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Genetics

Conservation of extended promoter regions of nodulation genes in *Rhizobium*

(symbiosis/gene expression/promoter sequence)

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ABSTRACT **A 47-base-pair (bp) conserved sequence in the 5′-flanking regions of three transcriptional units coding for nodulation functions (*nod*ABC, *nod*EFG, and *nod*H) has been identified in *Rhizobium meliloti* strain 41. The conserved region contains subsequences of 7 bp, 5 bp, and 25 bp. The conserved 25-bp sequence was synthesized and used as a hybridization probe; three additional copies of the sequence were identified in *R. meliloti* 41; all three were localized in the 135-kb *nod/nif* region of the symbiotic megaplasmid. Nucleotide sequence analysis of the six regions revealed that all contained the 47-bp conserved sequence but, with one exception, adjacent DNA regions did not have long conserved DNA stretches. The position of the 47-bp region was about 200–240 bp upstream of the translational start codons of the three *nod* genes. This conserved sequence is present in several other *Rhizobium* species and located adjacent to *nod* genes. We have demonstrated the involvement of this sequence in the expression of nodulation functions, which suggests that these extended promoter regions may have a role in the coordinated regulation of nodulation genes.**

The development of symbiotic nitrogen-fixing associations between *Rhizobium* and leguminous plants is a complex multistage process, which involves the expression of several bacterial and plant genes, some of which are activated only during the symbiotic process (see ref. 1 for review).

In *Rhizobium meliloti*, genes required for nodulation and nitrogen fixation (*nod* and *fix*) are located on a megaplasmid (2–4). A 135-kilobase (kb) segment of this plasmid in *R. meliloti* strain 41 was shown to carry all essential nodulation genes and some of the fixation genes (5). The essential nodulation genes are clustered on two *Eco*RI fragments. An 8.5-kb fragment carries the interspecifically highly conserved common nodulation genes (*nod*ABC, ref. 6) and a newly identified gene, *nod*D (ref. 7; unpublished results).

The second *nod* fragment of 6.8 kb codes for genes necessary for host-specific nodulation (*hsn*, ref. 6). Transposon 5 (Tn*5*) mutagenesis and nucleotide sequence analysis of this region have identified four genes involved in alfalfa nodulation, organized into probably two transcription units (unpublished results).

The number of identified *nod* genes is increasing, and it is apparent that most or probably all of these are turned on specifically during the symbiotic process. It is likely that the control of expression of the nodulation genes is tightly coordinated, and this may be reflected in the conservation of specific regulatory sequences in the promoter regions of the *nod* operons.

In this paper we present evidence identifying a 47-base pair (bp) conserved sequence in the 5′ region of a set of nodulation genes of *R. meliloti* 41. The 47-bp sequence is situated ca. 200–240 bp upstream of the translational start codons and consists of strictly conserved subsequences of 7 bp, 5 bp, and 25 bp. In addition, the interspecific conservation of the 25-bp sequence and its functional involvement in *nod* gene expression are shown.

MATERIALS AND METHODS

Strains and Plasmids. AK631 is a compact colony morphology variant of *R. meliloti* 41; ZB138, ZB157, ZB121, AK1212, AK635, ZB160, and ZB170 are deletion derivatives of AK631 (see Fig. 3 and ref. 2). AK1746, AK1745, AK1711, and BH91 are Tn*5* mutants in the *hsn* region of AK631 isolated in this laboratory (see Fig. 4). AK3216 and AK3217 are insertion mutants of AK631 (see Fig. 4). *R. meliloti* L5-30 and its deletion derivative *R. meliloti* L5-22 were obtained from J. Denarie. Wild-type *Rhizobium* strains *R. trifolii* RS10151, *R. phaseoli* RCR3610, and *R. japonicum* USDA122 were provided by F. Rodriguez-Quinoñes, W. J. Broughton, and H. Evans, respectively. *Rhizobium* sp. NGR234, and *R. leguminosarum* 300 were described earlier (8, 9). *Klebsiella pneumoniae* M5al and *Agrobacterium tumefaciens* Ach5 were from R. Dixon and C. Koncz. The *Escherichia coli* strains used were HB101 (10) and JM101 (11). The plasmid vectors and recombinant plasmids pEK10, pEK5121, pEK3148, and pEK5022 are described (ref. 5 and see Fig. 3). pEK17 and pEK18 are subclones of pEK10 (see Fig. 4). M13 phage vectors M13mp18 and M13mp19 were used for sequencing (12). Media, growth conditions, and genetic techniques were described (5, 11, 13, and 14).

