

Transcriptional Activation in Nuclei from Uninfected Soybean of a Set of Genes Involved in Symbiosis with *Rhizobium*

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Several plant genes coding for nodule-specific proteins, nodulins, are expressed in legume root nodules during symbiotic nitrogen fixation. We have found that three coordinately regulated nodulin genes of soybean are transcriptionally activated following incubation of nuclei from an uninfected tissue with nodule extract. Transcription in isolated nuclei was monitored following addition of *Escherichia coli* RNA polymerase, which appears to transcribe active regions of chromatin. The kinetics of induction were similar for nodulin-23, nodulin-24, and leghemoglobin c3 genes but different from nodulin-35, a gene induced in the uninfected cells later in nodule development. Detailed analysis of the transcripts from one of the nodulin genes showed that preferential transcription occurred in the 5' region, suggesting that a factor present in nodule extract is able to alter chromatin structural domains encoding nodulin genes that allow transcription from this region of the genome. *In vitro* activation of these nodulin genes is specific to nodule extract; free living *Rhizobium* and any other plant extracts or incubation conditions do not have any effect. These data indicate that a transactivating factor of bacterial or plant origin is produced following infection by *Rhizobium* and is involved in the induction of nodulin genes.

Additional key words: gene expression, *Glycine max*, nitrogen fixation, nodulins, transcription

The successful infection of leguminous plants by soil bacteria (*Rhizobium* and *Bradyrhizobium* spp.) results in a symbiotic association capable of reducing atmospheric dinitrogen to ammonia. *Rhizobium* infects root cortical cells and causes the formation of a tumor-like structure, the nodule, that provides the micro-environment necessary for the transformation of *Rhizobium* into a nitrogen-fixing bacteroid (Verma and Long 1983; Verma and Nadler 1984). Analysis of the host's contribution to this symbiosis has revealed the presence of several nodule-specific proteins called nodulins (Legocki and Verma 1980; Verma *et al.* 1986). These include leghemoglobins (Lb), a family of proteins required for nodule respiration (Kubo 1939; Appleby 1985) and enzymes involved in carbon/nitrogen assimilation, e.g., xanthine dehydrogenase, uricase (Bergmann *et al.* 1983; Christensen and Jochimsen 1983; Nguyen *et al.* 1985), sucrose synthase (Morell and Copeland 1985; Thummler and Verma 1987), and glutamine synthetase (Cullimore *et al.* 1984; Sengupta-Gopalan and Pitas 1986). Furthermore, several soybean genes encoding nodulins of as yet undetermined function have been cloned and characterized (Fuller *et al.* 1983; Fuller and Verma 1984; Katinakis and Verma 1985; Mauro *et al.* 1985; Verma *et al.* 1986; Fortin *et al.* 1987; and Jacobs *et al.* 1987).

Northern analysis of RNA from inoculated roots has suggested that the genes encoding nodulins-23 and -24 and Lbc3 are simultaneously induced shortly after infection of soybean by *Bradyrhizobium japonicum* (Fuller and Verma 1984). DNA sequence analysis has revealed several regions

of homology at the 5'-ends of these genes, indicative of possible cis-acting sequences responsive to common regulatory factors (Mauro *et al.* 1985). We report here the use of an *in vitro* transcription assay (Craine and Kornberg 1981a, 1981b) to investigate the possibility that transacting factors capable of activating these genes exist in the nodule. Nuclei from an uninfected tissue (embryonic axes) were preincubated with nodule extract, reisolated, and monitored for the accessibility of specific genes to *Escherichia coli* RNA polymerase, a measure of gene activity (Craine and Kornberg 1981a, 1981b; Luse *et al.* 1981; Samuels *et al.* 1982; Brown 1984; Tata and Baker 1985). We report that a subset of nodulin genes can be activated in this manner by a factor present in the nodule extract.

