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Induction and Expression of Nodule-Specific Host Genes in Effective and Ineffective Root Nodules of Soybean†

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ABSTRACT: Regulation of moderately abundant host sequences in soybean root nodules was studied by using purified complementary deoxyribonucleic acid (cDNA) probes prepared by hydroxylapatite fractionation of total nodule cDNA. A probe for a subset of moderately abundant sequences (M1-cDNA) hybridized with uninfected root polysomal RNA containing poly(adenylic acid) [poly(A)+] to 52% as compared to 77% with homologous (nodule) RNA, suggesting the presence of "nodule-specific" sequences within this RNA population. A nodule-specific cDNA probe (NS-cDNA) with a complexity of 2.3×10^4 nucleotides was then purified following hybridization of moderately abundant cDNA (M-cDNA) with uninfected root mRNA. NS-cDNA hybridized to over 95% with nodule mRNA and to only 15% with uninfected root mRNA. These sequences are of host origin as demonstrated by the hybridization of NS-cDNA with soybean DNA and its lack of hybridization with *Rhizobium* DNA. Hybridization of NS-cDNA with nuclear RNA from uninfected root, hypocotyl, and leaf suggested that these sequences either are not transcribed or are present at exceedingly low levels within these organs. They appear to be regulated at the transcriptional level since NS-cDNA reacts to the same extent with nuclear and polysomal RNAs from uninfected tissue. Their relative concentration increases in parallel with leghemoglobin during nodule development and reaches a pleateau when nitrogen fixation commences. At this stage, they represent about 7.5% of the mass of polyadenyated polysomal RNA in root nodules. The concentration of nodule-specific sequences is reduced to different extents in nodules developed due to the infection with ineffective strains of *Rhizobium*. This study demonstrated the presence of a set of nodule-specific host sequences which are induced following infection of *Rhizobium* and may be involved in symbiotic nitrogen fixation.

The molecular events leading to the successful infection and development of a root nodule symbiosis between legume hosts and *Rhizobium* spp. are largely unknown. The notion is emerging that a precisely timed, intricately coordinated expression of specific plant and bacterial genes is essential for the establishment of an effective, nitrogen-fixing nodule [see Verma (1980a) for review]. Several host genes have been implicated in this process, and some of them have been identified through classical genetic experiments (Nutman, 1956; Holl & LaRue, 1976; Caldwell & Vest, 1977). In addition to leghemoglobin (Lb), which is obligatory for symbiotic nitrogen fixation in legumes, a number of other "nodule specific" host proteins (nodulins), which may be involved in the development of root nodule symbiosis, have been detected (Legocki & Verma, 1980).

Since nodulins are present in relatively low concentration in nodules and are not detected in uninfected roots (Legocki & Verma, 1980), the mRNA sequences encoding these proteins may be absent or present in very low concentrations in roots prior to infection by *Rhizobium*. Comparison of the uninfected root and nodule mRNA populations by total cDNA/mRNA homologous and heterologous hybridizations revealed a marked shift in concentration of the superabundant (Lb) and moderately abundant mRNAs, but detection of a small mass fraction of nodule-specific sequences was beyond

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the sensitivity of such a kinetically heterogeneous probe (Auger et al., 1979). A cDNA probe partially enriched for moderately abundant sequences when hybridized with nodule and root mRNA resolved the moderately abundant RNA population into two kinetic components whose concentrations in the nodule were 20–30-fold higher than in the uninfected root (Verma, 1980b). Results obtained with a probe enriched for the more abundant kinetic component suggested that a small number of nodule-specific sequences are present within this mRNA population.

Fractionation of the moderately abundant cDNA population into the nodule-specific and common moieties confirmed the presence of a small number of nodule-specific sequences. We demonstrate that these sequences are of host origin and are not detectable in the nuclear and polysomal RNAs from the uninfected tissue and that their expression appears to be influenced by mutations in *Rhizobium* which result in the development of ineffective nodules.

Materials and Methods

Growth of Plant Tissues. Soybean seeds (*Glycine max*, var. Prize) were obtained from Strayer Seed Farms, Hudson, IO, inoculated with *Rhizobium japonicum* strain 61A76, and grown as described previously (Verma et al., 1974, Verma & Bal, 1976). Root nodules were harvested at different stages of development (9–35 days) and stored in liquid nitrogen. Ineffective (unable to fix nitrogen) nodules developed by mutant strains 61A24 (Nitragin Co., Milwaukee, WI) and SM5 (Maier & Brill, 1976) were harvested at 21 days after infection. Uninfected root tips (2–3 cm) were harvested from 3-day-old seedlings of soybean. Embryo axes were handpicked from imbibed seeds.

