Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 8578–8582, November 1988
Genetics

Rhizobium meliloti nodA and *nodB* genes are involved in generating compounds that stimulate mitosis of plant cells

(*nodABC* operon/antibodies/cellular location/plant protoplasts/mitosis-stimulating factor)

JÜRGEN SCHMIDT*, RUTH WINGENDER, MICHAEL JOHN, URSULA WIENEKE, AND JEFF SCHELL

Max-Planck-Institut für Züchtungsforschung, Abt. Schell, D-5000 Cologne 30, Federal Republic of Germany

Contributed by Jeff Schell, August 8, 1988

ABSTRACT **The *nodB* gene of *Rhizobium meliloti* encodes a 23.8-kDa protein that is conserved in several *Rhizobium* species. Monospecific polyclonal antibodies against NodB were used to localize this protein in the cytosol of *R. meliloti* and *Escherichia coli* cells containing *nodABC* genes. In comparison to the NodA and NodC proteins, NodB is synthesized in a disproportionately low amount. The NodA and NodB proteins are involved in generating small, heat-stable compounds that stimulate the mitosis of various plant protoplasts. Our experiments suggest that NodC is not involved in the synthesis of the factors. On the basis of their properties, we speculate that the factors are cytokinin-like substances.**

The formation of nodules on the roots of legumes and non-legumes requires the *nodABC* genes of *Rhizobium* (1–5), which are generally located on large (*sym*) plasmids (6, 7). These genes and other *nod* genes can be activated by the product of the *nodD* gene (8, 9) in the presence of flavonoid compounds that are exuded from the roots of the host plants (10). Mutations within the *nodABC* genes completely abolish root hair curling (Hac), one of the earliest steps in the symbiotic interaction, causing a Nod^- Hac^- phenotype (1, 11). There is increasing evidence that the proteins encoded by these genes produce factors that change the mode of growth of the plant roots (12, 13) and induce root cortical cell division (14).

Recent studies on the function of the Nod proteins of *Rhizobium meliloti* suggest that NodC is a cell-surface protein with a eukaryotic receptor-like structure, which may serve as a transducer of an intracellular bacterial signal to root cells (15, 16). Previously, we localized the NodA protein within the bacterial cell (17). In this paper, we show that the NodB protein is also located in the cytosol of *R. meliloti* and that it is produced in a disproportionately low amount. We further show that the NodA and NodB proteins are involved in the production of low molecular weight molecules that stimulate the mitosis of various plant protoplasts. Our data indicate that NodC is not necessary for generating the factors.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. These are shown in Table 1. Unless otherwise stated, the *E. coli* and *Rhizobium* strains were grown in M9 salts (26) supplemented with 0.2% Casamino acids and 0.4% glycerol.

Construction of *cI–nodB* Hybrid Gene. Recombinant DNA techniques were carried out essentially as described by Maniatis *et al.* (27). To overexpress the NodB protein, a gene fusion with *cI* repressor sequences of bacteriophage λ and the *nodB* gene of *R. meliloti* was constructed. For this purpose we used the *tac* promoter vector pEA305 (25), which directs the synthesis of high levels of the λ CI repressor. The strategy was similar to that reported for the construction of a *cI–nodC* gene fusion (15), except that a 10-mer *Hin*dIII linker was attached to the filled-in *Hin*dIII sites of pEA305, leading to the plasmid intermediate pEA305ΔHindIII-3. The 10-mer linker was inserted to ligate the *nodB* coding sequence in-frame with the λ *cI* initiation codon on the expression vector.

In pJS143 the *nodB* gene is contained within a 0.7-kilobase *Eco*RI fragment. This fragment was cut with *Nae* I and the protruding 5′ end was filled in with the Klenow enzyme. A 0.53-kilobase blunt-ended *nodB* fragment was isolated that encodes ≈86% of the NodB protein (28). Insertion of the *nodB*-containing fragment into the filled-in *Hin*dIII site of pEA305ΔHindIII-3 yielded plasmid pJS3028.

Protein Purification. The CI–NodB fusion protein was overproduced in *E. coli* W3110 carrying pJS3028 and purified from inclusion bodies as described for another CI–Nod fusion protein (17).