MATERIALS AND METHODS

Plant tissues. All soybean tissues were from *Glycine max* cv. Prize. Embryonic axes were manually dissected from seeds that had been imbibed in distilled water for 3 hr. Leaf tissue was obtained from 2-wk-old plants. Uninfected roots were from seeds germinated in the dark, in vermiculite, for 3 days at 27°C. Nodules were obtained from 3-wk-old plants inoculated with *B. japonicum* (61A76) and grown using a nitrogen-free nutrient solution (Verma *et al.* 1974). All tissues were frozen in liquid nitrogen and either used immediately or stored at -70°C until used.

Nuclei isolation. Tissue was ground in liquid nitrogen to a fine powder and thawed in 10 times excess Honda buffer (Honda *et al.* 1966) (0.44 M sucrose, 2.5% Ficoll 400,000, 5% Dextran T-40, 25 mM Tris HCl, 10 mM MgCl₂, 10 mM β-mercaptoethanol, 0.5% Triton X-100) containing 2 mM spermine. The suspension was filtered through several layers of Miracloth (Calbiochem). The filtrate centrifuged at 6,000 × g for 5 min at 4°C. The pellet was resuspended in Honda

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buffer without spermine (5–10% of the initial volume) and layered on top of a Percoll (Pharmacia Fine Chemicals) gradient consisting of a 2 M sucrose cushion topped by 80, 60, and 40% Percoll steps. All the solutions in the Percoll gradient contained 25 mM Tris HCl (8.5), 10 mM MgCl₂, and 0.45 M sucrose.

The gradients were centrifuged at 6,000 × *g* for 30 min at 4°C. Nuclei were recovered from the top of the 2 M sucrose cushion and washed with at least an equal volume of Honda buffer (no spermine) and recovered by centrifugation for 5 min at 6,000 × *g* at 4°C. This step was repeated once more. The nuclei were then washed with 5 mM Tris HCl (pH 8.5), 5 mM MgCl₂, 10 mM β-mercaptoethanol, and 20% glycerol (v/v). The nuclei were then suspended in a small volume of the wash buffer that contained 50% glycerol (v/v).

Preparation of extracts and preincubation conditions.

Extracts of root, nodule, and leaf tissues were all prepared by first grinding the tissue to a fine powder in liquid nitrogen and then following the protocol of Manley *et al.* (1980); 20 g of tissue had a packed volume of 25 ml. All buffers used in preparation of the extracts were kept at 4°C and contained 1 mM phenylmethylsulfonyl fluoride (PMSF) as a protease inhibitor.

Incubations of nuclei in extract typically used 90 μg of nuclei (DNA) in a 300–400 μl incubation volume consisting of 70% extract in water (containing 1 mM EDTA). Incubations were done at room temperature in Eppendorf tubes slowly rotating on a wheel. Incubations in buffer, DNase, or proteinase K were done using the same final buffer used in the extracts, i.e., 20 mM Hepes (7.9), 100 mM KCl, 12.5 mM MgCl₂, 0.1 mM EDTA, 2 mM dithiothreitol, 17% glycerol, and 1 mM PMSF.

Transcription assay in isolated nuclei. Nuclei were either preincubated or used directly. Before adding to the transcription reaction, nuclei were collected by centrifugation for 20 sec at 15,000 rpm. They were then suspended in a reaction mixture (300 μl) consisting of 100 mM KCl, 15 mM MgCl₂, 500 nM uridine triphosphate (UTP), 500 μM each of cytidine triphosphate (CTP), adenosine triphosphate (ATP), and guanosine 5'-triphosphate (GTP), 20 mM (NH₄)₂SO₄, 12.5 mM phosphoenol pyruvate, 500 ng/μl of pyruvate kinase, 40 mM Tris HCl (7.9), 100 nM EDTA, 5 mM dithiothreitol, 0.02 units/μl RNasin, up to 500 μCi α-³²P UTP (410 Ci/mMole) and typically 0.2 μg *E. coli* RNA polymerase per microgram of nuclei (DNA). The transcription reaction was optimized for various components.

