *nifH* gene was established, and the amino acid sequence of its gene product was deduced. The reading frame is 882 nucleotides long, corresponding to 294 amino acids which add up to a polypeptide with a molecular weight of 31,525. There was extensive sequence homology with *nifH* genes or gene products from other nitrogen-fixing bacteria. The transcription initiation site of the *R. japonicum nifH* gene was found to lie 153 nucleotides upstream from the coding region and was preceded by a characteristic promoter sequence. A potential terminator region was located 13 nucleotides downstream from the coding region.

In all nitrogen-fixing bacteria so far investigated, the nitrogenase enzyme complex consists of two components (16). Component 1 (also called nitrogenase MoFe protein or the actual dinitrogenase) is an α_2-β_2 tetramer in which the two nonidentical α and β subunits have similar molecular weights of 55,000 to 60,000. Component 2 (also called nitrogenase Fe protein or dinitrogenase reductase) is a dimer of two identical subunits, each having a molecular weight of ca. 35,000.

In *Klebsiella pneumoniae*, the three constituent polypeptides are encoded by genes *nifD* and *nifK* (for nitrogenase MoFe protein) and *nifH* (for nitrogenase Fe protein) (3, 11). These genes, together with a fourth gene *nifY* (17) of unknown function, are organized in one transcriptional unit, *nifHDKY* (3, 11, 17). A similar arrangement (*nifHDK*) was found in two fast-growing rhizobia, *Rhizobium meliloti* (23) and *Rhizobium leguminosarum* (25). In the slow-growing *Rhizobium japonicum*, we have recently found that *nifD* and *nifK* form one operon, and that *nifH* must be located elsewhere on the genome (5, 10). A similar observation was made with another member of the slow-growing rhizobia, *Rhizobium* sp. Parasponia: a *nifH* region was cloned and was found not to be linked to *nifD* (27). Yet a different arrangement was detected in the cyanobacterium *Anabaena* sp. 7120, in which *nifHD* is separated from *nifK* (19).

The separation of the *nifH* and *nifDK* genes in *R. japonicum* raised the interesting question of whether the expression of these genes is coordinately regulated and whether the respective operons share common DNA control regions. A necessary prerequisite for such an analysis is the molecular cloning of these genes and their control regions. We had previously reported on the cloning of an *R. japonicum* DNA fragment containing *nifD* and *nifK* (5, 8, 10). This report now describes the isolation of the *R. japonicum nifH* gene, the expression of its gene product, and the complete nucleotide sequence of the structural and regulatory regions.

MATERIALS AND METHODS

Bacterial strains and plasmids. The cloning vector pHE3 and the methods for the direct selection of recombinant DNA in *Escherichia coli* RR28 have been described recently (9). Other *E. coli* strains used were HB101 for transformations, 294 *cys*::Tn*5* for transposon mutagenesis, and DS410 for minicell production; these and the plasmids pBR322, pACYC184, and pACYC177 have been described in a previous paper (5). Hybrid *nif* plasmids containing the *K. pneumoniae* and *R. meliloti nifH* genes were pSA31 (11) and pRmR2 (22), respectively. The source for *R. japonicum* DNA was strain 110 *spc-4* (18). Growth conditions for *E. coli* and *Rhizobium* strains were as described previously (5, 18).

Plasmid constructions. Recombinant DNA work such as DNA isolations, restriction endonuclease digests, ligations, and transformations were performed by using established techniques (4). Transposon (Tn*5*)-induced mutant plasmids were obtained as described previously (5). Plasmids constructed in this work are shown in Fig. 1.