Enzymes and Isotopes. Restriction endonucleases and other enzymes were prepared at this Institute, except T4 polynucleotide kinase and Klenow enzyme that were from Bethesda Research Laboratories. [γ-^{32}P]ATP (1000–3000 Ci/mmol; 1 Ci = 37 GBq) was product of the Isotope Institute (Budapest, Hungary).

DNA Chemistry. DNA purifications, restriction endonuclease digestions, filling-in reactions, and ligations were carried out as described (14). pEK17 and pEK18 contain the 4-kb and 2-kb *Bam*HI fragments of pEK10, respectively, ligated into the *Bgl* II site of pRK290.

Synthesis of the Mixed Oligonucleotide Probe. A mixture of 25-mer oligodeoxyribonucleotides d(AT$^{AA}_{CC}$AAACAATCG-ATTTTACCAATC) was prepared according to the phosphate triester method (15), by using controlled pore glass as solid support. The coupling reactions were performed with an equimolar mixture of activated A and C monomers in the appropriate steps. The mixture of 25-mers was purified by polyacrylamide gel electrophoresis.

Southern Blotting and Hybridizations. DNA restriction fragments were transferred to nitrocellulose filters (Schleicher and Schuell) as described (16). Filters were baked

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Abbreviations: kb, kilobase(s); bp, base pair(s).

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at 80°C for 2 hr and prehybridized for at least 1 hr in 6× SSPE, 1% $NaDodSO_4$ (Sigma), and 5× Denhardt's solution. The hybridization solution was identical, except that it contained in addition 10% (wt/vol) dextran sulfate (Sigma) and 1–10 × 10^7 cpm of ^{32}P-labeled oligonucleotide. Hybridization was for 18 hr at 37°C. The 25-mer oligonucleotide was labeled with T4 polynucleotide kinase to a specific activity of 1–5 × 10^8 cpm/μg. Total DNA filters were washed in 6× SSC, plasmid DNA filters in 3× SSC at 37°C for 1.5–2 hr. [The compositions of standard saline phosphate EDTA (SSPE), standard saline citrate (SSC), and Denhardt's solutions are given in ref. 14].

DNA Sequencing. DNA sequences were determined by the dideoxy chain-termination method (12).

Plant Test. These were carried out as described (17).

RESULTS

Identification of a 25-bp Conserved Sequence in the 5'-Flanking Region of Three Nodulation Genes of *R. meliloti*. The complete nucleotide sequences of the *nod*ABC genes (6), the *nod*D gene (unpublished results), and the *nod*E, *nod*F, *nod*G, and *nod*H genes (unpublished results) have been established. Computer alignment of these sequences revealed the existence of a highly conserved sequence 25 bp in length, in the 5' region of *nod*A, *nod*E, and *nod*H. The position of the 25-bp sequence was typically about 210 bp upstream of the proposed translational start codons (Fig. 1).

***R. meliloti* 41 Genomic DNA Has Three Additional Restriction Fragments Homologous to the 25-mer Oligonucleotide.** Since the probability of the random occurrence of a 25-bp sequence is very low, it was of interest to examine whether the sequences we observed represent either a class of small repetitive sequences in the intergenic regions of the *R. meliloti* genome, or whether they play a role in the organization and/or regulation of some of the symbiotic genes. To ascertain the copy number of this sequence in the *R. meliloti* 41 genome, the mixed oligonucleotide d(AT$^{AA}_{CC}$AAACAATCGATTTTACCAATC) was synthesized and used as a hybridization probe.