Run-off transcription reactions were carried out in the same conditions without the bacterial polymerase, in buffer consisting of 75 mM (NH₄)₂SO₄, 15 mM MgCl₂, 500 μM each of CTP, ATP, and GTP, 12.5 mM phosphoenol pyruvate, 0.1 mg/ml of pyruvate kinase, 25 μM UTP, and up to 500 μCi α-³²P UTP (410 Ci/mMole).

Total incorporation was determined by precipitation of the nucleic acids in cold 10% trichloroacetic acid (TCA) followed by filtration through GFA (Whatman) glass fiber filters that were then washed with cold 5% TCA, dried, and counted in scintillation fluid.

An estimate of the amount of messenger RNA produced *in vitro* was obtained using a NaI nitrocellulose binding assay (Bresser *et al.* 1983). Working at template excess concentrations for *E. coli* RNA polymerase, we observed 0.1–0.6% of the counts behaving as messenger RNA as determined by filter binding in NaI (Bresser *et al.* 1983).

RNA purification and hybridization conditions.

Transcription reactions were stopped with the addition of

deoxyribonuclease I (120 units) for 2 min at 37°C. Sample buffers were adjusted to 0.5% NaDodSO₄ and 300 μg/ml proteinase K (Boehringer) and incubated for 1 hr at 37°C. Samples were then extracted with phenol/chloroform/isoamyl alcohol (24:24:1) followed by chloroform/isoamyl alcohol. Samples were adjusted to 0.2 M sodium acetate (pH 5.5), two volumes of ethanol were added, and the RNA was precipitated at –70°C. The ethanol pellet was washed once with 70% ethanol, dried, and resuspended in hybridization buffer consisting of 50% distilled formamide, 0.1 M Pipes (pH 6.4), 0.1% NaDodSO₄, 1 mM EDTA, 0.6 M NaCl, and 750 μg/ml of both poly (A) and tRNA.

The RNA, in a volume of 0.2 ml, was hybridized simultaneously to all filters (6 mm in diameter) containing 50 μg of bound recombinant plasmids or pBR322 (used as a measure of background levels). In the transcript mapping experiments, filter-bound M13 clones (in mp8 and mp9 vectors) were used. DNA was immobilized on Genescreen (New England Nuclear). The filters were prehybridized in the hybridization buffer without probe for a minimum of 4 hr at 45°C. The RNA/hybridization mix was heated for 5 min at 70°C before being added to the filters. Hybridization proceeded at 45°C for 36–48 hr. Filters were washed at 50°C. The first wash was in hybridization buffer with 0.05 M Pipes (pH 6.4) for 30 min followed by a 20-min wash in 0.5% NaDodSO₄, 0.01 M Pipes (pH 6.4), and 60 mM NaCl. Five 5-min washes in 0.5% NaDodSO₄, 3 mM Pipes (pH 6.4), and 18 mM NaCl, were followed by five 5-min washes in the same buffer without NaDodSO₄. The final wash was 10 min at room temperature in 20 mM Tris (pH 7.6). The RNA was then eluted from the filters by boiling for 1 min in 300 μl of H₂O, and counted. TCA precipitation of the eluted RNA gave the same values as direct counting, so later experiments were done using only direct counting. The values obtained using hybrid selection with filter-bound pBR322 were subtracted from those obtained with the other probes. The background levels were relatively constant and did not change with different incubations.

Plasmids. All plasmids used were CsCl purified with the exception of the M13 clones that were prepared as described (Mauro *et al.* 1985). The probes used for detecting various sequences are: for nodulin-23, 2-kb *Hind*III-*Eco*RI fragment described earlier (Mauro *et al.* 1985); for nodulin-24, pNod18 (Katinakis and Verma 1985), and for nodulin-35, the 6.9-kb *Eco*RI genomic clone described by Nguyen *et al.* (1985). Lbc3 and Lbc2 were detected using pLb11-3.7 (Brisson and Verma 1982) and p60E-15, a genomic subclone (Lee *et al.* 1983). The probe used for detecting the soybean actin gene was pSAC3 (Shah *et al.* 1982). The root specific sequence was pRt57 from Fuller *et al.* (1983).