Antibodies. Antiserum was raised in rabbits against purified CI-NodB fusion protein. Antibodies were affinity-purified by using the hybrid protein coupled to CNBr-activated Sepharose 4B as a matrix (29). Preparation of affinity-purified antibodies against the NodA protein (17) and the NodC protein (15) has been described.

Electrophoresis and Immunoblotting. $NaDodSO_4$/PAGE was performed in 12% polyacrylamide gels (30). Proteins were electrophoretically transferred to nitrocellulose (31) and the membranes were incubated with antibodies and washed as described (17). Bound antibodies were localized with ^{125}I-labeled protein A (5 μCi; 1 μCi = 37 kBq; Amersham).

Preparation of Protoplasts. Protoplasts were prepared from a soybean suspension-culture line derived from callus material of *Glycine max* cv. Corsoy. Suspensions were grown in the presence of light on a rotary shaker at 26°C in 40 ml of Murashige–Skoog medium (32) supplemented with 0.25 mg of 2.4-dichlorophenoxyacetic acid per liter. After 3 days, samples of cells (from 10- to 20-ml suspensions) were resuspended in 10 ml of a solution (pH 5.5) containing 0.6 M mannitol, 1% cellulase "Onozuka" RS, 0.5% macrozyme R-10 (both enzymes from Yakult Honsha, Nishinoma, Japan), and 0.05% pectolyase (Sigma). This suspension was incubated for 2 hr with gentle shaking and protoplasts were isolated by passing the cells successively through 300-, 100-, and 50-μm sieves. Protoplasts were washed twice with sterile seawater, centrifuged, and then resuspended at a final concentration of 5×10^5 cells per ml in B5 medium (33) containing 0.6 M mannitol and 0.1 mg of 2,4-dichlorophenoxyacetic acid, 0.1 mg of 1-naphthaleneacetic acid, and 0.1 mg of 6-benzyladenine per liter.

The same procedure was used to prepare tobacco and alfalfa protoplasts from suspension cultures provided by Hans-Henning Steinbiss (Max Planck Institute, Cologne,

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

*To whom reprint requests should be addressed.

Table 1. Bacterial strains and plasmids

Name	Characteristics	Source or ref.
Strains		
R. meliloti		
1021	Nod$^+$, Fix$^+$, wild type, Sm^r derivative of strain SU47	18
AK631	Nod$^+$, Fix$^+$, wild type, compact-colony variant of wild-type *R. meliloti* 41	6
AK635	Nod$^-$ deletion of *nodDABC* genes obtained by heat treatment	6
AK1655	Nod$^-$, Km^r, Tn*5* insertion in *nodA*	19
AK1657	Nod$^-$, Km^r, Tn*5* insertion in *nodC*	19
AK1679	Nod$^-$, Km^r, Tn*5* insertion in *nodB*	19
MG107	Nod$^+$-delayed, Km^r, Tn*5*-induced insertion in the *nodD1* gene	20
E. coli		
DS410	Minicell-producing strain	21
HB101	*hsdS hsdM pro leu thi gal lacY recA str*	22
W3110	*lacI*^{q} L8 host for *lac* and *tac* promoter-containing plasmids	23
Plasmids		
pIN-II-A2	Ap^r, vector with tandemly inserted *lpp* and *lac* promoters	24
pEA305	Ap^r, Tc^r, plasmid carrying the *c*I gene of phage λ under *tac* promoter control	25
pEA305ΔHindIII-3	Ap^r, Tc^r, *Hin*dIII deletion derivative of pEA305	This work
pJS120	Tc^r, pACYC184 with the *nodA, -B, -C* genes	11
pJS143	Tc^r, pACYC184 with the *nodB* gene	This work
pJS201	Ap^r, pIN-II-A2 with the *nodA, -B, -C* genes	11
pJS204	Ap^r, pIN-II-A2 with the *nodA* gene	11
pJS205	Ap^r, pIN-II-A2 with the *nodA* and *-B* genes	11
pJS209	Ap^r, pIN-II-A2 with the *nodC* gene	15
pJS2023	Ap^r, Tc^r, plasmid pEA305ΔHindIII-2 carrying a *c*I–*nodA* gene fusion	17
pJS3028	Ap^r, Tc^r, plasmid pEA305ΔHindIII-3 carrying a *c*I–*nodB* gene fusion	This work

Ap, ampicillin; Km, kanamycin; Sm, streptomycin; Tc, tetracyclin.