Total DNA was isolated from wild-type *R. meliloti* 41 (strain AK631), digested with restriction enzyme *Eco*RI, electrophoresed, and Southern blotted. The filter was hybridized to the 5'-labeled mixed oligonucleotides. As seen in Fig. 2, lane 1, five *Eco*RI fragments, with molecular sizes of 9.4, 8.5, 6.8, 3.5, and 0.7 kb were detected. As the *nod*ABC genes are located on the 8.5-fragment and the *nod*E and *nod*H genes are located on the 6.8-kb fragment, this hybridization pattern indicated that the genomic DNA has three additional regions that have homology to the 25-bp sequence. Hybridizations to several other digests of the genomic DNA confirmed that each *Eco*RI fragment had only one copy of the 25-bp sequence except for the 6.8-kb fragment that had two copies (5' ends of *nod*E and *nod*H; data not shown).

A more detailed analysis of the 6.8-kb fragment confirmed that only the expected sequences of the fragment gave a hybridization signal. It was known from the nucleotide sequence data that two more parts of the 6.8-kb fragment have 47% and 56% homology to the 25-bp sequence (70% homology when calculated to 29 bp). When the four homologous parts were separated in different restriction fragments only two of them, those with the high level of homology, hybridized.

Thus, the additional three *Eco*RI fragments in the genome (i.e., 9.4, 3.5, and 0.7 kb) represent new loci of the 25-bp homology.

By the use of the deletion mutants and overlapping cosmid clones shown in Fig. 3, these fragments could be localized in the well-known *nod/nif* region of the symbiotic plasmid pRme41b of *R. meliloti* (5). All hybridizing fragments were mapped in the region delineated by the right breakpoints of the deletions present in strains ZB170 and ZB121 (data not shown). Hybridizations to *Eco*RI-digested DNA from over-