Preparation of RNA. Poly(A)+ RNA[1] was isolated from total polysomal RNA as described previously (Verma et al., 1974; Auger et al., 1979). Nuclear RNA from uninfected root tissue was prepared by isolating nuclei (Goldberg et al., 1978) and extracting the RNA as by Glisin et al. (1974). The nuclear pellets were resuspended (2.5 mL/g of tissue) in 100 mM Tris-HCl, pH 8, and 4% (w/v) sodium laurylsarcosinate, and solid $CsCl_2$ (3 g/g tissue) was added. The homogenate was centrifuged at 15000 rpm for 15 min at 15 °C, and the supernatant was filtered through two layers of Miracloth (Calbiochem). The filtered supernatant (4 mL) was layered over 0.9 mL of a 5.7 M $CsCl_2$, 100 mM Na_2EDTA, and 50 mM NaOAc, pH 5.5, cushion in a cellulose nitrate tube and centrifuged in a Beckman SW56 rotor at 34000 rpm for 18–20 h at 20 °C. The clear RNA pellet was dissolved in chelexed water, made 0.3 M in NaOAc, pH 5.5, and precipitated with 2 volumes of ethanol at −20 °C.

Total poly(A)+ RNA from uninfected tissue was prepared by homogenizing tissue in RNA extraction buffer (Verma et al., 1974) followed by 3 phenol extractions and isolation of the polyadenylated moiety from the ethanol precipitated RNA by oligo(dT)–cellulose chromatography.

Preparation of DNA. DNA was prepared from *Rhizobium japonicum* (strain 61A76) as by Marmur (1961) and sonicated to an average size of 500–1000 base pairs. Its kinetics of reassociation, assayed by hydroxylapatite chromatography, were essentially identical with those reported by Sutton (1974), and 90% reassociation was achieved by a C_0t = 100.

Nuclei were isolated from soybean embryos as described above, and DNA was purified by two ethidium bromide–cesium chloride centrifugations as reported by Bendich et al. (1980). The DNA was sonicated to a size of 500–1000 base pairs as determined by the ethidium bromide staining pattern of an agarose gel (Sharp et al., 1973).

Preparation of cDNA Probes. A flow diagram of various steps involved in fractionation of nodule cDNA is in Figure 2. All phosphate buffers (equimolar mono- and dibasic sodium phosphate) were chelexed, and glassware and the jacketed hydroxylapatite (HAP) column were siliconized to minimize losses during the fractionation procedures. Elutions from the HAP column were in a stepwise fashion with 2-mL aliquots of phosphate buffer. The approximate mass fraction of total nodule cDNA comprised by the various cDNA probes are indicated as percent values. About 1×10^8 cpm of ^{3}H-labeled total nodule cDNA, synthesized as described previously (Auger et al., 1979), with a specific activity of 2×10^7 cpm/μg was hybridized with a 10-fold excess of nodule poly(A)+ polysomal RNA to EROT = 0.1. Following hybridization, samples were diluted and passed over a 0.5-mL HAP (DNA grade, Bio-Rad) column in 12 mM PO_4 buffer. The double-stranded moiety (20%), comprising mainly Lb sequences, was removed. The single-stranded fraction eluted with 120 mM PO_4, comprising the moderately abundant and rare sequences, was recovered by centrifugation at 50000 rpm for 8 h and rehybridized with additional nodule mRNA to EROT = 10.0. The double-stranded moiety (17%), containing moderately abundant sequences (M-cDNA), was eluted from the HAP column with 400 mM PO_4 buffer and recovered as above. Following hydrolysis of the RNA in 0.3 N NaOH for 60 min at 60 °C, the cDNA was recovered by ethanol precipitation. The nodule-specific and common moieties were separated by hybridizing M-cDNA with at least 2000-fold excess of either poly(A)+ polysomal or total poly(A)+ RNA from uninfected root to EROT = 2000. Following HAP fractionation, both the single-stranded (NS-cDNA, 7.5%) and double-stranded (common M-cDNA, 9%) moieties were recovered as above, and the hybridization cycle of NS-cDNA to uninfected root RNA was repeated. After hydrolysis of the RNA, the cDNA was recovered by ethanol precipitation, dissolved in chelexed double-distilled water, and stored at −20 °C. NS-cDNA recovery was about 1–2% of the starting material.

Solution Hybridizations. cDNA/RNA hybridization reactions were carried out as described previously (Auger et al., 1979) except that following S_1 nuclease digestion quantitation of hybrid formation was performed by using Whatman DE-81 filter paper disks (Maxwell et al., 1978). cDNA/DNA reassociations were performed as described by Baulcombe & Verma (1978), with DNA excesses of 5×10^5 and 2×10^6 with *Rhizobium* and soybean DNAs, respectively. Briefly, 1000–2000 cpm of [^{3}H]cDNA was incubated with RNA or DNA in 0.6M NaCl, 10 mM Hepes, pH 7.8, and 10 mM EDTA at 68 °C in 1.5-mL polypropylene micro test tubes (Bio-Rad) overlaid with liquid paraffin. Hybridizations were started by boiling the reaction mixture for 2 min, quick-cooling in an ice–water bath, and incubating at 68 °C for the required times. Reactions were terminated by immersion of the tube in liquid nitrogen. The unannealed cDNA was digested for 90 min with 50 μL of S1 nuclease buffer as described previously (Auger et al. 1979). The samples were spotted onto

[1] Abbreviations used: poly(A)+ RNA, RNA containing poly(adenylic acid); "nodule-specific" sequences, poly(A+) RNA sequences present in root nodule polysomes but undetectable in uninfected root RNA; M-cDNA, complementary DNA for moderately abundant nodule sequences; M1-cDNA, complementary DNA for a subset of moderately abundant nodule sequences; NS-cDNA, complementary DNA for moderately abundant nodule-specific sequences; common M-cDNA, complementary DNA for moderately abundant nodule sequences present in both root and nodule tissue; EROT and ECOT, products of RNA and DNA concentrations, respectively (mol L^{-1}), and time(s) corrected to equivalent salt concentration of 0.18 M Na (Britten et al., 1974).