Molecular biology techniques. A number of techniques were used in this work which are now well established: nick translation (12), nitrocellulose filter hybridizations (4), Southern blotting (30), and colony hybridization (6). Interspecies hybridizations were done at 65°C in the following buffers. (i) For Southern blot hybridizations, 6× SSC (1× SSC, 0.15 M NaCl plus 0.015 M sodium citrate, pH 7.2), 1× Denhardt solution (0.2 mg each of bovine serum albumin, polyvinylpyrrolidone, and Ficoll per ml), 5 g of sodium dodecyl sulfate (SDS) per liter, and 1 mM EDTA were used. (ii) For colony hybridizations, 6× SSC–3× Denhardt solution was used. DNA sequencing was done by the method of Maxam and Gilbert (13) or by the method of Sanger et al. (24), with phage M13 derivatives (14) for cloning. Nuclease S1 mapping was done by the method of Berk and Sharp (1), with *R. japonicum* bacteroid mRNA prepared by previously described methods (32). Methods for the isolation and radioactive labeling of *E. coli* minicells and the analysis of plasmid-encoded polypeptides by SDS-polyacrylamide gel electrophoresis have been previously described in detail (5).

RESULTS

Cloning of the *nifH* gene. Southern blot hybridization experiments with total DNA of *R. japonicum* revealed that a *Pst*I fragment of ca. 12 to 14 kilobase pairs (kb) specifically hybridizes to radioactively labeled *nifH* DNA from *K.*

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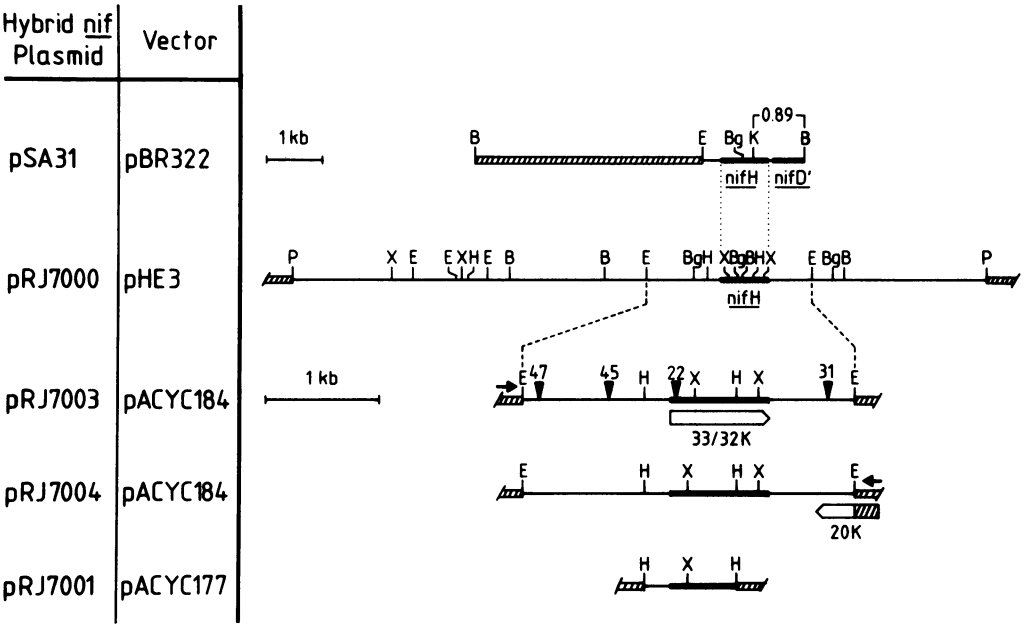

FIG. 1. The cloned *nif* DNA fragments used in this work. pSA31 contains *K. pneumoniae nifH* and part of *nifD* (*nifD'*). The other plasmids contain *R. japonicum nifH* or fragments thereof. pSA31 and pRJ7000 are drawn to scale, whereas the other plasmids are enlarged by a factor of two. Restriction sites are: *Bam*HI (B), *Bgl*II (Bg), *Eco*RI (E), *Hin*dIII (H), *Kpn*I (K), *Pst*I (P), *Xho*I (X). Cloned fragments are drawn in thin line with *nif* genes emphasized in bold line. Vectors are shown as hatched bars; except for pSA31, the vectors are not drawn to completion. The bars underneath the DNAs denote polypeptides expressed in minicells and the direction of expression. Gene products encoded by cloned DNA are drawn as open regions, and the peptide specified by a fraction of the antibiotic resistance gene of the vector is drawn as hatched regions. Expression starts from promoters outside the cloned region (indicated by small right- or leftward-oriented arrows). The positions of four different Tn*5* insertions in pRJ7003 are marked by numbered vertical arrowheads. K, Kilodaltons.

