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Preparation of a complementary DNA for leghaemoglobin and direct demonstration that leghaemoglobin is encoded by the soybean genome

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ABSTRACT

In soybean root nodules, leghaemoglobin (Lb) accounts for 25-30% of the total soluble protein but is not detected in other tissues. In order to determine whether the Lb genes are plant or bacterial in origin a cDNA probe for Lb was prepared from 9S poly (A) containing mRNA of root nodules. Although this 9S mRNA directed synthesis of predominantly three forms of Lb in vitro, the kinetics of hybridisation of cDNA and the 9S mRNA showed a transition at about 30% hybridisation which suggested that the 9S-cDNA was not pure Lb-cDNA. The abundant, Lb-cDNA was prepared by two cycles of hybridising 9S mRNA and cDNA to a Rot of 3 X 10^{-3} and isolation of the hybridised cDNA on hydroxyapatite. The Lb-cDNA was homogeneous in hybridisation analysis with 9S mRNA and electrophoresis in 98% formamide gels. This cDNA hybridised with soybean DNA and not with Rhizobium DNA, thus directly demonstrating that Lb genes are of plant origin. Titration of Lb-cDNA with soybean DNA showed that Lb genes are reiterated about forty-fold per haploid genome.

INTRODUCTION

The paucity of information from higher plants regarding the mechanisms of expression of nuclear genes is partly due to the lack of hybridisation probes for defined mRNAs. The usefulness of a high specific activity cDNA transcript of a mRNA is illustrated by work with globin cDNA, which has been used, for example, to monitor globin mRNA levels in the nucleus and cytoplasm of erythropoietic and non-erythropoietic cells (1) and to detect the accumulation of globin mRNA during induction of Friend cells by dimethylsulphoxide (2). A globin cDNA probe has been used to determine the number of globin genes in human cells (3) and the repetition of the sequences next to the globin genes in the duck genome. (4). Double stranded globin cDNA has been amplified by insertion into an E. coli plasmid and the amplified insert used to determine the nucleotide sequence of the β-globin mRNA (5).

Although several plant mRNA species have been identified as poly A(+)

molecules (6-10), only two, leghaemoglobin mRNA (6) and zein mRNA (7, 10) have been prepared in any purity. This communication describes the preparation of a cDNA for leghaemoglobin from a partially pure mRNA.

Leghaemoglobin is synthesized exclusively in the nitrogen fixing root nodules which develop due to the symbiotic association of a bacterium (Rhizobium sp.) with legumes (11). In soybean nodules it accounts for 25-30% of the total soluble protein and is encoded by a 9S poly A(+) mRNA (6) which is translated on free polysomes in the plant cell cytoplasm (12). There are three major forms of leghaemoglobin in soybean, two of which have very similar amino acid sequences (13, 14). Indirect evidence suggests that leghaemoglobin may be a plant gene product (12, 15, 16). By showing that leghaemoglobin cDNA hybridised to soybean DNA and not to Rhizobium DNA we have now demonstrated directly that leghaemoglobin is encoded by the plant genome. The reiteration of the leghaemoglobin genes is about forty-fold, as shown by saturation hybridisation analysis.

MATERIALS AND METHODS

Growth of Plants: Soybean seeds (Glycine max. var. Kanrich) were germinated in moist vermiculite. Three day old seedlings were infected with Rhizobium japonicum ('Nitragin', the Nitragin Co., Milwaukee, Wisconsin) and replanted in vermiculite. Plants were grown under a 16 hr light (28°C), 8 hr dark (21°C) regime in constant 70% humidity. The nodules were harvested 3 weeks after infection and stored immediately in liquid nitrogen.