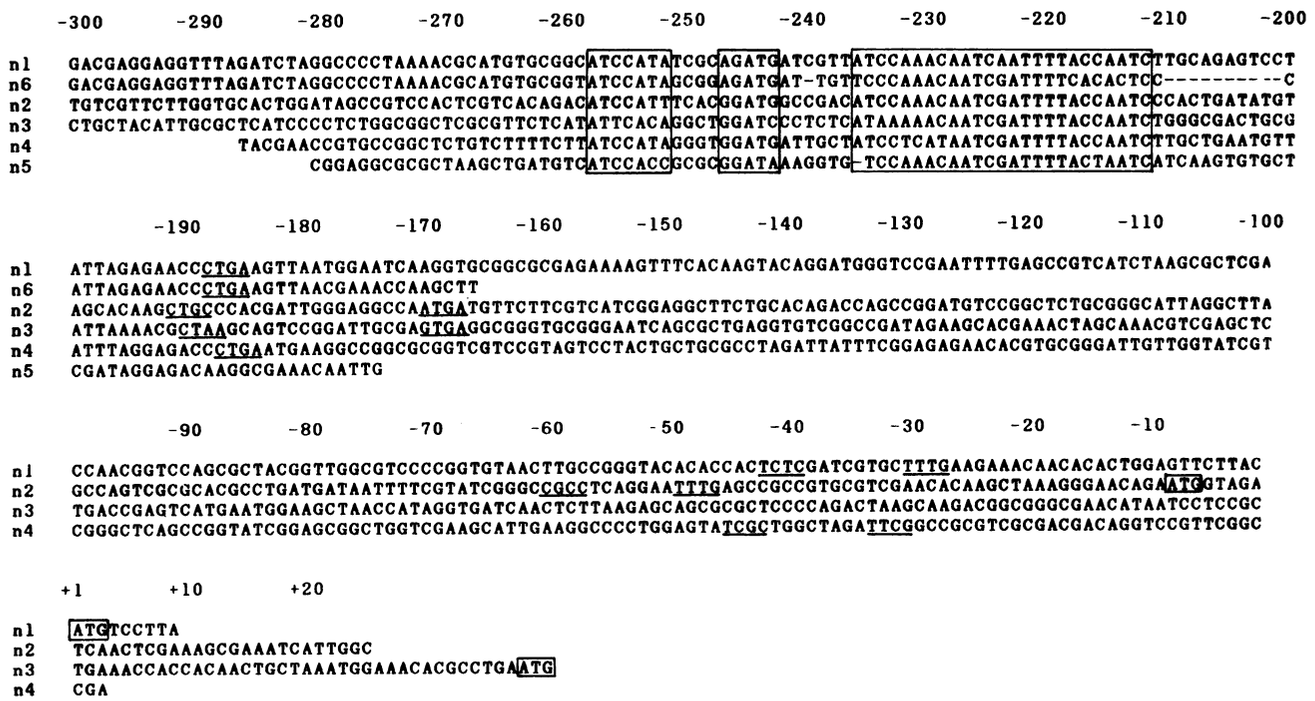

FIG. 1. Comparison of DNA sequences in the 5'-flanking regions of three nodulation genes and of three additional DNA segments located in the *nod/nif* region of *R. meliloti* 41. The scale follows 310 nucleotides of the 5' region of *nod*A (n1); +1 marks the first nucleotide of the translational start codon. The corresponding *nod*E (n2) and *nod*H (n3) sequences and additional sequences (n4, n5, and n6) were aligned to the *nod*A sequence at the maximal homologous regions, which are boxed. The translational start codons (ATG) in the individual sequences are also boxed, the presumptive "*nif*-promoter like" sequences are underlined. Sequences read 5' to 3' from left to right.

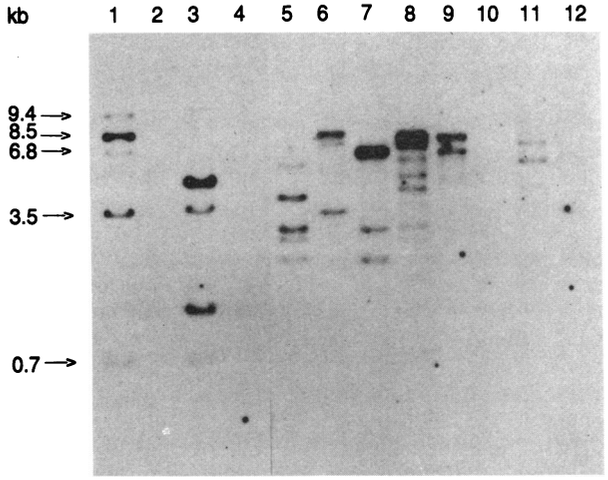

FIG. 2. Hybridization of the ^{32}P-labeled mixed 25-mer oligonucleotide to *Eco*RI-digested genomic DNA from *R. meliloti* 41 (lane 1), *R. meliloti* ZB138 (lane 2), *R. meliloti* L5-30 (lane 3), *R. meliloti* L5-22 (lane 4), *R. japonicum* USDA122 (lane 5), *R. trifolii* RS1051 (lane 6), *R. leguminosarum* 300 (lane 7), *R. phaseoli* RCR3610 (lane 8), *Rhizobium* sp. NGR234 (lane 9), *K. pneumoniae* (lane 10), *A. tumefaciens* Ach5 (lane 11), and *E. coli* HB101 (lane 12). The differences in the intensity of the individual bands within a lane are due to the formation of secondary structures of the DNA during filter preparation. Digestion with a second restriction enzyme cleaves the structures that block hybridization.

lapping cosmid clones of this region confirmed that the insert present in pEK5121 contains the 3.5-kb, 8.5-kb, and 6.8-kb fragments, pEK5022 carries the 0.7-kb, and pEK3148 carries the 0.7-kb and 9.4-kb fragments. The precise locations of the 25-bp sequences in the *nod/nif* region of pRme41b are shown in Fig. 3.

The 25-bp Sequence Is Conserved in Many *Rhizobium* Species. Studies have shown that the common nodulation genes are highly conserved both at the structural and functional levels among diverse *Rhizobium* species (5, 18–22).

The 25-bp sequence may also be interspecifically conserved, if we suppose that it forms a part of the *nod*ABC promoter region. Furthermore, the 25-bp sequence may represent the signal for the coordinated expression of genes coding for early nodulation functions.

Lanes 3–9 in Fig. 2 show that genomic DNAs of the various *Rhizobium* species have 1–6 discrete restriction fragments that exhibit sequence homology to the 25-mer oligonucleotide. To identify these regions of homology, cloned DNA of a species with known nodulation function was used as control.