pneumoniae and *R. meliloti* (10). To clone this fragment, size-fractionated *R. japonicum* DNA (*Pst*I fragments between 10 and 16 kb) was ligated with *Pst*I-digested pHE3, and the recombinant DNA was transformed into *E. coli* RR28 (9). As this cloning system enables the direct selection of recombinant plasmids, a total of 730 transformants were picked and screened for the presence of the *R. japonicum nifH* gene by interspecies colony hybridization. One colony contained the desired recombinant plasmid (named pRJ7000) with a 12.1-kb insert that clearly hybridized to both the ^{32}P-labeled *nifH*-containing *R. meliloti Hin*dIII fragment of pRmR2 (22) and the ^{32}P-labeled *nifH*-containing *K. pneumoniae Eco*RI-*Kpn*I fragment of pSA31 (11, 26; cf. Fig. 1). A restriction enzyme cleavage map of the 12.1-kb *R. japonicum* fragment of pRJ7000 is shown in Fig. 1. The region of *nifH* homology is indicated by dotted lines, and the results described in the following sections confirm this interpretation.

Interspecies hybridization. Most of the *nifH* homology regions were found to be confined to a 0.82-kb *Hin*dIII fragment (data not shown), which was then subcloned into pACYC177 to give pRJ7001 (Fig. 1). To confirm the *nifH*-specific interspecies homology, this *Hin*dIII fragment itself was used as radioactive DNA to probe restriction digests of pSA31 (Fig. 2). and pRmR2 (not shown). The results of Fig. 2 show that the fragments of pSA31 which hybridize are all those which contain parts of the *K. pneumoniae nifH* gene, except for the 0.89-kb *Bam*HI-*Kpn*I fragment containing the terminal part of *nifH* (Fig. 2, lane c; cf. Fig. 1).

Expression in *E. coli* minicells. The polypeptides encoded by the *R. japonicum nifH* region were analyzed in minicells of *E. coli*. For this purpose, a *nifH*-containing *Eco*RI fragment was cloned into the chloramphenicol resistance gene of

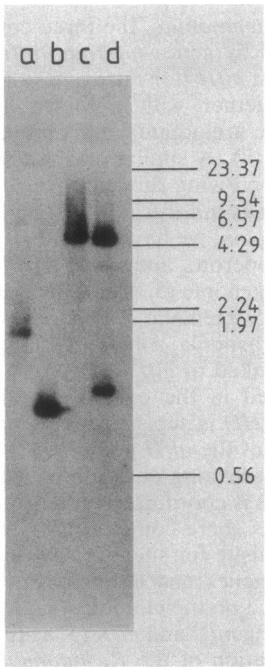

FIG. 2. Autoradiogram of a nitrocellulose filter showing the *nifH*-specific interspecies hybridization. Restriction digests of pSA31 were done with *Eco*RI and *Bam*HI (lane a), *Eco*RI and *Kpn*I (lane b), *Bam*HI and *Kpn*I (lane c), and *Bam*HI and *Bgl*II (lane d). Restriction fragments were separated on an agarose gel and blotted onto a nitrocellulose filter. The filter was then hybridized with the ^{32}P-labeled *Hin*dIII insert of pRJ7001. Molecular weight standards of *Hin*dIII-digested λ DNA (in kb) are given on the right margin.