Messenger RNA Preparation: Messenger RNA was prepared from nodule polysomes. The nodules were ground to a fine powder in liquid N_2 and then homogenized in high Tris, high pH buffer (6) (150 mM Tris- HCl (pH 8.5, 0°C), 50 mM KCl, 20 mM $Mg(OAc)_2$, 0.3 M sucrose, 10 mM β mercaptoethanol, 0.4% Nonidet P-40 (Shell Chem. Co.)). After centrifugation (15,000 rpm, 20,000 gav) for 10 min, the supernatant was layered over 1 M sucrose in 50 mM Tris-HCl (pH 8.5, 0°C), 50 mM KCl, 10 mM $Mg(OAc)_2$. The polysomes were collected by centrifugation for 2 hr at 60,000 rpm (Beckman Ti 60 rotor) and total RNA was prepared (6). Poly A(+) mRNA was isolated by oligo dT-cellulose chromatography (17). 50 mg of RNA was dissolved in 10 mM Tris pH 7.6 (20°C), 10 mM EDTA, 400 mM NaCl and passed twice over a column of oligo dT-cellulose (2 cm x 1.5 cm). Bound RNA was eluted in 10 mM Tris-HCl pH 7.6, 10 mM EDTA and collected by centrifugation for 16 hr

at 50,000 rpm (Beckman Type 65 rotor). The 9S poly A(+) mRNA which contains leghaemoglobin mRNA (6) was prepared from total nodule poly A(+) mRNA by sucrose gradient centrifugation. The gradients were 5 to 20% sucrose in 10 mM Tris-HCl pH 7.5, 1 mM EDTA, and were centrifuged at 55,000 rpm (Beckman SW56 rotor) for 6 hr at 0°C. The final yield of 9S mRNA was about 40 µg per 100 g of nodules.

Rabbit globin mRNA was prepared from rabbit reticulocytes (Gibco). Polysomes were prepared from the lysed reticulocytes by centrifugation through 1M sucrose and 9S globin mRNA was prepared as described above for nodule 9S poly A(+) mRNA.

cDNA Preparation: cDNA for 9S-mRNA was prepared using avian myeloblastosis virus reverse transcriptase. Reaction mixtures contained in 50 µl: 50 mM Tris-HCl pH 8.3, 50 mM KCl, 8 mM $MgCl_2$, 4 mM dithiothreitol, 0.2 mM dATP, 0.2 mM dTTP, 0.2 mM dGTP, 0.2 mM [^{3}H]-dCTP (20 Ci/mmole, New England Nuclear), 30 µg ml^{-1} oligo dT_{10}, 4 mM sodium pyrophosphate, 20 µg ml^{-1} mRNA and 450 units ml^{-1} of reverse transcriptase (18). The mixture was preincubated without the enzyme for 10 min at 0°C and then incubated with the enzyme for 1 hr at 37°C. The reaction was stopped by addition of 1% sarkosyl and 10 mM EDTA. RNA was hydrolysed by the addition of 0.3M NaOH and incubation for 16 hr at 37°C. After neutralization, the unincorporated nucleotides were removed from the cDNA by passage through a Sephadex G50 column (15 x 0.7 cm) and the cDNA was collected by ethanol precipitation. Rabbit globin-cDNA was prepared by the same procedure using rabbit globin 9S-mRNA.

cDNA/RNA Hybridisation: All hybridisations were carried out in ethanol washed 1.5 ml conical test tubes (Brinkman) overlayed with liquid paraffin. Hybridisations contained RNA and cDNA, 0.18 M or 0.6M NaCl, 10 mM HEPES, 10 mM EDTA (pH 7.8) at 60°C (0.18 M NaCl) or 70°C (0.6M NaCl). The RNA: cDNA ratio was at least 100:1. The rate of reactions in 0.6M NaCl was corrected to standard (0.18M Na^+) conditions (19). Hybridizations were started after boiling for 3 min and stopped by rapid freezing in liquid N_2. The amount of hybridisation was assayed by digestion in 1 ml of 0.3M NaCl, 4.5 mM $ZnSO_4$, 30 mM NaOAc (pH 4.5), 5% glycerol with 25 µg of native and denatured calf thymus DNA (Sigma) and 1000 U ml^{-1} of S1 nuclease (Miles). Undigested material was TCA precipitated onto GF/A filters (Whatman). Samples were solubilized with Protosol (New England Nuclear) and counted in toluene based scintillant. Each experiment included zero Rot and undigested controls.