F.R.G.) and Adam Kondorosi (Biological Research Center, Szeged, Hungary), respectively.

Protoplast Bioassay. Bacterial cells were suspended in water and disrupted by sonication. After centrifugation (30 min at 48.000 × *g*) the supernatant was passed through an ultrafiltration membrane (YM5, Amicon) to remove high molecular weight material. For sorbent extraction of cell division factors, an aliquot of the ultrafiltrate was applied to a solid-phase extraction tube containing octadecyl (C_{18})-bonded silica (Supelco, Bellefonte, PA). The column bed was then washed with water. The retained fraction was eluted with a small volume of methanol, evaporated to dryness, dissolved in water, and bioassayed for cell division activity.

Samples (5–100 μl) of the isolated fraction were added to 3-day-old protoplast suspensions (2 ml). The protoplasts were cultured at 26°C for 24 hr in the presence of light, and the dividing cells were counted microscopically with a hemocytometer.

Other Procedures. The NodB protein, which was expressed and labeled with [^{35}S]methionine in *E. coli* minicells containing *nod* genes of *R. meliloti*, was immunoprecipitated as described (15). Cytosol and inner and outer membrane fractions of *E. coli* HB101 were prepared as described (34), and this procedure has been modified for the fractionation of *Rhizobium* cells (17).

Protein concentrations were determined by the method of Bradford (35) with bovine immunoglobulin as the standard. *R. meliloti* was induced with 5 μM luteolin (Roth, Karlsruhe, F.R.G.; ref. 17). Plant nodulation experiments were carried out as described (15).

RESULTS

Induced Synthesis of the NodABC Proteins in *R. meliloti*. Monospecific polyclonal anti-NodB antibodies were prepared and their reactivity and specificity were tested by immunoprecipitation of the NodB protein synthesized and labeled in *E. coli* minicells. The antibodies specifically precipitated the 23.8-kDa NodB protein, which is produced from pJS120 carrying *nodABC* (data not shown).

We determined the amounts of NodABC proteins in induced cells of *R. meliloti* 1021 by immunoblotting (Fig. 1). At the end of exponential cell growth the protein level of NodA was ≈5-fold above the level of NodC. Interestingly, only disproportionately low amounts of the NodB protein were present within the bacterial cells. Since NodB was not found in the culture medium, we assume that this protein is not secreted. The nucleotide sequence of the *nod* genes revealed that in the sequence ATGA the *nodA* stop codon overlaps the *nodB* start codon (28). Moreover, the tetranucleotide GGAG, representing a potential Shine–Dalgarno sequence (36) of *nodB*, lies within the *nodA* coding sequence and is separated from the ATG initiation codon by only 3 base pairs. Such an overlap of regulatory signals suggests a functional coupling between the *nodA* and *nodB* genes. It has been reported that the spacing between the Shine–Dalgarno sequence and the ATG codon affects the amount of protein synthesized (37–39). We assume that the disproportionately low level of *nodB* gene expression may be due to the suboptimal spacing of 3 base pairs between the ribosome binding site and the ATG translational start for NodB. This mechanism of regulation might serve to coordinate the expression of *nodA* and *nodB* very closely and may be a way to ensure that the two proteins are produced in proper amounts.

Cellular Location of the NodB Protein. For the cellular localization of the NodB protein, we used *E. coli* HB101 cells harboring pJS143 (see Table 1) and the *nodD*::Tn*5* *R. meliloti* mutant MG107 (20), which has been used for the localization of NodA and NodC (17). We fractionated cells into cytosol and membrane fractions by sucrose gradient centrifugation. NaDodSO$_4$/PAGE of the fractions and subsequent labeling of the immunoblots with anti-NodB antibodies revealed that the NodB protein is present in the cytosol fraction of *E. coli* carrying pJS143 (data not shown) and *R. meliloti* MG107 (Fig. 2, lane 2).