RESULTS

Assay for gene activation. The assay used to monitor nodulin gene activation is based on their relative accessibility to *E. coli* RNA polymerase and is similar to the approach used to study other eucaryotic genes (Craine and Kornberg 1981a, 1981b.; Luse *et al.* 1981; Samuels *et al.* 1982; Brown 1984; Tata and Baker 1985).

In this study, *E. coli* RNA polymerase was used to transcribe RNA from isolated nuclei in the presence of α-³²P UTP. Embryonic axes were chosen as a source of nuclei because they do not express the nodulin genes. These nuclei were able to synthesize RNA on their own but showed a large increase in incorporation of radioactive label with the addition of *E. coli* RNA polymerase (Fig. 1). This

synthesis was found to be DNA dependent (DNase 1 sensitive) and RNA polymerase directed. Incorporation of the radioactive label was not the result of an end-labeling activity; exclusion of any one rNTP resulted in no incorporation. This was further supported by the lack of incorporation when polymerase inhibitors were included in the reactions. Endogenous and exogenous polymerase activities were distinguished using α -amanitin and rifampicin to inhibit the eucaryotic polymerases and the bacterial polymerase, respectively. The final product of α - 32 P UTP incorporation was RNA, as demonstrated by its sensitivity to RNase.

Incorporation of α - 32 P UTP into RNA continued for several hours and was proportional to the concentration of *E. coli* RNA polymerase up to approximately 0.22 μ g of RNA polymerase per 1.0 μ g of nuclei DNA (Fig. 1B). A preliminary experiment showed that those genes that were monitored (e.g. nodulin-23, nodulin-24, Lbc3, a soybean actin gene, pSAc3, and a gene expressed in early root tissue, pRt57) were correctly determined as active or inactive by differential transcription in isolated nuclei from embryonic axes. This assay is therefore accurate in assessing the actual transcriptional status of these genes as judged by hybrid selections with run-off transcripts (data not shown). A preferential transcription of active genes has been observed in other systems (Tan and Miyagi 1970; Chiu *et al.* 1975; Craine and Kornberg 1981a, 1981b).

Transactivation of nodulin genes. The presence of transacting factors modulating the expression of the nodulin genes was sought by preincubating nuclei with an extract made from nodules. The nuclei were reisolated and assayed for gene activation by transcription and hybrid selection of the RNA with various filter-bound probes.

An increase in the total incorporation of α - 32 P UTP was observed with increasing times of preincubation of nuclei in nodule extract (Fig. 2A). In order to monitor the expression of individual genes, hybrid selections were performed with various specific probes (Fig. 2B). Nodulin-23, nodulin-24, and Lbc3 genes, postulated to be coordinately regulated, together with the leghemoglobin c2 gene, were all found to be activated with very similar kinetics, i.e., maximal activation occurring after approximately 1 hr of incubation. The nodulin-35 gene, on the other hand, coding for a

subunit of the nodule-specific enzyme uricase (Bergmann *et al.* 1983; Nguyen *et al.* 1985), displayed different activation kinetics in this assay. Since the nodulin-35 gene is expressed in uninfected cells of the nodules and at a later time in nodule development than the genes encoding nodulin-23, nodulin-24, and Lbc3 (Nguyen *et al.* 1985), the data suggest that it may be controlled by different transacting factors.