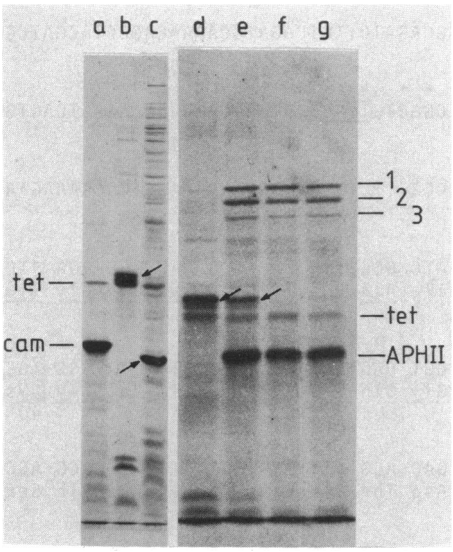

FIG. 3. Autoradiogram of the SDS gel electrophoresis analysis of ^{35}S-labeled extracts from minicells: plasmid-directed synthesis of the *R. japonicum nifH* gene product. On the left gel, polypeptides are shown encoded by (a) vector pACYC184, (b) recombinant plasmids pRJ7003 (arrow indicates 33,000-32,000-molecular-weight doublet), and (c) pRJ7004 (arrow indicates 20,000-molecular-weight polypeptide). The gel on the right shows polypeptides encoded by (d) pRJ7003 and the transposon insertion mutants (e) pRJ7003::Tn*5*-31, (f) pRJ7003::Tn*5*-22, and (g) pRJ7003::Tn*5*-45. The left and right margins of the gels designate the following proteins: tetracycline resistance gene product (tet), chloramphenicol acetyltransferase I (cam), Tn*5*-encoded aminoglycoside 3′-phosphotransferase II (APHII), and further Tn*5*-encoded transposase proteins (1 to 3). The arrows designate those proteins (or fusion proteins) encoded by *R. japonicum* DNA. These proteins, together with their molecular weights, are included in Fig. 1.

pACYC184 in both possible orientations to give pRJ7003 and pRJ7004 (cf. Fig. 1). Expression was thus mediated by the strong promoter of the gene for chloramphenicol acetyltransferase. pRJ7003 coded for the synthesis of two proteins with molecular weights of 33,000 and 32,000 (Fig. 3, lanes b and d). In the opposite orientation (pRJ7004), a 20,000 protein was made (Fig. 3, lane c); this suggests the presence of a long open reading frame in that direction (cf. Fig. 1). Tn*5* insertion derivatives of pRJ7003 were constructed and were analyzed to determine which of the mutations exerted a polar effect on the expression of the encoded proteins. The 33- and 32-kilodalton doublet was synthesized by the mutant plasmid pRJ7003::Tn*5*-31 (Fig. 3, lane e), but not by pRJ7003::Tn*5*-22 and pRJ7003::Tn*5*-45 (Fig. 3, lanes f and g, respectively) or pRJ7003::Tn*5*-47 (not shown). This indicates that the 33- and 32-kilodalton proteins were encoded by the DNA region between the Tn*5* insertions 45 and 31.

DNA sequence analysis of the coding region. The complete nucleotide sequence of the *R. japonicum nifH* gene was established using both the chemical method of Maxam and Gilbert (13), and the chain termination method of Sanger et al. (24). Figure 4 gives an outline of the sequencing strategy employed. All restriction endonuclease sites shown in Fig. 4 were confirmed by overlapped sequencing. Both DNA strands of the gene were sequenced (see the legend to Fig. 4). The nucleotide sequence is shown in Fig. 5, together with the predicted amino acid sequence of the *nifH* gene product (the polypeptide of the nitrogenase Fe protein). From the presumptive start codon (ATG) to the stop codon (TAA) there is an open reading frame of 882 nucleotides, corresponding to 294 amino acids. The molecular weight of the predicted polypeptide is 31,525, which agrees fairly well with the molecular weight of the gene product obtained by expressing pRJ7003 in *E. coli* minicells (cf. Fig. 1 and 3). Eight nucleotides upstream from the start codon (Fig. 5) there is a sequence 5′-TGGA-3′, which is a potential ribosome-binding site (so-called Shine-Dalgarno sequence [29]).