When *R. meliloti* AK631 was inoculated onto its host plant, *Medicago sativa* (alfalfa), in the presence of antibodies directed against NodB, normal nodulation occurred and there was no difference in the number of nodules formed in

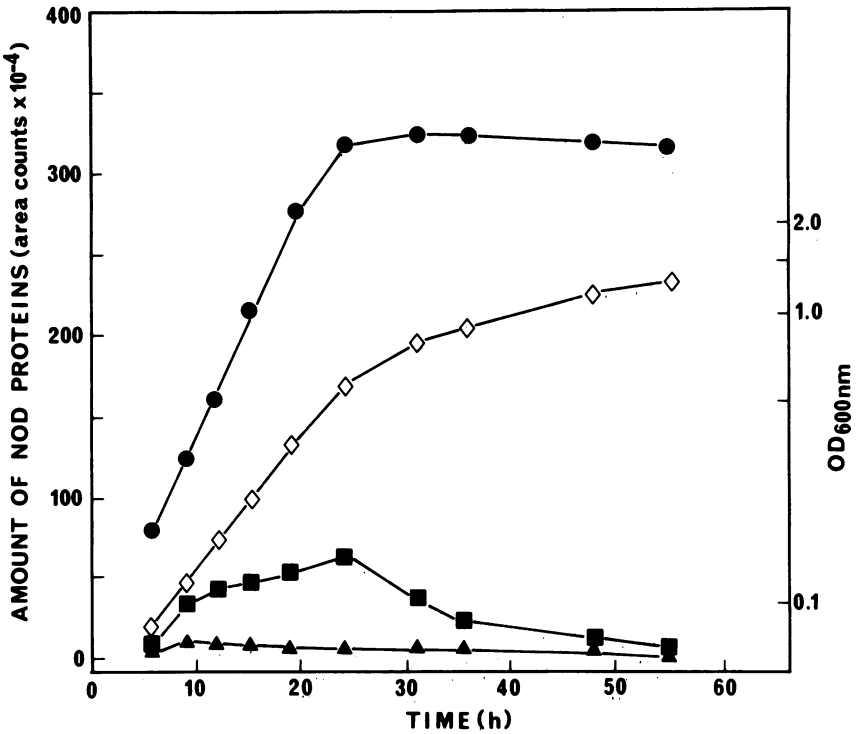

FIG. 1. Induced synthesis of NodA, -B, and -C proteins in *R. meliloti* 1021. Extracts of luteolin-induced *R. meliloti* cells were analyzed by $NaDodSO_4$/PAGE, immunoblotting, and autoradiography. Autoradiograms were scanned and peak areas corresponding to the Nod protein bands were determined with an integrator. ●, NodA; ▲, NodB; ■, NodC. Bacterial growth was monitored by measuring the OD at 600 nm (◇).

comparison with the control experiments (data not shown). This result shows that in contrast to similar experiments with antibodies against the *R. meliloti* NodC cell-surface protein (15), the cytoplasmic NodB protein could not be reached by the antibodies, since the bacterial membrane is impermeable to large molecules.

Effect of NodA and NodB Proteins on the Synthesis of Mitosis-Stimulating Factors. Experiments on the cocultivation of soybean root tissue with *Bradyrhizobium japonicum*

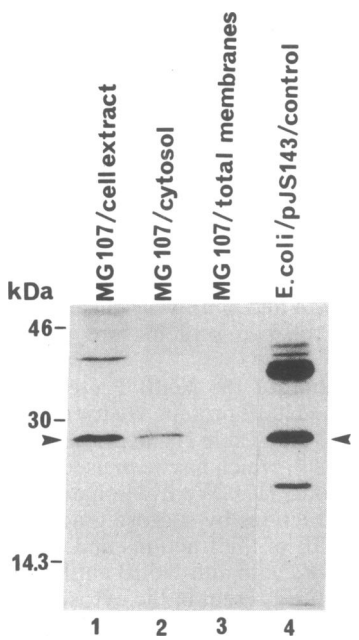

FIG. 2. Cellular location of the NodB protein in *R. meliloti* MG107. Cytoplasmic and membrane fractions were prepared as described in the text. Proteins were analyzed by $NaDodSO_4$/PAGE, transferred to nitrocellulose, and detected with anti-NodB antibodies. The position of the NodB protein is indicated by an arrowhead.