In order to prepare the leghaemoglobin cDNA, 9S-cDNA was incubated with a twenty-fold excess of 9S poly A(+) mRNA to a Rot of 2 x 10^{-3} in 1.0 ml. The reaction was diluted to 10 ml and passed over 0.5 ml column of hydroxyapatite (DNA grade, BioRad) at 65°C. Unhybridised material was eluted with 0.12M phosphate and the cDNA/RNA hybrids with 0.4M phosphate. The cDNA hybrids were passed over Sephadex G50 and collected by ethanol precipitation. The hybridisation and hydroxyapatite fractionation was repeated twice and the hybridised cDNA was purged of RNA by alkaline treatment (0.3M NaOH for 16 hr at 37°C). This cDNA was again passed over hydroxyapatite and eluted in 0.12M phosphate to remove any extensively double-stranded molecules. The leghaemoglobin cDNA was then collected after passage through Sephadex G50 by ethanol precipitation.

DNA Preparation: DNA was prepared from embryos of water embibed seeds as described by Scott and Ingle (20) except that batchwise binding and elution from hydroxyapatite replaced CsCl centrifugation as the final purification step. Nodule DNA was prepared by the same method from 15,000 rpm pellet material produced during polysome extractions. The proportion of soybean DNA, in the nodule DNA preparation was determined by CsCl equilibrium centrifugation.

Rhizobium japonicum (strain 61A76) was grown in culture to late log phase (21) and DNA was prepared by the method of Marmur (22).

Nick Translation of DNA: In vitro labelling of DNA by nick translation was by the method of Rigby et al (23). The reaction (0.1 ml) contained 5 mM Tris-HCl pH 7.8, 5 mM $MgCl_2$, 1 mM dithiothreitol, 50 µg ml^{-1} bovine serum albumin, 20 µM each of dATP, dGTP, dTTP and [^{3}H]-dCTP (20 Ci/mmole, New England Nuclear), 1 µg of soybean DNA and 5U of E. coli DNA polymerase I (Boehringer, Mannheim). No additional DNase was necessary. The reaction was incubated for 2 hr at 16°C, to substitute 30% of the dCTP with [^{3}H]dCTP. The nick translated cDNA was about 500 bases long as determined by alkaline sucrose gradient sedimentation with marker 9S-cDNA (24).

DNA Hybridisation: DNA samples were sheared to a length of 500-1000 bases by sonication. Their size was measured by alkaline sucrose gradient fractionation with 9S-cDNA as marker (24). cDNA and nick translated DNA were hybridised with soybean DNA or Rhizobium DNA in 0.6M NaCl buffer at 70°C and the rate of hybridisation was corrected for the effect of salt (19). The DNA : cDNA ratio in kinetic experiments was 10^6 : 1 (Rhizobium DNA) or 5 x 10^6 : 1 (soybean DNA).

Cell-Free Protein Synthesis: Messenger RNA was translated in a modified wheat germ system (25). In order to reduce protein synthesis due to endogenous wheat germ mRNA, the S.23 was dialyzed and digested with Ca^{++} dependant micrococcal nuclease (26, 27). To 200 µl of S.23 were added 2 µl of 0.1M $CaCl_2$ and 4 µl of micrococcal nuclease (7500 U ml^{-1}, P.L. Biochemicals) and the mixture was incubated for 10 min at 20°C. The nuclease reaction was halted by the addition of 4 µl of 0.1 M EGTA. Cell-free protein synthesis was carried out as described (12) except that K(OAc) was 90 mM, Mg $(OAc)_2$ was 2.5 mM and the reactions also contained 40 µM spermidine tetrahydrochloride (neutralized). The incubation was for 60 min at 25°C. Ribosomes and unreleased peptides were removed by centrifugation for 60 min (50,000 rpm Beckman Type 65 rotor). In vitro synthesized proteins were analyzed on SDS slab gels after treatment with heme and $K_3Fe(CN)_6$ (Verma, manuscript in preparation) using the discontinuous buffer system of Laemmli (28) or on nondenaturing gels (29). Gels were stained with Coomassie blue to detect markers and fluorographed (30) using prefogged film (31).