Table I: Sequence Complexity Analysis of Nodule-Specific and Common M-cDNA Probes

probe	mass fraction of total N-cDNA	$R_0t_{1/2}$ obsd (mL^{-1} s)	$R_0t_{1/2}$ pure[a] (mL^{-1} s)	complexity[b] (nucleotides)	no. of sequences[c]	molecules/cell/ sequence[d]
NS-cDNA	0.075	0.24	1.35×10^{-2}	2.3×10^4	19 (40)	2400 (1145)
common M-cDNA	0.09	4.7	0.3	5.4×10^5	440	125

[a] Corrected for 25% rRNA contamination and for the fraction of each probe presented in nodule mRNA. [b] Complexity is compared to globin mRNA of complexity 1200 and our determined $R_0t_{1/2}$ value 7×10^{-4}. [c] Value in preceding column divided by number average size of total mRNA (1240 nucleotides). The values in parentheses in this and the following column represent the number of sequences and molecules/cell/sequence based upon the number average size of nodule-specific mRNA which was experimentally determined to be 567 nucleotides by hybridization of NS-cDNA to electrophoretically fractionated mRNA. [d] (Fraction of N-cDNA × number of poly(A) + RNA molecules) divided by value in the preceding column (see Auger et al., 1979).

Whatman DE-81 filter paper disks, which were first washed with ether to remove paraffin oil and then washed repeatedly with 0.5 M phosphate buffer, distilled water, and ethanol as described by Maxwell et al. (1978). The filters were dried and counted in toluene-based scintillant. Each experiment included duplicates of all R_0t values and undigested controls. After subtraction of the zero R_0t background from each sample, the fraction of [^{3}H]cDNA hybridized was calculated as the ratio of filter-bound radioactivity in the sample to the undigested controls.

Computer Analysis. The cDNA/RNA reassociations were analyzed and plotted as described previously (Auger et al., 1979), using a program of Pearson et al. (1977). The cDNA/DNA hybridization data were analyzed with another option of Pearson's program by employing a modified second-order reaction and plotted by using a HP 9830A plotter.

Results

Identification of Moderately Abundant Nodule-Specific Sequences and Preparation of a cDNA Probe. Hybridization kinetics of a probe partially enriched for moderately abundant nodule sequences, with nodule and root mRNAs, revealed differences in concentration of this RNA population during nodule development (Verma, 1980b). A probe, M1-cDNA, for a subset of moderately abundant sequences enriched for the faster kinetic component, hybridized to only 52% with uninfected root mRNA ($R_0t_{1/2}$ = 45), as compared to 77% with its homologous (nodule) mRNA ($R_0t_{1/2}$ = 0.84) (Figure 1). The 25% differential hybridization of M1-cDNA between nodule and uninfected root mRNA suggested the existence of "nodule-specific" sequences within this population.

In order to obtain a nodule-specific cDNA probe, we first removed the most and the least abundant sequences of nodule cDNA by kinetic fractionation, following hybridization with homologous RNA (Figure 2). The resulting moderately abundant cDNA (M-cDNA) was then hybridized with uninfected root mRNA to a R_0t = 2000 and fractionated on a hydroxylapatite column. The sequences common to root and nodules (common M-cDNA) were recovered from hybrids while unhybridized sequences, sequences specific to nodules (NS-cDNA), were further purified by another cycle of rehybridization. The results of this fractionation are shown in Figure 3 and quantified in Table I.

NS-cDNA hybridizes to more than 95% with its homologous (nodule) mRNA ($R_0t_{1/2}$ = 0.24) and to less than 15% with uninfected root mRNA ($R_0t_{1/2}$ = 83) (Figure 3a). The almost identical kinetics obtained by using two probes prepared at different times from different tissue samples (closed and open circles, Figure 3a), indicate the reproducibility of the procedure. To ensure that the low hybridizability of NS-cDNA to uninfected root mRNA was not caused by degradation of the NS-cDNA, homologous (nodule) mRNA was added to the reaction mixture at the highest R_0t point (3700,

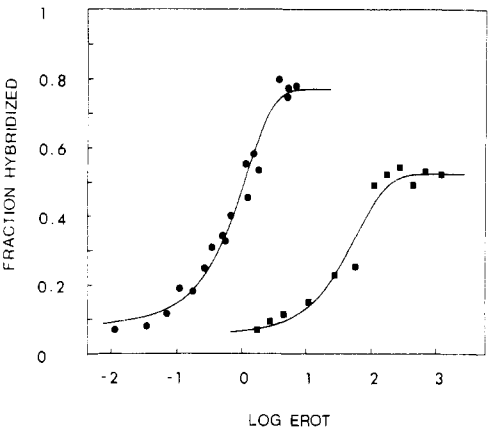

FIGURE 1: Hybridization of M1-cDNA to poly(A)+ mRNA from nodules and uninfected roots. All symbols here and in subsequent figures represent experimental data, and continuous lines are computer fits to the data. M1-cDNA hybridized to only 50% with uninfected root RNA (■) as compared to almost 80% with its homologous (nodule) RNA (●).