showed a stimulation of plant cell differentiation (40). It has been observed recently that mitosis of cultured soybean protoplasts is stimulated by the presence of *Rhizobium* cells (R.W., unpublished results). By using various *R. meliloti* strains with mutations in the *nod* region, we found that *nod* gene expression affects cell division of soybean protoplasts. We used this as a bioassay to elucidate the function of the *R. meliloti* Nod proteins. Purified extracts prepared from cytosols of various bacterial strains were added to protoplasts, and after 1 day the dividing cells were counted.

Induction of the *R. meliloti* wild-type strain with luteolin resulted in a significant increase in cell division of plant protoplasts (Fig. 3, bars 1 and 2). A deletion mutation in the *nod* region (bar 3) and a Tn*5* insertion in *nodA* (bar 4) abolished this effect. In the induced strain AK1679, carrying the transposon Tn*5* in *nodB*, the NodA protein is synthesized (17). Analysis of this strain showed only a slight increase in mitosis stimulation (bar 5). When the NodA and NodB proteins were produced and *nodC* was not expressed due to Tn*5* insertion, a high level of mitosis-stimulating activity was found (bar 6). The culture supernatants of the *Rhizobium* strains shown in Fig. 3 were also bioassayed, and activity was found in the media of those strains in which biological activity in the cytosol was detected (data not shown). This indicates that the mitosis-stimulating compounds are secreted into the medium.

In a further experiment we incubated the cytosol from the induced *R. meliloti* deletion mutant AK635 (Δ*nodABC*) with protein extracts of *E. coli* strains in which the *nodABC* genes were expressed under the control of strong promoters. The NodA protein alone was sufficient to produce a factor with mitosis-stimulating activity (Fig. 4, bars 2 and 3). The activity was increased when both NodA and NodB proteins were present in the incubation mixture (bar 5). In the soybean protoplast assay, NodB and NodC alone did not show a significant increase in activity (bars 4 and 6). From these data we speculate that the NodB protein may convert the factor generated by NodA into another more active compound. We

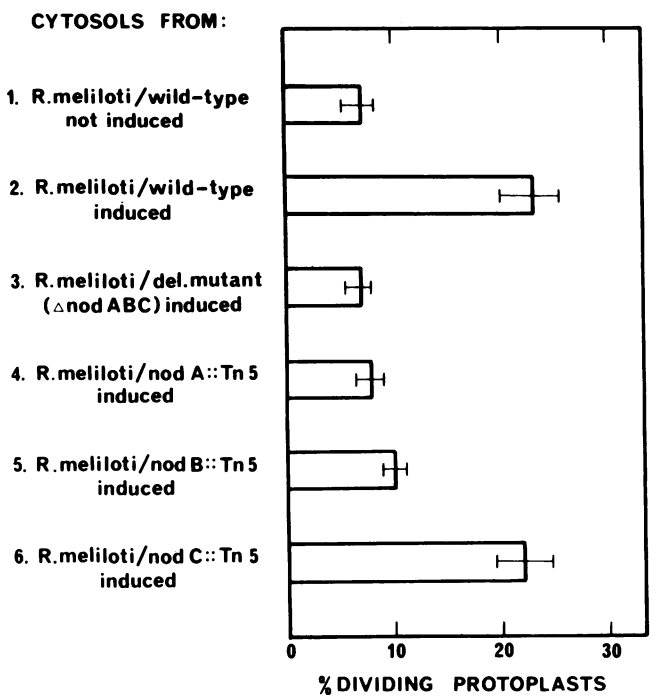

FIG. 3. Effect of NodA and NodB proteins on the synthesis of factors that stimulate mitosis of cultured soybean protoplasts. Samples prepared from cytosols of *R. meliloti* wild-type and mutant strains (see text) were added to protoplast suspensions. After 24 hr the dividing cells were counted microscopically. Bars 1 and 2, *R. meliloti* AK631; bar 3, *R. meliloti* AK635; bar 4, *R. meliloti* AK1655; bar 5, *R. meliloti* AK1679; bar 6, *R. meliloti* AK1657. Standard deviations are marked by error bars.