R. meliloti L5-30, a *Rhizobium* species of European origin nodulates both *Medicago* and *Macroptilium*. Some of its nodulation genes have been isolated in clones *nod*-1, *nod*-11, and on an R prime (unpublished results). Clone *nod*-1 carries *nod*ABC, *nod*-11 contains partial *nod*ABC sequences, and a large (16 kb) *Eco*RI fragment present on an R prime complements *hsn* (*nod*H) mutants of *R. meliloti* 41.

The *Eco*RI digest of the total DNA from *R. meliloti* L5-30 shows five bands hybridizing to the probe sequence (Fig. 2, lane 3); four of these correspond to identically sized *Eco*RI fragments of the described nodulation clones. *R. meliloti* L5-22 is a *nod/nif*-deletion mutant of strain L5-30 (Fig. 2, lane 4). Based on the hybridization data (lanes 3 and 4), all the known nodulation genes and the 25-bp hybridizing bands can be localized within the region missing in L5-22 (plasmid hybridizations are not shown). Thus, the *nod/nif* region of *R. meliloti* L5-30 resembles that of *R. meliloti* 41 as defined by the deletions present in L5-22 and ZB138.

Three *Eco*RI fragments of the genomic DNA from *R. leguminosarum* 300 showed hybridization to the 25-mer oligonucleotide. This pattern of hybridization is identical with that obtained after hybridization with pIJ1089 DNA (the *nod* clone of *R. leguminosarum* 300, ref. 23). The homologous fragments are of 6.6, 3.2, and 2.2 kb (Fig. 2, lane 7). The first two fragments have been shown to code for common nodulation and *hsn* gene functions (23, 18, respectively).

Rhizobium sp. NGR234, the broad host range *Rhizobium* species also has three *Eco*RI fragments with sequence homology to the 25-mer oligonucleotide (Fig. 2, lane 9). One of these fragments (7.9 kb) carries the *nod*C gene (20); functions linked to the other two fragments are not known yet. For the rest of the *Rhizobium* species tested, hybridizations could not be correlated with functional data (lanes 5, 6, and 8).

Lanes 10, 11, and 12 show hybridization of the 25-bp sequence to genomic DNAs of *K. pneumoniae*, *A. tumefaciens*, and *E. coli* HB101. Faint bands can be observed in the *Agrobacterium* lane, which are suggestive of specific hybridizations.

Comparison of Nucleotide Sequences of the 5′-Flanking Regions of *nod*A, *nod*E, *nod*H, and Additional Homologous Regions Present on the 3.5-kb, 0.7-kb, and 9.4-kb *Eco*RI