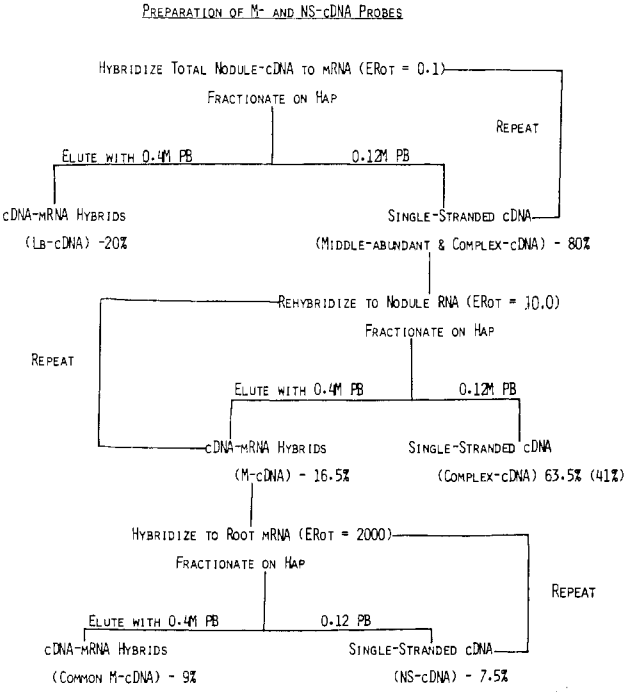

FIGURE 2: Flow diagram for the procedure to purify various cDNA probes (see Materials and Methods for details). The approximate mass fraction of total nodule cDNA comprised by each moiety is indicated as a percent value. Lb-cDNA, 20%, comprises the most abundant nodule mRNA class and M-cDNA, 16.5%, the moderately abundant mRNA population, is subdivided into the nodule-specific (7.5%) and common M (9%) moieties, which are also kinetically distinct (see Table I and Figure 3). Complex cDNA is only 65% reactive with nodule mRNA and, hence, contains some unhybridizable elements. Its corrected mass fraction, 41% (0.65 × 0.635), is indicated in parentheses.

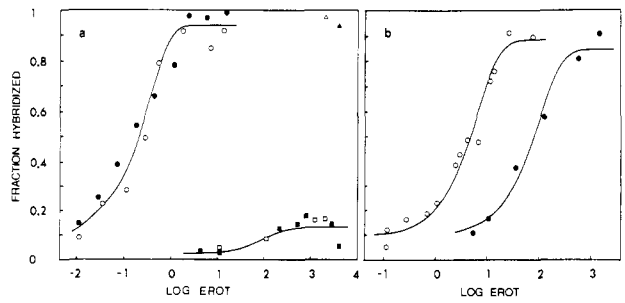

FIGURE 3: Hybridization of NS-cDNA and common M-cDNA to poly(A)+ RNA from nodules and uninfected root. (a) NS-cDNA was purified from M-cDNA (Figure 2). Open symbols represent a NS-cDNA probe prepared following hybridization with total poly(A)+ RNA from uninfected root and assayed by Cl_3AcOH precipitation, and closed symbols represent a different NS-cDNA probe preparation assayed by using Whatman DE-81 filters. About 1000 cpm of NS-cDNA were hybridized to 2000-fold excess nodule poly(A)+ RNA (●, ○) and 140000-fold excess uninfected root poly(A)+ RNA (■, □). At the termination of the hybridization to root RNA, homologous (nodule) RNA was added and allowed to hybridize to EROT = 10, whereupon NS-cDNA hybridized to greater than 95% with its homologous RNA (▲, △), compared to about 15% with uninfected root RNA. (b) Common M-cDNA (see Materials and Methods), freed of the nodule-specific moiety, hybridized to root-RNA (●) and with nodule RNA (○).