The pattern of the nodulin gene activation and the lack of activation of the root (pRt57) and actin (pSAc3) genes suggest that these results do not merely reflect the increase in total incorporation with increasing preincubation time. A number of control experiments demonstrated the specificity of nodule extract in activating the nodulin genes (Fig. 3). Incubation of nuclei with extracts prepared from leaf, uninfected root, embryonic axes, or with the extraction buffer alone did not cause the activation of any of the genes probed. Therefore, the *in vitro* activation of the genes encoding nodulin-23, nodulin-24, nodulin-35, Lbc2, and Lbc3 was specifically caused by the nodule extract and was not the result of nonspecific plant factors or an artifact of incubation. Incubation of the nuclei in either 0.5 or 5 units of DNase 1 or with up to 100 units of proteinase K for 1 hr

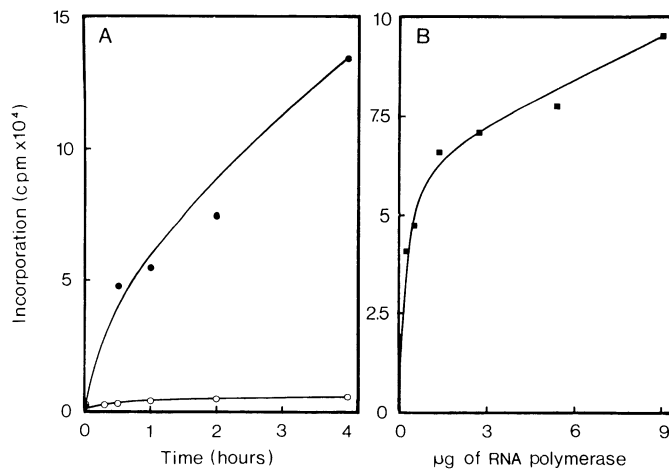


Fig. 1. Transcription in isolated nuclei from embryonic axes of soybean. **A**, Time course of incorporation of α - 32 P UTP in RNA in the presence (●) or absence (○) of 1.5 μ g of *Escherichia coli* RNA polymerase. **B**, Dependence of incorporation of α -UTP in RNA on RNA polymerase concentration. Reactions were carried out for 1 hr. Each reaction contained 2.3 μ g of nuclei (DNA) and 16 μ Ci α - 32 P UTP.