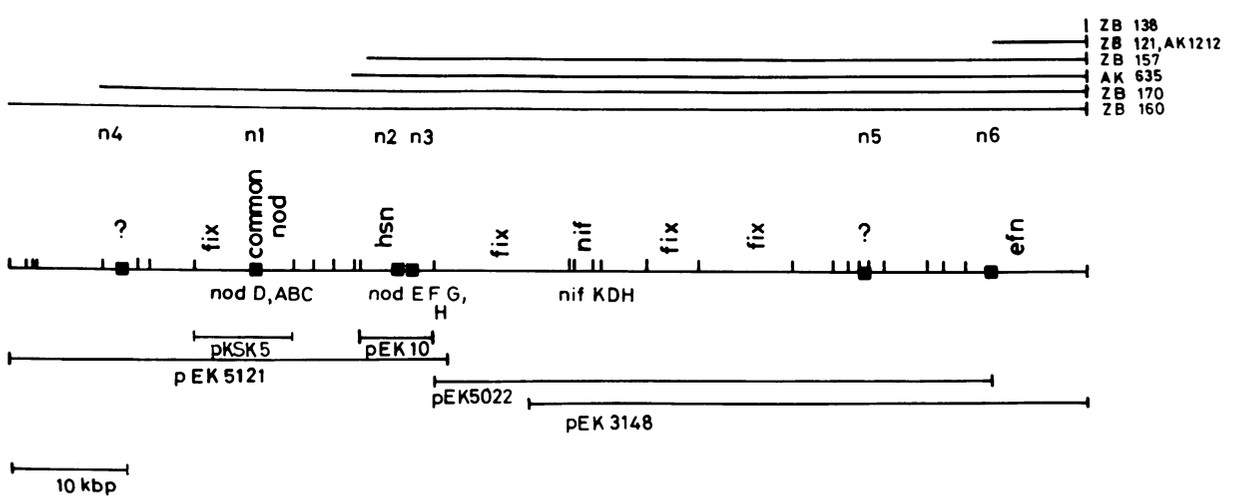

FIG. 3. The physical–genetic map of the 135-kb *nod/nif* region of *R. meliloti* 41. Restriction sites are shown only for *Eco*RI, a more detailed physical map is reported in (5). The horizontal lines indicate the region present in the mutants. pEK10 contains *nod* sequences in pRK290, the three large overlapping fragments were inserted into pJB8. The locations of the 25-bp sequence is marked on the physical map (■) and above the functional signs (n1–6).

Fragments. The nucleotide sequences of the relevant regions of the 3.5-kb (n4), the 0.7-kb (n5), and the 9.4-kb (n6) *Eco*RI fragments were determined and aligned, together with the *nod*E (n2) and *nod*H (n3) sequences, with the 5'-flanking sequence of *nod*A (n1) at their regions of maximal homology (Fig. 1). These regions of homology are 7 bp, 5 bp, and 25 bp long, clustered in a 47-bp segment of DNA. The overall homologies between the individual 5' sequences (ca. 300 nucleotides) are 37.8% between n4 and n1, 31.6% between n3 and n1, 35.8% between n2 and n1, and 33.5% between n2 and n3. For n5 and n6, sequences of 107 and 126 nucleotides, respectively, of the 5' region have been determined. The 25-bp sequence is present in both. Over the corresponding region, n5 has 48.6% homology to n1, while n6 has an unexpectedly high, 84.9% homology to n1. The similarity between n6 and n1 seems to increase upstream from the 25-bp sequence, in the locality of the possible overlap of the *nod*A and *nod*D genes. The translational start codon for *nod*D is situated in the 7-bp segment of the 47-bp region. Therefore, it is not unlikely that the 9.4-kb fragment carries a gene similar to the *nod*D gene. The n4 region of the 3.5-kb *Eco*RI fragment also has more matching nucleotides to the n1 region (mainly in the 47-bp region) than n3 or n2 but the comparatively low level of overall homology makes n4 an unlikely derivative of n1. However, there is no biological data yet available to suggest that n4, n5, and n6 belong to functional genes.

The 25-bp Conserved Sequence Is Essential for the Expression of Downstream *nod* Genes. The major tool in the functional characterization of the two nodulation regions of pRme41b has been directed Tn*5* mutagenesis (5).

In a separate study (unpublished results) the 3.5-kb *Sal* I–*Bam*HI fragment of the *hsn* nodulation region was saturated with Tn*5* insertions and shown to code for genes *nod*E and *nod*H (Fig. 4). These genes are transcribed in opposite directions and are preceded by the conserved 25-bp sequences (n2 and n3 in Figs. 1 and 3). Tn*5* inserted into the coding sequence of *nod*E causes delayed nodulation ($Nod^{+/-}$). Similar insertions in the *nod*H gene lead to the loss of nodulation ability (Nod^{-}, for example see AK1746 and AK1745; constructions d and b in Fig. 4). Contrary to expectation, Tn*5* insertions located in between the 25-bp sequence and the translational start codon of either gene gave an unaltered nodulation phenotype (AK1711 and BH91; constructions c and e in Fig. 4). The explanation of this phenomenon could be that (*i*) these DNA regions are not essential for the proper functioning of *nod*E and *nod*H, or (*ii*) promoter-like sequences located in Tn*5* uncoupled the expression of the genes from their own regulatory sequences. Although Tn*5* is known to cause chiefly strong polar mutations, the nonpolarity of certain Tn*5* insertions has already been demonstrated in *E. coli* (24) and in *R. meliloti* (25). In these cases, promoter-like sequences located near the ends of the inverted repeats in Tn*5* served as promoters for genes distal from the insertion.