Formamide Gel Electrophoresis: Slab gels containing 3.6% polyacrylamide and 98% formamide were prepared as described by Maniatis et al (32) and allowed to polymerise overnight. Electrophoresis was for 5 hr at 100V. Radioactivity was detected using fluorography (30) on prefogged film (31). Hind II fragments of [^{32}P] labelled S13RFI DNA (a gift of Klaus Harbers) were used as molecular weight standards. The Hind II digestion was for 1 hr in 10 mM Tris HCl pH 7.6, 50 mM NaCl 10 mM $MgCl_2$, 15 mM dithiothreitol at 37°C.

RESULTS

In order to obtain leghaemoglobin-mRNA enriched template for reverse transcription, we isolated the 9S poly A(+) RNA from 3 week old root nodules (6). Sucrose gradient analysis of this RNA showed a broad peak of approximately 9S (Fig. 1A). This RNA was very active in a wheat germ in vitro protein synthesis system, giving 50 to 100 fold stimulation over background (minus RNA) activity. Analysis of the products of in vitro translation of the 9S poly A(+) mRNA on SDS gels demonstrated a single major component which comigrated with authentic leghaemoglobin (Fig. 1B). The identity of the major component as leghaemoglobin has been established previously by immunoprecipitation and partial N-terminal sequencing (6, 27). By electrophoresis in a non-denaturing gel it is possible to resolve two major forms of leghaemoglobin which have similar molecular weights and comigrate on SDS gels. Analysis of 9S poly A(+) mRNA translation products

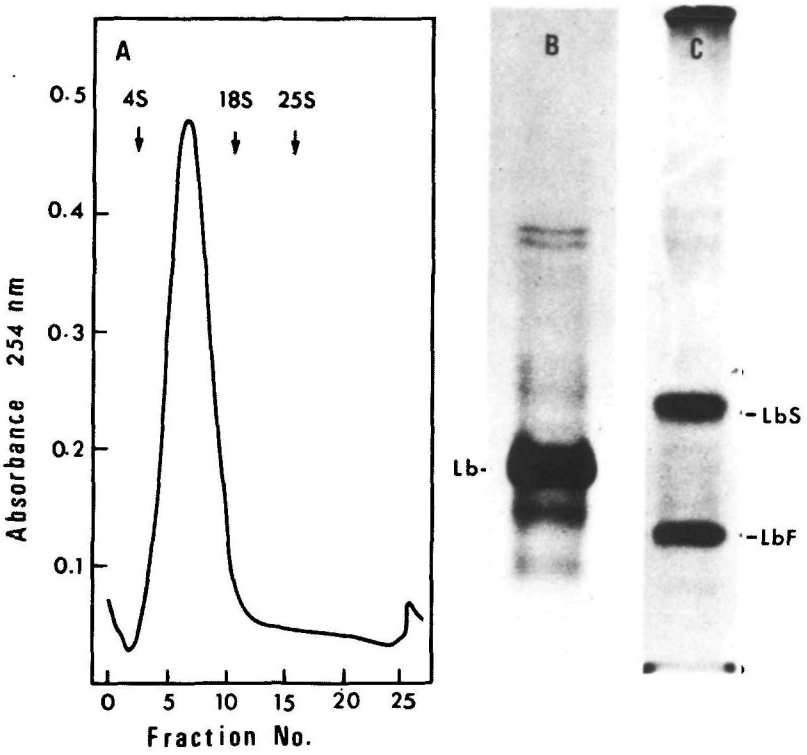

Fig. 1 Properties of 9S poly A(+) mRNA from root nodules. (A) RNA was centrifuged in a 5-20% sucrose gradient (55,000 rpm for 6 hr). Sedimentation was from right to left and marker tRNA and rRNA were run in a parallel tube. Translation products of 9S poly A(+) mRNA were electrophoresed in SDS/polyacrylamide gels (B) or non-denaturing polyacrylamide gels (C). The position of molecular weight markers and authentic leghaemoglobin (Lb) or leghaemoglobin subforms (LbS and LbF) in parallel slots is indicated.