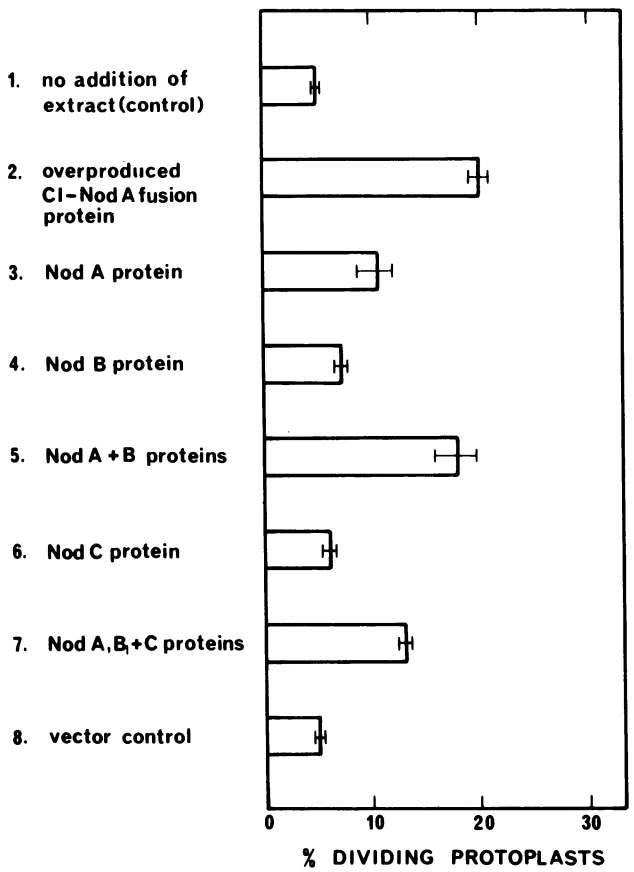

FIG. 4. Incubation of cytosol from the induced *R. meliloti* deletion mutant AK635 (Δ*nodABC*) with extracts from *E. coli* cells synthesizing Nod proteins. The mixtures were incubated for 30 min at 30°C, and isolates purified by sorbent extraction were analyzed by the protoplast bioassay as described in the text. The *E. coli* strains used in these experiments carried the following plasmids: bar 2, pJS2023; bar 3, pJS204; bar 4, pJS143; bar 5, pJS205; bar 6, pJS209; bar 7, pJS201; bar 8, pIN-II-A2.

also assume that NodC is not involved in generating the mitosis-stimulating factors.

Properties of Factors. The factors produced by the NodA and NodB proteins of *R. meliloti* have a molecular mass of <1000 Da, since they pass through an ultrafiltration membrane with a 1000-Da cutoff (YM2, Amicon). After boiling for 1 hr no loss of activity was observed. The factors are soluble in water and are retained on a C_{18} reverse-phase column. This indicates that the active compounds are partially hydrophobic. A purified active fraction that was eluted with 35% methanol from a C_{18} reverse-phase HPLC column (Beckman Ultrasphere ODS, 250 × 10 mm) was lyophilized, dissolved in water, and assayed for the presence of carbohydrates with the orcinol/sulfuric acid reagent (41). Since the assay was positive, we assume that this active compound may be glycosylated.

The factors stimulated not only the mitosis of soybean protoplasts but also cell division of protoplasts from alfalfa, barley, carrots, tobacco, and wheat to various extents (data not shown). High concentrations of the factors seem to be inhibitory in the bioassay, so that the number of dividing protoplasts is reduced.

DISCUSSION

Recent studies have shown that the *nodABC* gene cluster is absolutely essential for root hair curling (1) and the induction of root cortical cell division (14, 42), which leads to the formation of the nodule meristem. We have shown that the proteins encoded by the *nodA* and *nodB* genes are located in the cytoplasm of *Rhizobium* and that both proteins are necessary to produce small diffusible factors that stimulate, at low concentrations, the mitosis of various plant protoplasts. The bacterial factors are heat-stable and probably glycosylated compounds with some hydrophobic character.