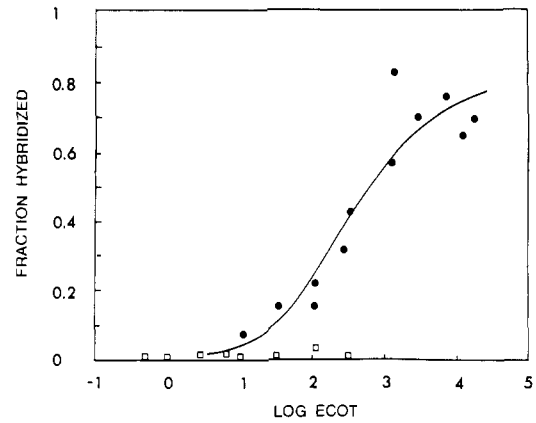

FIGURE 4: Hybridization of NS-cDNA to soybean axes DNA and *Rhizobium* DNA. Hybridizations were carried out in DNA excess; *Rhizobium* DNA/cDNA was 10^5:1 and soybean DNA/cDNA 2×10^6:1. There was no reassociation of NS-cDNA with *Rhizobium* DNA (□) whereas NS-cDNA hybridized (assayed with S_1 nuclease) to 75% with soybean DNA(●), $C_0t_{1/2}$ = 360.

i.e., end of the incubation) and allowed to hybridize to a R_0t of about 10. NS-cDNA hybridized to greater than 95% with nodule mRNA when added to the root mRNA hybridization reaction.

The 15% reactivity of NS-cDNA with uninfected root mRNA may be due to contaminating common and/or leghemoglobin (Lb) sequences present within NS-cDNA. Preliminary data on the hybridization of NS-cDNA with a Lb-cDNA clone (pLb1; D. Sullivan and D. P. S. Verma, unpublished results) indicated that Lb sequences may comprise 10% of this probe. Contamination could occur either from incomplete hybridization of all common sequences to root RNA during the preparative hybridization or from destabilization of some of the cDNA/mRNA hybrids during the HAP fractionation, resulting in their elution with the single-stranded NS-cDNA moiety [see Hastie & Bishop (1976)]. If this is the case, the lack of hybridization beyond the 15% level could imply that nodule-specific sequences are absent from or are present below the level of detection in root polysomal mRNA. However, the possibility remains that the 15% hybridization of NS-cDNA with uninfected root mRNA could represent the limits of detection of extremely rare sequences present in only a small number of root cells. Due to the above limitations, the values in table I should be regarded as approximate estimates of the number and mass fraction of these sequences in nodule cDNA.

The kinetics of hybridization of common M-cDNA to nodule mRNA ($R_0t_{1/2}$ = 4.7) and its complexity are similar to those of the middle abundant class of nodule mRNA sequences reported in Auger et al. (1979), although its mass fraction is lower due to the resolution of M-cDNA into two components. Common M-cDNA hybridizes to greater than 90% with uninfected root mRNA, indicating that these moderately abundant sequences are present in the uninfected tissue. The difference in kinetics of common M-cDNA hybridized to mRNA from uninfected root ($R_0t_{1/2}$ = 75) and nodule tissue ($R_0t_{1/2}$ = 5) indicates that the relative concentration of these sequences increases significantly (15-fold) during nodule development.

Host Origin of Nodule-Specific Sequences. NS-cDNA is composed of the second most abundant class of mRNA sequences in the nodule poly(A)+ polysomal mRNA population. Although this RNA was obtained from host polysomes that are substantially free of bacteroidal ribosome contamination (Verma & Bal, 1976; Legocki & Verma, 1980), the possibility of these sequences being encoded by the *Rhizobium* genome could not be ruled out. Accordingly, NS-cDNA was hybridized to *Rhizobium* DNA (Figure 4) in DNA excess (500000). No hybridization was observed, even up to C_0t 300, i.e., over 3 times greater than that needed for the complete reassociation of *Rhizobium* DNA [see Sutton (1974)]. As shown in Figure 4, NS-cDNA hybridized to soybean embryo DNA to greater than 75% with a $C_0t_{1/2}$ = 360, following S1 nuclease kinetics (a modified second-order reaction). Because the reassociation may not have reached saturation by the highest C_0t value (10000) in these experiments and NS-cDNA may be contaminated with slightly reiterated (Lb) sequences, a precise copy number for nodule-specific sequences cannot be established from the kinetics of this reaction. However, similar kinetics of hybridization were observed by using total root cDNA/soybean embryo DNA ($C_0t_{1/2}$ = 410; S. Auger, unpublished results). Total cDNA/DNA reactions appear to follow single or low copy kinetics (Goldberg et al., 1978; Kiper et al., 1979). The lack of hybridization of NS-cDNA to *Rhizobium* DNA and its extensive hybridization to soybean DNA clearly demonstrate the host origin of the nodule-specific sequences.