on a non-denaturing gel shows (Fig. 1C) that the two forms of leghaemoglobin, LbS and LbF were synthesized in vitro. LbF can be further resolved into two subcomponents by DEAE-cellulose chromatography (Verma, manuscript in preparation). Thus, although somewhat heterogenous in size, the 9S poly A(+) mRNA from nodules directed almost exclusively the synthesis of 3 forms of leghaemoglobin in vitro.

Complementary DNA synthesized from 9S poly A(+) mRNA (9S-cDNA) was hybridised to the template RNA in a large RNA excess. In RNA excess the rate of hybridisation of a cDNA and the homologous RNA is directly proportional to the complexity of the RNA (33). For a mixed population of RNA and cDNA, the

rate of hybridisation of any one cDNA and the homologous RNA is also influenced by the concentration of RNA (33). The expectation therefore, based on the in vitro translation data, was that 9S mRNA and 9S-cDNA would hybridise with at least biphasic kinetics, showing a rapid component representing the hybridisation of the abundant leghaemoglobin mRNA and cDNA and a slower component(s) representing the hybridisation of less abundant sequences. In fact the rapid phase of the reaction ($Rot_{1/2} \approx 7 \times 10^{-4}$) accounted for only 30% of the total hybridisation (Fig. 2A). The 9S-cDNA was therefore too heterogenous to be useful as a probe for leghaemoglobin sequences. By comparison with the kinetics of globin mRNA- cDNA hybridisation (Fig. 2A) and assuming accurate reverse transcription of nodule mRNA abundance classes we have calculated that the complexity of the RNA driving the rapid component was about 310,000 daltons. This value is very close to the molecular weight of leghaemoglobin mRNA (260,000 daltons (34)) and is consistent with the

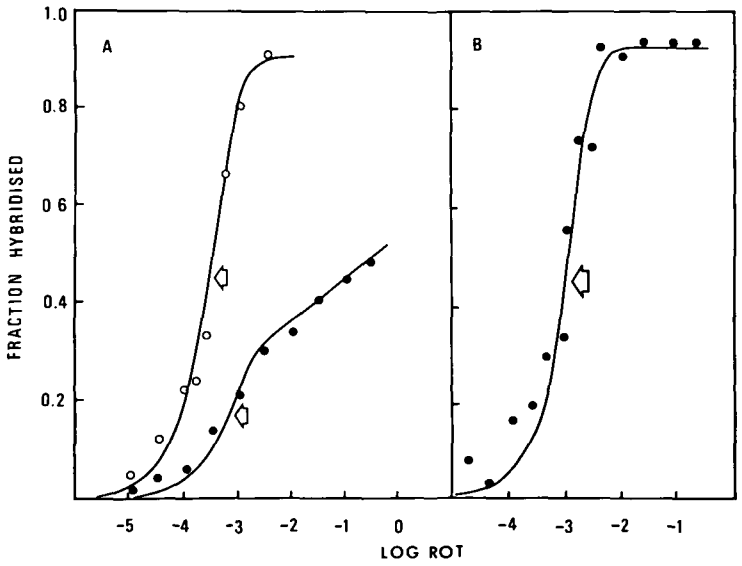

Fig. 2 Hybridisation of cDNA with mRNA. (A) Hybridisation of 9S poly A(+) mRNA with 9S cDNA (●) and globin cDNA with globin mRNA (0). (B) Hybridisation of 9S poly A(+) mRNA with leghaemoglobin cDNA (●). The leghaemoglobin cDNA had been purified by low Rot hybridisation and hydroxyapatite fractionation, as described in Materials and Methods. The $Rot_{1/2}$ values for the globin cDNA (3.3×10^{-4}), leghaemoglobin cDNA (1.0×10^{-3}) and rapid component of 9S-cDNA (7.0×10^{-4}) are indicated by arrows. The lines shown are the best fit values to the data for a pseudo-first order reaction. Hybridisation was assayed with S1 nuclease.