On the basis of these properties we speculate that the active compounds may be cytokinin-like substances. It has been shown recently by others that dialyzable, heat-stable compounds that contain neutral sugars are present in culture filtrates of *Rhizobium trifolii*. These compounds, together with other substances, are involved in white clover root hair deformation (43). Our data suggest that NodA alone is involved in generating a molecule that is active in the cell division assay. This compound may be modified by NodB to a molecule with enhanced biological activity (Figs. 3 and 4). The nucleotide sequence of the *nodA* and *nodB* genes revealed an overlap of translational regulatory signals (28), which supports our assumption that the proteins encoded by these two genes are functionally linked (see *Results*).

Since mutations in *nodC* abolish root hair curling (1, 11), the mitosis-stimulating factors produced by NodA and NodB are not sufficient to induce hair curling. Apparently, the presence of the NodC cell-surface protein (16) as a potential signal transducer is therefore additionally required. The simple bioassay described here did not allow us to evaluate the role of NodC in the more complex process of nodule induction, which involves cortical cell division and root hair curling. The NodA, NodB, and NodC proteins are present in the nodule (16, 17), and it is possible that the mitosis-stimulating factors are also needed during later stages of nodule development.

It still has to be elucidated whether NodA and NodB are directly involved in the synthesis of the mitosis-stimulating factors. The properties mentioned above may provide a basis

for the further purification of the factors and the elucidation of their structure.

We appreciate the skillful technical assistance of Heinz-Dieter Krüssmann. We thank Frans de Bruijn and Adam Kondorosi for bacterial strains, discussions, and critical reading of the manuscript. Horst Lörz, Hans-Henning Steinbiss, and Bruno Gronenborn kindly provided the barley, carrot, and wheat protoplast cultures. We thank Dietrich Bock for photographic work. This work was supported by a grant from Bundesministerium für Forschung und Technologie (BCT 03652/Projekt 8). R.W. was supported by a grant from the European community [BAP-0087-D (B)].