The predicted amino acid sequence is highly homologous to the corresponding sequences of the *nifH* gene products from other nitrogen-fixing bacteria. A total of 146 amino acids (which are underlined in Fig. 5) occur at identical positions in all *nifH* gene products sequenced to date. Five cysteine residues, at positions 40, 87, 99, 133, and 185, are believed to be of functional importance in the complexing of FeS clusters. Based on the amino acid sequences we have calculated the similarity coefficients (S_{AB}) for all individual pairs of *nifH* gene products (Table 1). The highest degree of homology was found for two members of the slow-growing rhizobia, *R. japonicum* (this work) and *Rhizobium* sp. Parasponia (27). Only 9 of 294 amino acids are different. On the nucleotide sequence level, however, the difference was more pronounced as 94 of the *nifH* codons are changed in the wobble position. The members of the fast-growing rhizobia,

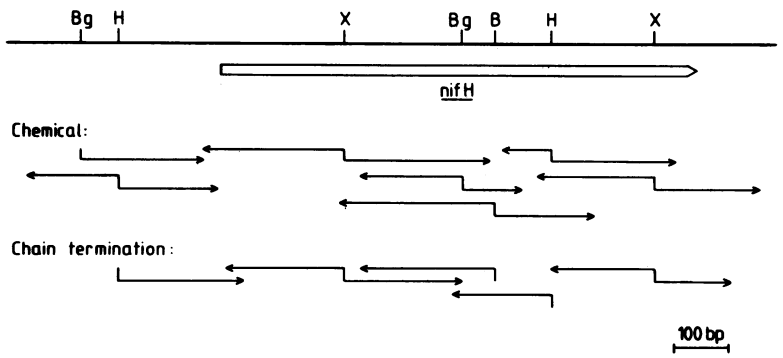

FIG. 4. Sequencing strategy for the *R. japonicum nifH* gene. Sequences established by the chemical method (13) or by the chain termination method (24) are indicated. The direction and extent of sequence determinations are given by arrows. Both DNA strands were sequenced: whenever the strand sequenced by the Maxam-Gilbert and Sanger techniques was the same (i.e., sequencing from 5′ to 3′), we have also sequenced the complementary strand from 3′ to 5′ by the Maxam-Gilbert technique; in such cases, the 3′ end was ^{32}P-labeled by fill-in reactions on sticky ends employing Klenow DNA polymerase. Restriction sites are as in Fig. 1.

FIG. 5. Complete nucleotide sequence of the *R. japonicum nifH* gene and flanking regions. The noncoding DNA strand is shown. The derived amino acid sequence of the nitrogenase Fe protein is shown below the nucleotide sequence. Nucleotide positions upstream from the coding region and those of amino acids are numbered. Underlined amino acids are conserved in all species in which corresponding sequences have been established (Table 1). Conserved cysteine residues are in boxes. Structural features in the nucleotide sequence are marked as follows: *, identical nucleotides found in the *R. japonicum nifDK* promoter; boxed region, consensus *K. pneumoniae nif* promoter sequence; vertical arrowhead, transcription start site; overscored and underlined region, ribosome-binding site; horizontal arrows, inverted repeat structure forming a potential transcriptional terminator.

R. meliloti and *R. trifolii*, also show high *nifH* homology (S_{AB} = 0.91). Interestingly, the similarity between the fast-growing and slow-growing rhizobia was only 0.76 to 0.78 S_{AB} units. A remarkably high similarity was found between *K. pneumoniae* and *Azotobacter vinelandii* (S_{AB} = 0.88). In all cases, the lowest degree of homology existed with *Clostridium pasteurianum*.

Promoter and terminator regions. The transcriptional start point of the *R. japonicum nifH* gene was determined by nuclease S1 mapping (1). RNA was isolated from soybean root nodule bacteroids. In addition, RNA was also prepared from free-living, microaerobic cultures of *R. japonicum* 110 (under these conditions the bacteria derepress nitrogenase activity ex planta). The RNA isolates were hybridized to a *Hin*dIII-*Hin*fI fragment (5′ ^{32}P labeled at the *Hin*fI site) which extended from position −201 of the 5′ *nifH*-flanking region to nucleotide +41 of the coding region (cf. Fig. 5). The hybrid was digested with nuclease S1, and the protected DNA was electrophoresed adjacent to a Maxam-Gilbert sequencing ladder of the same *Hin*dIII-*Hin*fI fragment. One example of such experiments is presented in Fig. 6. Usually, two to three stronger bands appeared on the autoradiogram (Fig. 6, lane 3). The use of higher nuclease S1 concentrations or prolonged incubation in the presence of the nuclease enhanced the signal of the band marked by an arrowhead in lane 3 of Fig. 6, whereas the other bands were more faint (not shown). The intense band matches up with a cytosine of the coding strand (Fig. 6) which corresponds to the guanine of the noncoding strand located 153 nucleotides upstream from the beginning of *nifH* (marked by an arrowhead in Fig. 5). The appearance of several bands in the S1 experiment might be explained by the assumption that the terminal two guanine-cytosine pairs stabilize the end of the RNA-DNA hybrid and partially protect extending nucleotides from S1 digestion.