initial rapid phase of the hybridisation involving only leghaemoglobin mRNA and leghaemoglobin cDNA. In order to isolate the leghaemoglobin cDNA, 9S-cDNA was subjected to three cycles of preparative scale hybridisation to a Rot value of 3×10^{-3} followed by hydroxyapatite chromatography to isolate the hybridised cDNA. The cDNA was purged of RNA by extended alkaline hydrolysis. This purified leghaemoglobin cDNA now hybridised to 9S poly A(+) mRNA as a single component (Fig. 2B). The $Rot_{1/2}$ value (10^{-3}) was similar to the $Rot_{1/2}$ value for the rapid phase of the unfractionated cDNA hybridisation (7×10^{-4}) showing that the fractionated leghaemoglobin cDNA was homologous with the most abundant mRNA in the 9S poly A(+) mRNA fraction.

To further demonstrate the homogeneity of this leghaemoglobin cDNA, we subjected an aliquot to electrophoresis in 98% Formamide. Most of the sample electrophoresed as a single band of molecular weight 250,000 daltons (Fig. 3B).

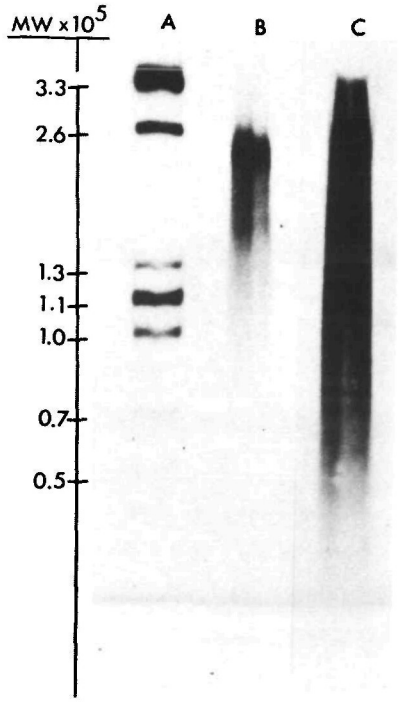

Fig. 3 Formamide gel electrophoresis of cDNA. (A) Hind II digest S13 RFI DNA, (B) leghaemoglobin cDNA and (C) 9S cDNA. The molecular weight of the restriction fragments (daltons x 10^{-5}) is indicated. Electrophoresis was for 5 hr at 100V constant voltage. The figure shows a fluorograph of the gel.

This compares with the heterodisperse electrophoretic profile given by unfractionated 9S-cDNA (Fig. 3C).

The genomic origin of leghaemoglobin mRNA was probed by hybridisation of the leghaemoglobin-cDNA to soybean DNA from uninfected tissue (embryos) and to DNA from cultured Rhizobium japonicum. Even after hybridisation with Rhizobium DNA to Cot of 10^3, which is 5 fold greater than that needed for complete reassociation of Rhizobium DNA (21), no leghaemoglobin cDNA had been duplexed (Fig. 4). However, the leghaemoglobin cDNA hybridised extensively with soybean embryo DNA demonstrating the presence of leghaemoglobin genes in the plant genome (Fig. 4). For comparison, the reassociation of total soybean DNA is also shown. Although the total soybean DNA reassociation data are too sparse to allow detailed analysis they do show that the lowest repetition component of the soybean DNA was completely reassociated