To test if the inactivation of regulatory sequences in our mutants was masked by Tn*5*-promoted transcription, the central *Hin*dIII fragment of Tn*5* (coding for kanamycin resistance) was inserted into the *Cla* I site of the 25-bp sequence preceding *nod*E (Fig. 4). The ends of the Tn*5*-derived fragment were filled by using the Klenow enzyme, and *Cla* I linkers were joined to them to produce compatible ends. The mutated *nod*E gene was introduced into wild-type *R. meliloti* in the mobilizable plasmid pRK290, then homogenotized with the help of the incompatible plasmid pPHIJI. The homogenote (AK3216) showed delayed nodulation ($Nod^{+/-}$), which is typical for *nod*E Tn*5* mutants located within the coding region of the gene (construction f, Fig. 4). The same procedure was carried out with construction g (Fig. 4) where the replacement of the central *Cla* I fragment of the 3.3-kb *Sal* I–*Bam*HI fragment with the kanamycin gene of Tn*5* eliminated intact 25-bp sequences preceding *nod*E and also *nod*H. The mutant bacteria (AK3217) failed to nodulate alfalfa, indicating that intact 25-bp sequences are essential for the expression of *nod*E and *nod*H. The expression of the *nod*E and *nod*H genes is not influenced by each other as confirmed by complementation analysis. Mutations affecting either gene can be suppressed by pEK10 or by the respective wild-type alleles pEK17 and pEK18. AK3217, having mutations both in *nod*E and *nod*H, can be fully complemented only by pEK10; pEK18 restores nodulation but the $Nod^{+/-}$ phenotype of the *nod*E mutation remains unsuppressed while pEK17 cannot restore the nodulation ability of AK3217.

DISCUSSION

The *nod/nif* region of *R. meliloti* contains six copies of a conserved 47-bp sequence (*nod-box*), three of which are located upstream of nodulation genes *nod*ABC, *nod*E, and *nod*H, respectively, the other three in DNA regions with unidentified functions. The major part of the 47-bp region is a 25-bp sequence that we found to be essential for the expression of *nod*E and *nod*H.

The conservation of this sequence is extended to various *Rhizobium* species. One common locus of the 25-bp sequence is the region of common nodulation genes (*nod*ABC) in *R. meliloti* 41, *R. meliloti* 2011 (sequence from ref. 7), *R. meliloti* L5-30, *R. leguminosarum* 300, and *Rhizobium* sp. NGR 234.

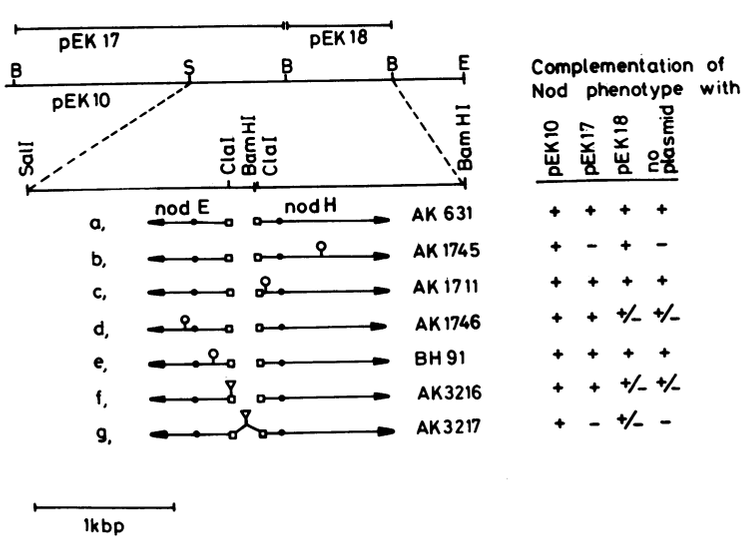

FIG. 4. Physical–genetic map of the central part of the 6.8-kb *Eco*RI fragment (present in pEK10) of pRme41b. Genes *nod*E and *nod*H are transcribed in opposite directions as marked by the arrows (construction a). The 25-bp sequences (□) are located 203 bp and 250 bp upstream from the proposed translational start points (●), respectively. Transposon Tn*5* mutants (♀) were generated downstream (constructions b and d) and upstream (constructions c and e) of the ATG codons. Constructions f and g have the kanamycin resistance gene of Tn*5* inserted into the *Cla* I sites of *nod*E and *nod*H (▽).