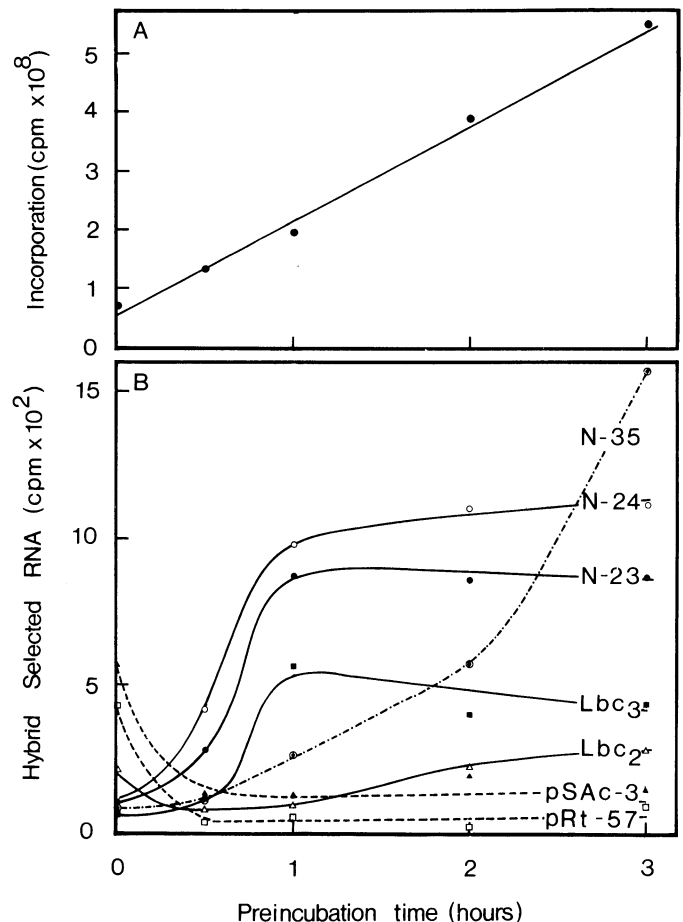


Fig. 2. Kinetics of activation of nodulin genes by nodule extract. Nuclei from embryonic axes were preincubated with nodule extract, reisolated and added to the transcription reactions from the time points indicated. **A**, Total incorporation following different times of preincubation in nodule extract. An aliquot was removed at each time point and total incorporation was measured. **B**, Hybrid selection of RNA with various DNA probes. Nuclei (90 μ g DNA) following different preincubations with nodule extracts were incubated with 250 μ Ci α - 32 P UTP and 18 μ g *E. coli* RNA polymerase, and the level of specific transcript was monitored by hybridization of the transcripts with filter-bound probes. N-35, nodulin-35; N-24, nodulin-24; N-23, nodulin-23; Lbc2 and Lbc3 leghemoglobin c2 and c3; pSAc3, soybean actin gene; pRt57, a soybean root sequence.

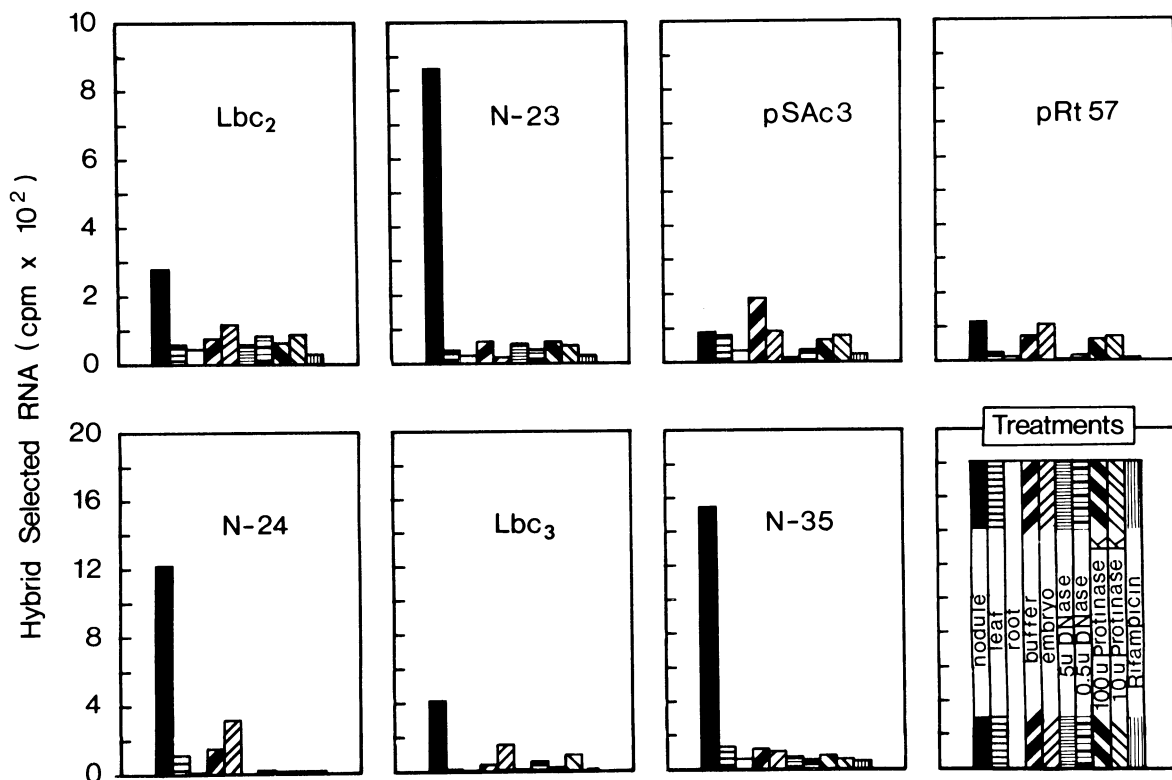


Fig. 3. Control preincubation experiments demonstrating the specificity of nodule extract in activating nodulin genes. Experiments were performed as described in Figure 2 with a 2-hr preincubation in the extracts or with buffer alone. The preincubations with DNase I or Proteinase K were for 1 hr. The rifampicin treatment refers to nuclei preincubated in nodule extract and transcribed in the presence of 50 $\mu\text{g/ml}$ rifampicin. Each box represents hybrid selection with one probe (as indicated) under the various preincubation conditions.

before transcription did not result in the activation of any genes. This suggests that the activations were not the result of DNase or protease activities in the nodule extract.