Transcriptional Regulation of Nodule-Specific Sequences. The lack of hybridization of NS-cDNA (above the 15% level) to the poly(A)+ polysomal RNA from uninfected root suggested that these sequences are either absent from or extremely rare within the root polysomal polyadenylated RNA (see above). For determination of whether these sequences were present in the nuclear RNA fraction, total nuclear RNA was prepared from uninfected roots. Since the extent of hybridization of NS-cDNA with root polysomal poly(A)+ RNA is known (15%), contamination of the nuclear RNA with cytoplasmic RNA, if any, would not prevent detection of these sequences in the nuclear-RNA fraction, providing sufficient RNA excess is used. Root cDNA prepared from root poly(A)+ RNA and nodule common M-cDNA were also hybridized to the root nuclear RNA (Figure 5). Both probes hybridized to 90%, indicating that root cytoplasmic as well as nodule moderately abundant common sequences are detectable in this RNA. As shown in Figure 5, both probes exhibit similar heterogeneous kinetics within the root nuclear

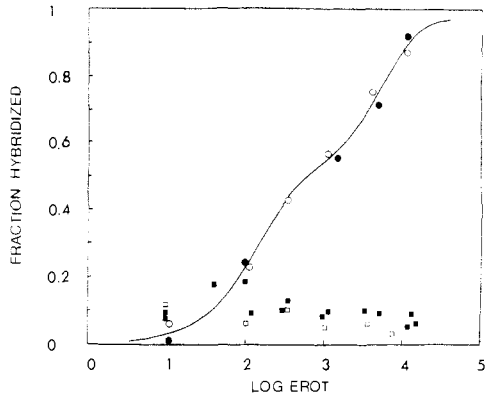

FIGURE 5: Hybridization of total root cDNA, common M-cDNA, and NS-cDNA with uninfected root total nuclear RNA. Root cDNA (○), common M-cDNA (●), and NS-cDNA (■). Hybridization of NS-cDNA with leaf total nuclear RNA (□).

RNA, indicating that the majority of these RNA transcripts are present at similar concentrations within the nuclear RNA [see also Goldberg & Kamalay (1978)]. The heterogeneity in kinetics of the nuclear RNA reaction could be the result of contamination of the nuclear RNA with cytoplasmic sequences or may reflect frequency differences of the transcripts. Total root cDNA and common M-cDNA sequences were also well detected in nuclear RNA from hypocotyl and leaf tissues (data not shown), implying that the genes for most structural mRNAs are constitutively transcribed within various organs [see also Kamalay & Goldberg (1980)]. However, NS-cDNA hybridized to only about 15% with nuclear RNA from root, leaf (Figure 5), and hypocotyl tissues (data not shown), similar to its reaction with root poly(A)+ polysomal RNA, even though great RNA excess (360 000-fold) was used and the reaction carried to a R_0t of more than 10000. That NS-cDNA reacted to the same extent with root nuclear and polysomal RNAs implies a primarily transcriptional mode of regulation of nodule-specific sequences.

The possibility that nodule-specific transcripts are present at exceedingly low levels within nuclear RNAs of uninfected tissue cannot be ruled out. However, that the 15% reaction may be due to contaminating common sequences within the probe is a more likely explanation (see above). The lack of hybridization beyond this level implies either that nodule-specific sequences are not transcribed or are beyond the level of detection or that they are transcribed but rapidly turned over, preventing their detection within the nuclear RNAs from root, hypocotyl, and leaf tissues.

Expression of Nodule-Specific Sequences in Ineffective Nodules. For determination of whether the nodule-specific sequences were present in ineffective nodules developed by infection with *Rhizobium japonicum* strains SM5 and 61A24, NS-cDNA was hybridized to RNA from these tissues (Figure 6). The hybridizations of NS-cDNA to 90% or greater with RNA from both ineffective nodules indicate that these sequences are present in nodules irrespective of their effectiveness in nitrogen fixation. The shift in kinetics and the biphasic nature of the hybridizations indicate that, in addition to being less abundant, these sequences are present at varying concentrations within the RNA populations of ineffective nodules (cf. the pseudo-first-order kinetics observed in the wild type, Figure 3a). The concentration of nodule-specific sequences is more reduced in 61A24 than in SM5-induced nodules, as is the case with Lb sequences, although the degree of reduction is not as marked [see Verma et al. (1980)].

In order to determine whether the expression of other mRNA sequences is influenced by ineffective strains of

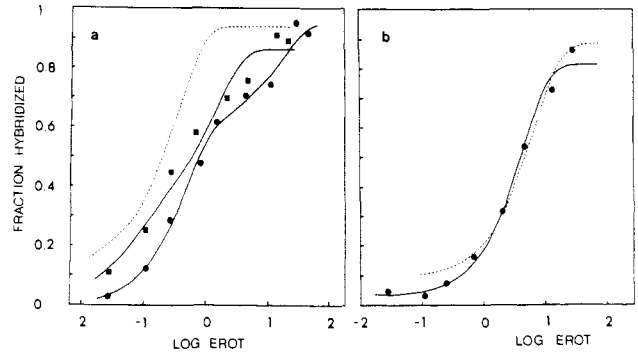

FIGURE 6: Expression of NS-cDNA (a) and common M-cDNA (b) sequences in poly(A)+ RNA from ineffective nodules. (a) Hybridization of NS-cDNA to poly(A)+ RNA from SM5 (■) and 61A24 (●) induced ineffective nodules. The data could not be fit to a single component. The dotted line represents the kinetics of reassociation of NS-cDNA to wild-type nodule poly(A)+ RNA (Figure 3a). (b) Hybridization of common M-cDNA to 61A24 nodule poly(A)+ RNA (●). The dotted line represents the kinetics of reassociation of common M-cDNA to wild-type nodule poly(A)+ RNA (Figure 3b).