1. Kondorosi, E., Banfalvi, Z. & Kondorosi, A. (1984) *Mol. Gen. Genet.* **193,** 445–452.
2. Djordjevic, M. A., Schofield, P. R., Ridge, R. W., Morrison, N. A., Bassam, B. J., Plazinski, J., Watson, J. M. & Rolfe, B. G. (1985) *Plant Mol. Biol.* **4,** 147–160.
3. Downie, J. A., Knight, C. D., Johnston, A. W. B. & Rossen, L. (1985) *Mol. Gen. Genet.* **198,** 255–262.
4. Fisher, R. F., Tu, J. K. & Long, S. R. (1985) *Appl. Environ. Microbiol.* **49,** 1432–1435.
5. Marvel, D. J., Torrey, J. G. & Ausubel, F. M. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 1319–1323.
6. Banfalvi, Z., Sakanyan, V., Koncz, C., Kiss, A., Dusha, I. & Kondorosi, A. (1981) *Mol. Gen. Genet.* **184,** 318–325.
7. Rosenberg, C., Boistard, P., Denarie, J. & Casse-Delbart, F. (1981) *Mol. Gen. Genet.* **184,** 326–333.
8. Mulligan, J. T. & Long, S. R. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 6609–6613.
9. Rossen, L., Shearman, C. A., Johnston, A. W. B. & Downie, J. A. (1985) *EMBO J.* **4,** 3369–3373.
10. Peters, N. K., Frost, J. W. & Long, S. R. (1986) *Science* **233,** 977–980.
11. Schmidt, J., John, M., Kondorosi, E., Kondorosi, A., Wieneke, U., Schröder, G., Schröder, J. & Schell, J. (1984) *EMBO J.* **3,** 1705–1711.
12. van Brussell, A. A. N., Zaat, S. A. J., Canter Cremers, H. C. J., Wijffelman, C. A., Pees, E., Tak, T. & Lugtenberg, B. J. J. (1986) *J. Bacteriol.* **165,** 517–522.
13. Zaat, S. A. J., Wijffelman, C. A., Spaink, H. P., van Brussell, A. A. N., Okker, R. H. J. & Lugtenberg, B. J. J. (1987) *J. Bacteriol.* **169,** 198–204.
14. Dudley, M. E., Jacobs, T. W. & Long, S. R. (1987) *Planta* **171,** 289–301.
15. John, M., Schmidt, J., Wieneke, U., Kondorosi, E., Kondorosi, A. & Schell, J. (1985) *EMBO J.* **4,** 2425–2430.
16. John, M., Schmidt, J., Wieneke, U., Krüssmann, H.-D. & Schell, J. (1988) *EMBO J.* **7,** 583–588.
17. Schmidt, J., John, M., Wieneke, U., Krüssmann, H.-D. & Schell, J. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 9581–9585.
18. Meade, H. M., Long, S. R., Ruvkun, G. B., Brown, S. E. & Ausubel, F. M. (1982) *J. Bacteriol.* **149,** 114–122.
19. Kondorosi, A., Kondorosi, E., Banfalvi, Z., Putnoky, P., Török, I., Stepkowski, T., Schmidt, J. & John, M. (1984) in *Proceedings of the 14th International Symposium on the Chemistry of Natural Products*, ed. Zalewski, A. (Elsevier, Amsterdam), pp. 643–654.
20. Göttfert, M., Horvath, B., Kondorosi, E., Putnoky, P., Rodriguez-Quinones, F. & Kondorosi, A. (1986) *J. Mol. Biol.* **191,** 411–426.
21. Dougan, G. & Sherratt, D. (1977) *Mol. Gen. Genet.* **151,** 151–160.
22. Boyer, H. W. & Roulland-Dussoix, D. (1969) *J. Mol. Biol.* **41,** 458–472.
23. Brent, R. & Ptashne, M. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 4204–4208.
24. Nakamura, K. & Inouye, M. (1982) *EMBO J.* **1,** 771–775.
25. Amann, E., Brosius, J. & Ptashne, M. (1983) *Gene* **25,** 167–176.
26. Miller, J. H. (1972) *Experiments in Molecular Genetics* (Cold Spring Harbor Lab., Cold Spring Harbor, NY), pp. 3369–3373.
27. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
28. Török, I., Kondorosi, E., Stepkowski, T., Posfai, J. & Kondorosi, A. (1984) *Nucleic Acids Res.* **12,** 9509–9524.
29. De Mey, J. R. (1983) in *Immunohistochemistry*, ed. Cuello, A. C. (Wiley, Chichester, U.K.), pp. 347–372.
30. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
31. Towbin, H., Staehelin, T. & Gordon, T. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.
32. Murashige, T. & Skoog, F. (1962) *Physiol. Plant* **15,** 473–497.
33. Gamborg, O. L., Miller, R. A. & Ojima, K. (1968) *Exp. Cell Res.* **50,** 151–158.
34. Ito, K., Sato, T. & Yura, T. (1977) *Cell* **11,** 551–559.
35. Bradford, M. (1976) *Anal. Biochem.* **72,** 248–254.
36. Shine, J. & Dalgarno, L. (1974) *Proc. Natl. Acad. Sci. USA* **71,** 1342–1346.
37. Backman, K. & Ptashne, M. (1978) *Cell* **13,** 65–71.
38. Roberts, T. M., Kacich, R. & Ptashne, M. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 760–764.
39. Guarente, L., Roberts, T. M. & Ptashne, M. (1980) *Science* **209,** 1428–1430.
40. Veliky, I. & La Rue, T. A. (1967) *Naturwissenschaften* **54,** 96.
41. Svennerholm, L. (1956) *J. Neurochem.* **1,** 42–53.
42. Calvert, H. E., Penel, M. K., Pierce, M., Malik, N. S. A. & Bauer, W. D. (1984) *Can. J. Bot.* **30,** 2375–2384.
43. Bhuvaneswari, T. V. & Solheim, B. (1985) *Physiol. Plant* **63,** 25–34.