The transcription start point is immediately preceded by the promoter region (Fig. 5) containing the sequence 5′-TTGG-8 base pairs (bp)-TTGCT-3′, which is homologous to the consensus sequence (5′-CTGG-8 bp-TTGCA-3′) found in *K. pneumoniae nif* promoters (2). Furthermore, the region from positions −193 to −163 contains 22 nucleotides found at identical positions in the promoter region of the *R. japonicum nifDK* operon (K. Kaluza and H. Hennecke, Mol. Gen. Genet., in press).

The region flanking the 3′ end of the *R. japonicum nifH*-coding region was examined for any possible termination signals. A very obvious inverted repeat structure was found 13 nucleotides downstream from the last *nifH* codon (inverted arrows in Fig. 5). mRNA transcribed from this region could potentially form a characteristic stem and loop terminator structure. The potential stem is formed by 11 consecutive bp, 8 of which are guanine-cytosine pairs. The structure would be considerably stable with a free energy or ΔG° (25°C) = −26.2 kcal as estimated by the method of Tinoco et al. (34). On the 3′-flanking side, the inverted repeat structure is followed by a cytosine-rich region.

DISCUSSION

This report confirms and extends the previous observation that the gene (*nifH*) for the nitrogenase Fe protein of slow-growing rhizobia, including *R. japonicum*, is controlled by its own promoter (10, 27) and is thus separated from the *nifDK* operon (5, 10) encoding the nitrogenase MoFe protein subunits. Both the *nifH* promoter region and a potential *nifH* terminator region of *R. japonicum* were identified, which makes it likely that *nifH* forms an operon with just one single gene encoded. The *nifH* promoter sequence differs from the *nifDK* promoter region in only a few bp (K. Kaluza and H. Hennecke, Mol. Gen. Genet., in press). It will be of interest as to whether these differences could account for a possibly different regulation of the *nifH* and *nifDK* promoters. Unlike typical *E. coli* promoters the *R. japonicum nifH* promoter presented here has a different −10 region and no obvious −35 region (21). Rather, it contains a characteristic region between nucleotides 9 and 25 upstream from the transcription start site with typical elements of *K. pneumoniae nif* promoters (2) which were also found in *R. meliloti* (32) and *Rhizobium* sp. Parasponia (27) *nifH* promoters. As some of these elements were supposed to be implied in *nif* regulation (2, 32) it cannot be excluded that *nif* control circuits (or remnants of them) similar to those in *K. pneumoniae* may also operate in the symbiotic *R. japonicum*. The postulated guanine-cytosine-rich terminator region is of the *E. coli* type, except that the stem and loop structure is followed by a cytosine-rich region (Fig. 5) rather than a thymine-rich region (21). The postulated ribosome-binding site is also similar to those of *E. coli* genes (29), which explains why it has been possible to translate *nifH* fusion mRNA into a *nifH*-specific polypeptide(s) in *E. coli* minicells (Fig. 3). These experiments have revealed a protein doublet, with molecular weights of 33,000 and 32,000, encoded by the same DNA region. At present, it is unknown whether this reflects protein processing in *E. coli* minicells or the presence of two overlapping open reading frames. At 12 bp upstream from the *nifH* coding region, and in frame with it, there is a second possible ATG initiator codon which, however, is not preced-

TABLE 1. Similarities between the amino acid sequences of nitrogenase Fe proteins from different nitrogen-fixing bacteria[a]