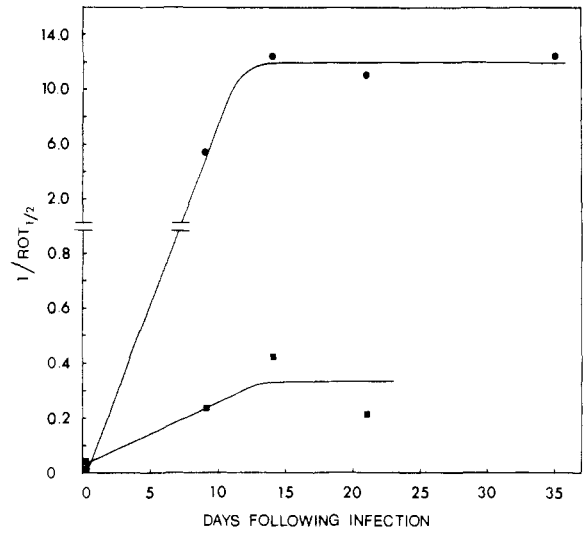

FIGURE 7: Increase in relative concentrations of NS-cDNA and common M-cDNA sequences during root nodule development. NS-cDNA (●) and common M-cDNA (■) probes were hybridized to nodule RNAs at various times following infection. The relative concentration of these sequences at each stage of development is indicated by the reciprocal of the $R_0t_{1/2}$ value of the reaction.

Rhizobium, common M-cDNA was hybridized to poly(A)+ RNA from 61A24 induced nodules (Figure 6). Although considerable shifts in concentration of Lb and nodule-specific sequences are observed in these nodules, the expression of common moderately abundant sequences is virtually unchanged from that of the effective nodule.

Expression of Nodule-Specific Sequences during Root Nodule Development. Figure 7 summarizes the data that show an increase in the relative concentration of nodule-specific and common moderately abundant sequences during root nodule development. The physiological parameters of the young, rapidly proliferating nodule tissue change extensively as the nodule matures. The amount of mRNA/cell and the number of cells/nodule may not remain constant throughout development, and consequently changes in $R_0t_{1/2}$ values may not be directly reflective of changes in the absolute number of molecules per sequence per cell. Nevertheless, these data show that both nodule-specific and common moderately abundant sequences increase in concentration relative to other nodule sequences during early nodule development. Both these populations exhibit similar pattern of development to that of

Lb mRNA sequences (Auger et al., 1979) with a maxima obtained by about day 13 or 14 following infection with *Rhizobium*. The magnitude of increase differs, however, with the common moderately abundant sequences increasing about 15-fold, while nodule-specific sequences increase to 2000-fold during the same period. Since NS-cDNA hybridized to over 90% with 9-day nodule RNA, nodule-specific sequences are induced prior to the expression of nitrogenase which occurs at day 10 or 11 (Verma et al., 1979, 1980), and thus appear to be independent of nitrogen fixation. This is also confirmed by their presence in ineffective nodules, although at a reduced level. These data and that of Lb-mRNA show that the specific expression of certain host genes occurs early in the development of the root nodule.

Discussion

Fractionated cDNAs and single-copy DNA probes have been used in many systems to estimate the number of "tissue-specific" sequences (Hastie & Bishop, 1976; Axel et al., 1976; Galau et al., 1976; Ernst et al., 1979; Kamalay & Goldberg, 1980). The enhanced sensitivity of the fractionated probes used in this study enabled the detection of qualitative and quantitative differences in moderately abundant sequences of uninfected root and nodule tissues. Assuming an average size of 1240 nucleotides per sequence, the complexity = 2.3×10^4 of the nodule-specific probe (NS-cDNA) corresponds to a population of 19 sequences; however, since the average size of NS-mRNA is smaller (567 nucleotides), the number of nodule-specific sequences may be higher (see Table I). These estimates should be regarded as tentative since possible contamination by other sequences could influence the kinetics and mass fraction estimate of NS-cDNA. A small number of nodule-specific polypeptides was detected independently by the immunoprecipitation of nodule–polysome translation products with a "nodule-specific" antibody (Legocki & Verma, 1980). There is no direct evidence that the nodule-specific sequences encode the nodulins, but since only the abundant newly synthesized proteins can be detected on gels [see Davidson & Britten (1979)] and since both cDNA and antibody probes were prepared by removing common sequences or proteins, it is likely that some of these sequences code for nodule-specific proteins.

The host origin of the nodule-specific sequences was demonstrated by the hybridization of NS-cDNA with soybean DNA from uninfected tissue and its lack of reassociation with *Rhizobium* DNA. NS-cDNA reacted to the same limited extent with both nuclear and polysomal RNAs from uninfected root, hypocotyl, and leaf, suggesting that these genes are either repressed or expressed at very low levels in these tissues. This implies that their principal mode of regulation following infection is transcriptional, as seems to be the case for other abundant gene products following activation [ovalbumin, Ono & Getz (1980); fibroin, Suzuki (1975)]. This contrasts with the constitutive transcription and primarily posttranscriptional mode of regulation of structural genes encoding rare class mRNAs (Kamalay & Goldberg, 1980; Ernst et al., 1979).