Transcription of the nodule extract alone, without nuclei, gave very little incorporation (less than 0.04% of that obtained in the presence of nuclei) and gave no hybrid-

selected counts with any probes. This argues against the possibility that the results were due to the labeling of contaminating DNA or RNA in the extract. The results of these control experiments show that the observed effects are due to a factor(s) in the nodule extract acting on the isolated nuclei to specifically activate the chromatin region comprising the nodulin genes.

In order to determine if the activations were in direct response to a *Rhizobium* factor(s), nuclei were preincubated with an extract made from a free-living *B. japonicum* (61A76) culture. The results of the hybrid selections suggest that the activation of the nodulin genes is not directly due to a *Rhizobium* factor from the free living cells (data not shown). We have, however, not ruled out the possibility that this factor requires an interaction between the two organisms or is produced by *Rhizobium* only after it has entered the plant cell.

Specificity of the transcripts. The specificity of the nodulin gene activations was further investigated by examining the distribution of transcripts along both strands of one of the activated genes. The nodulin-23 gene was chosen for detailed study. Single-stranded M13 probes covering both strands were bound to filters and used for the hybrid selection experiments. Preincubation of embryonic nuclei in nodule extract followed by *E. coli* RNA polymerase transcription and hybrid selection to M13 probes yielded a nonuniform distribution of transcripts (Fig. 4). Most of the transcription was initiated at the 5' end of the gene generating transcripts 100–900 bases in length, probably initiated at regions of altered chromatin structure where polymerase accessibility would be greatest. This may explain why there is little transcription into the coding region of the gene. Similar results were obtained by Craine and Kornberg (1981a) in their studies of the *Drosophila* heat

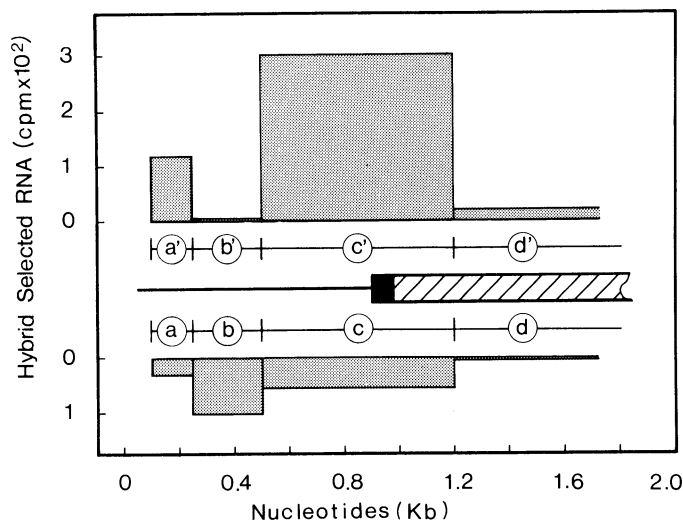


Fig. 4. Mapping of the nodulin-23 gene for transcripts produced in isolated nuclei. Nuclei were preincubated with nodule extract for 2 hr and transcription was as described in Figure 2. The RNA was hybrid-selected to filter-bound M13 clones (Mauro *et al.* 1985), washed, eluted, and counted. Control values were obtained from nuclei preincubated for 2 hr in buffer and were subtracted from values with nodule extract. The nodulin-23 gene coding region is represented as a hatched box. The 5' noncoding region is the black area and the 5' flanking region is a solid line. The various M13 probes are indicated by the lines labeled a-d (clones from the coding DNA strand) and a'-d' (clones from the noncoding strand).

shock genes. The transcription observed from the normally nontranscribed strand may be the result of transcription from a potential "antisense promoter" reported on this gene by Wong and Verma (1985). The significance of this result, however, is not clear at this stage and other nodulin genes need to be tested.