Bacterium	S_{AB}[b] with:							
	Rj	RP	Rm	Rt	Kp	An	Av	Cp
Rj		0.97	0.77	0.78	0.74	0.70	0.75	0.61
RP			0.76	0.77	0.74	0.70	0.74	0.61
Rm				0.91	0.67	0.69	0.68	0.61
Rt					0.68	0.69	0.69	0.62
Kp						0.70	0.88	0.69
An							0.71	0.60
Av								0.68

[a] The sequences of the *nifH* gene product from the following sources were compared: Rj, *Rhizobium japonicum* (this work); RP, *Rhizobium* sp. Parasponia (27); Rm, *Rhizobium meliloti* (35); Rt, *Rhizobium trifolii* (28); Kp, *Klebsiella pneumoniae* (26, 31); An, *Anabaena* sp. 7120 (15); Av, *A. vinelandii* (7); Cp, *Clostridium pasteurianum* (33).

[b] S_{AB} for two organisms A and B are calculated as follows: S_{AB} = 2 × (number of identical amino acids between A and B)/(number of total amino acids in A) + (number of total amino acids in B).

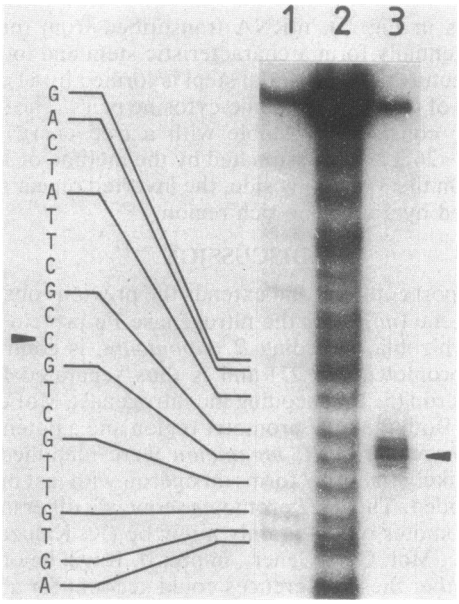

FIG. 6. Determination of the transcription start site by nuclease S1 mapping. The autoradiogram shows, as a reference, the Maxam-Gilbert sequencing ladder of a *Hin*dIII-*Hin*fI fragment (see text). Only the bands after guanine modification (lane 1) and guanine and adenine modification (lane 2) are shown. Lane 3 shows the electrophoresis of the protected DNA. For this experiment, nodule bacteroid RNA was used and the digestion with nuclease S1 (1,000 U) was for 40 min at 37°C. The more-intense band is marked by an arrowhead. On the left margin, the partial nucleotide sequence of the coding strand is shown; it is complementary (from top) to the sequence between −163 and −143 of the noncoding strand (cf. Fig. 5).

ed by a characteristic Shine-Dalgarno sequence (Fig. 5). It is relevant that two similar forms of the *K. pneumoniae* nitrogenase Fe protein were found in vivo (20) and that the expression of the *R. meliloti nifH* gene has also led to the synthesis of two proteins with similar molecular weights (36).

The predicted amino acid sequence of the *R. japonicum* nitrogenase Fe protein is highly conserved, and bears structural features, such as the conserved cysteine residues, which have been discussed in previous publications (7, 15, 31, 33). The evolution of the *nifH* gene is certainly channeled in part by the strict structural requirements of the nitrogenase Fe protein for catalytic functioning. Despite this fact, the amino acid sequences from eight different organisms known to date are divergent enough to classify them into an evolutionary scheme (27) which is not conceptually different from the phylogenetic relationships of the respective organisms harboring the *nifH* gene. One exception seems to be the systematic position of the two slow-growing rhizobia, *R. japonicum* and *Rhizobium* sp. Parasponia. Based on their *nifH* sequences they are almost as far distant from the fast-growing rhizobia as from N_2-fixing members of gram-negative, nonsymbiotic bacteria (Table 1 and reference 27). Hence, the placement of fast-growing and slow-growing rhizobia within the same genus does not seem to be appropriate.

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