The two operons and the single gene preceded by the 25-bp sequence on pRme41b contain those nodulation genes that are absolutely necessary for nodule formation: mutations in *nod*ABC and *nod*H lead to Nod$^-$ phenotype, while *nod*EFG mutations cause delayed nodule appearance. There are only two more regions on the megaplasmid pRme41b that seem to have important roles in alfalfa nodulation: *nod*D, which may have a 5′-flanking region overlapping regulatory sequences of *nod*A and *efn*, a region influencing the efficiency of nodulation (5).

The proposed translational start site of *nod*D is situated in the 7-bp subsequence of the 47-bp region of *nod*A; the genes are transcribed in opposite directions. Interestingly, *nod*D is the only known nodulation gene with its own transcriptional signals that lacks a proximal 47-bp conserved region. The overlapping part of *nod*A and *nod*D and the N-terminal region of *nod*D is repeated on the 9.4-kb *Eco*RI fragment (*efn*). Further data are required to ascertain the extent of the repetition as well as to show whether a specific *efn* gene is coded downstream of the partial *nod*D and the 47-bp region. *nod*D, having a distinct 5′-flanking sequence and being constitutively expressed (unpublished results) reflects a different regulatory mechanism than that seen with genes harboring the 47 bp that may have common regulatory routes.

Any genes encoded by the 3.5-kb and 0.7-kb *Eco*RI fragments are clearly not essential in nodule appearance, but may be involved in other nodulation functions like maintenance or senescence. The 5′-flanking sequences described here have little overall homology with each other (except for that between n6 and n1) or with 5′ regions of *nif* genes.

As shown in this paper, the 25-bp sequence is involved in the expression of nodulation genes in *R. meliloti* 41. When Tn*5* was inserted into the *nod* promoters the nodulation ability of the mutants was not affected indicating that the promoter activity present in Tn*5*, which allows transcription of adjacent genes, is sufficient for the full expression of these genes. However, when the central part of Tn*5*, coding for kanamycin resistance, was cloned into the 5′ region of *nod* genes, these genes became inactivated. This confirmed that the promoter activity present in Tn*5* is not derived from the central part of the transposon but from the ends and, further, that the interrupted 25-bp sequence is strictly required for *nod* gene expression.

The transcriptional start sites of the various nodulation genes have not been established yet, so the precise positioning as regards transcriptional function of the 25-bp sequence is not possible. From the analysis of the nucleotide sequence data it seems that there are no similarities among sequences just upstream (up to −30) of the start codons of the nodulation genes. Sequences suggestive of *nif* promoters can be seen in the −30 to −50 and the −180 to −200 regions. On this basis the 25-bp sequence should either be located at around −170 to −190 or at −20 to −40 from the transcriptional start sites. In the first case, the 25-bp sequence would represent a distantly located regulatory region of the *nod* promoters, similar to regulatory sites reported in *E. coli* (26), *K. pneumoniae* (27), and *R. meliloti* (28), which are thought to mediate coordinated expression of distal genes.

Alternatively, if transcription starts at −20 to −40, the 25-bp sequence may be the primary recognition site of transcription. The extent of the conserved sequence (47 bp) indicates a possible involvement of the subsequences in *nod* gene regulation. Further studies on the promoter regions of *nod*ABC and on the additional three segments containing the 25-bp homology should provide answers to the following questions: (*i*) whether the 25-bp sequence is a primary site of gene regulation (may serve as recognition site, a protein binding site, or a site of conformational changes) and (*ii*) whether the 25-bp sequence is a signal for the coordinated regulation of a set of nodulation genes.

Note Added in Proof. Based on nucleotide sequence comparison, the *nod*E and *nod*F genes of *R. meliloti* 41 correspond to the *nod*F and *nod*E genes of *R. leguminosarum* 300, respectively.

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