The observation that some of the strand-specific probes only hybrid-selected a few labeled RNA molecules raised the concern that these probes might be incapable of hybridizing to RNA. To test this, cRNA molecules were synthesized by transcribing the genomic clone for nodulin-23 with *E. coli* RNA polymerase *in vitro* and carrying out hybrid selections. All of the probes were capable of hybrid-selecting several thousand counts of RNA. Therefore the results were due to a lack of hybridizable sequences as opposed to an inability of the probes to hybridize with respective sequences. Transcription of the nodulin-23 gene in response to nodule extract, therefore, appears to be a gene-specific response and not the result of other chromosomal events allowing transcription throughout this region of DNA.

DISCUSSION

Nodulins are encoded by a set of genes that are expressed only in nodule tissue following infection of a leguminous plant by *Rhizobium*. They code both for structural proteins (Verma *et al.* 1986) and enzymes involved in nodule-specific carbon (Thummler and Verma 1987) and nitrogen metabolism (Nguyen *et al.* 1985; Cullimore *et al.* 1984; Sengupta-Gopalan and Pitas 1986). Various nodulin genes appear to be regulated by different signals at different stages of nodule development as deduced by studies of various *Rhizobium* mutants (Stanley *et al.* 1986; Fortin *et al.* 1987; Morrison and Verma 1987). There are two distinct groups of nodulins; those that are induced early in nodule development, e.g. nodulin-75, which does not require the physical presence of bacteria inside the root tissue (Franssen *et al.* 1987) and the late nodulins, which clearly require the physical presence of infection thread containing *Rhizobium* (Fortin *et al.* 1987; Morrison and Verma 1987; Verma and Delauney 1987). The results we have obtained using an *in vitro* transcription assay suggest the existence of transacting regulatory factor(s) in soybean nodules that may be involved in the coordinate regulation of a set of nodulin genes during symbiosis with *Rhizobium*. The regulation of the nodulin-35 gene appears to be different than those of nodulin-23, -24, and Lbc3 genes and a different transacting factor may be involved. This gene is expressed at a later time in nodule development (Nguyen *et al.* 1985), it does not carry the 5' consensus sequences (Verma *et al.* 1986), and it displays different activation kinetics in the *in vitro* activation assay.

Various lines of evidence suggest that structural differences exist between inactive and actively transcribing chromatin. For example, polytene chromosome puffing has been correlated to gene activity in insect salivary glands (Pelling 1964; Tissieres *et al.* 1974; Lamb and Daneholt 1979; Sass, 1982; Simon *et al.* 1986). Active chromatin has also been shown to be more accessible to those proteins that bind to DNA. These include DNase I, which preferentially digests genes that are active (Emerson *et al.* 1985; Romain *et al.* 1986), and *E. coli* RNA polymerase, which preferentially transcribes active genes (Tan and Miyagi 1970; Chiu *et al.* 1975; Craine and Kornberg 1981a, 1981b). The data presented here suggests that the specific regions of chromatin in nuclei from uninfected soybean tissue becomes

accessible for transcription following treatment with a nodule factor(s).

Further studies may reveal the mechanism by which *Rhizobium* maneuvers the host plant to change its pattern of gene expression and activate a set of nodulin genes that are involved in the formation and function of the nodules. By purifying and studying the molecules that regulate the expression of the nodulin genes, we may eventually be able to isolate their genes. Knowledge of these regulatory genes will be relevant to the successful engineering of novel plants that can respond correctly to *Rhizobium* infection leading to nitrogen fixation.

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