Lb, nodule-specific, and common moderately abundant sequences are differentially expressed during the early stages of infection (i.e., up to about day 14). Expression of other abundant plant gene products has been shown to be quantitatively regulated during development (Higgens et al., 1976; Link et al., 1978; Tobin, 1978). The accumulation of nodule sequences in the cytoplasm following infection could be the result of increased rate of transcription and/or a variety of posttranscriptional mechanisms such as a higher rate of processing and transport of RNA from the nucleus and its increased stability (Tobin, 1979). Increase in the concentration of common moderately abundant sequences appears to be modulated by auxins (S. Auger and D. P. S. Verma, unpublished data), known to be present in nodule tissue (Dullaart, 1967).

Similar to Lb, nodule-specific sequences are present in the ineffective nodules produced by SM5 and 61A24 strains of *Rhizobium*, but at variably reduced concentrations [cf. Verma et al. (1980) and Figure 6a). If this reduced frequency of nodule-specific sequences in ineffective nodules was due to nitrogen starvation and/or a lower proportion of infected cells, all nodule sequences should be similarly affected. However, as indicated in Figure 6b, the concentration of common moderately abundant sequences is unchanged in ineffective nodules. The selective influence of ineffective strains of *Rhizobium* on the expression of Lb and nodule-specific genes is consistent with the notion that nodule-specific sequences may encode proteins which have a role in nodulation and/or processes related to nitrogen fixation (Verma 1980a). The induction of Lb and nodule-specific sequences appears to be independent of nitrogenase function. The cellular distribution of these sequences is unknown, but, if they are directly involved in symbiotic nitrogen fixation, their location may be restricted to the infected cells of the nodules.

Root nodule development involves a coordinated expression of specific genes of the two organisms for the establishment of an effective symbiosis (Verma, 1980a). Qualitative and quantitative regulation of host gene expression may play a major role in this highly complex process. The availability of nodule-specific recombinant molecules will facilitate the investigation of questions concerning their structural organization, regulation of transcription, and specific interaction with *Rhizobium*.

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Cysteine Auxotrophy of Human Leukemic Lymphoblasts Is Associated with Decreased Amounts of Intracellular Cystathionase Protein†

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ABSTRACT: A series of human lymphoblastoid cell lines derived from nonleukemic donors are known to be cysteine prototrophs (*cys*$^+$), while several lymphoblastoid lines derived from leukemic donors are cysteine auxotrophs (*cys*$^-$). We have tested representative cell lines of each type for their content of cystathionase enzyme activity by a specific catalytic assay and their total cystathionase protein content by immunoprecipitation of in vivo labeled protein. There was a close correlation between the cellular content of the enzyme as determined in the two assays. Specifically, those *cys*$^+$ lines having readily measurable enzyme by catalytic assay were found to contain significantly higher levels of immunoprecipitable M_r 43 000 cystathionase subunit than those *cys*$^-$ lines tested which were depleted in active enzyme. Thus, the absolute cysteine requirement of the leukemic, *cys*$^-$ cell lines tested is likely due to an intracellular reduction of cystathionase protein.

During the acquisition of a leukemic phenotype, numerous changes are presumed to occur in the membrane composition and metabolic chemistry of a hematopoietic cell. For example, it has been shown that human peripheral blood leukemic cells have markedly different requirements for specific amino acids when compared to their nonleukemic counterparts (Ohnuma et al., 1971). In one case, certain human and rodent hematopoietic tumor cell lines are auxotrophic for L-cysteine (*cys*$^-$) when compared to nontumored cell lines of similar origin (Livingston et al., 1976; Iglehart et al., 1977). Furthermore, extracts of these leukemic (*cys*$^-$) cell lines contain markedly reduced levels of detectable cystathionase catalytic activity when compared to their nonleukemic (*cys*$^+$) counterparts. As a result, *cys*$^+$ cell lines are capable of normal growth in the absence of preformed cysteine when cystathionine is present in the medium, while *cys*$^-$ cell lines are growth-arrested under these conditions. These in vitro studies complement the observation that a reduction in murine thymocyte cystathionase activity occurs during thymic leukemogenesis whether induced by exogenous administration of a type C virus or arising spontaneously, as is the case in AKR mice (Livingston et al., 1976). Additional evidence suggests that in the majority of fresh human lymphoid and myeloid leukemic cells, obtained by bone marrow aspiration, there is a reduction in the concentration of cystathionase enzyme activity compared to that present in extracts of normal human bone marrow mononuclear cells (Glode et al., 1979). Thus, there is a strong correlation between the presence of the leukemic phenotype and depletion of cystathionase enzyme activity in cells of both lymphoid and myeloid origin. In none of these cases could the reduction in measurable cystathionase activity be accounted for by the presence of a soluble inhibitor of this enzyme.

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