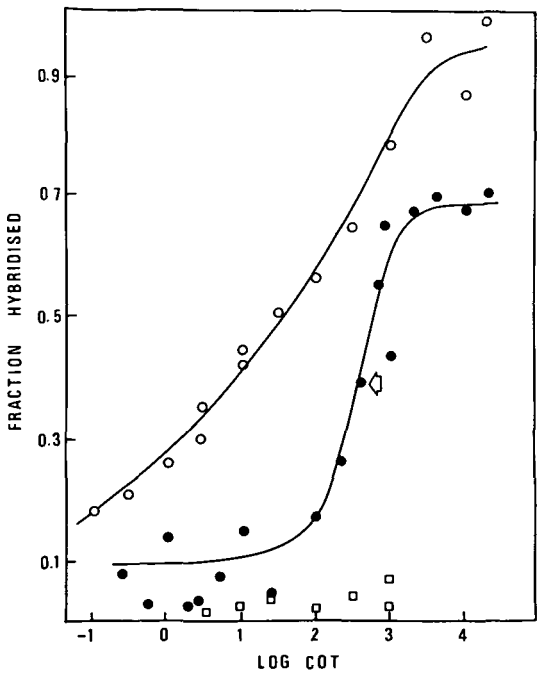

Fig. 4 (A) Hybridisation of leghaemoglobin-cDNA with Rhizobium DNA (□) or soybean embryo DNA (●). The hybridisations were carried out in large DNA excess. Rhizobium DNA:cDNA was 10^6:1 and soybean DNA:cDNA 5 x 10^6:1. The renaturation of soybean DNA with a trace of nick translated DNA is also shown (○). The $Cot_{1/2}$ value for leghaemoglobin-cDNA hybridisation (330) is indicated with an arrow.

more slowly than the leghaemoglobin cDNA hybridisation ($Cot_{1/2} \approx 330$). This indicates that the leghaemoglobin genes are of low repetition in the soybean genome. To determine the repetition of leghaemoglobin sequences more precisely, increasing amounts of leghaemoglobin cDNA were titrated against a fixed amount of soybean DNA. The maximal value of cDNA which hybridises in such an experiment indicates the amount of leghaemoglobin gene sequences in the soybean genome. The data (Fig. 5) show that both soybean embryo and nodule DNA hybridised about 12 pg of leghaemoglobin cDNA per µg of DNA. This value corresponds to about 40 gene copies per 1.4 pg constitutive soybean genome (35).

DISCUSSION

The procedure for cDNA purification outlined above, involving partial purification of a mRNA followed by isolation of the most rapidly hybridising cDNA may be generally applicable if the sequence of interest is more than a

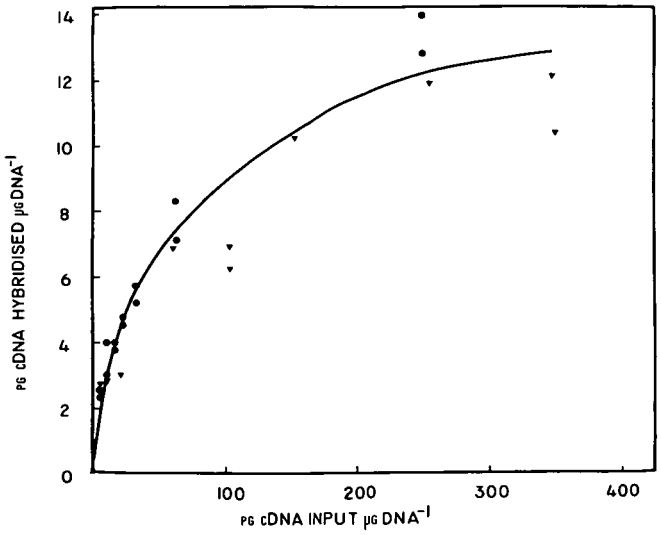

Fig. 5 Saturation hybridisation of soybean DNA with leghaemoglobin cDNA. Aliquots of 3 µg embryo DNA or 10 µg nodule DNA were incubated in 0.6M Na^+ buffer at 70°C to soybean DNA Cot of 10^4 moles l^{-1} sec^{-1} with increasing amounts of leghaemoglobin cDNA. The nodule DNA was 42% sobyean DNA as determined by equilibrium centrifugation of nodule DNA in CsCl. The amount of cDNA hybridisation was estimated with S1 nuclease. Soybean DNA (●), nodule DNA (▼).

few percent of the poly A(+) mRNA. It is of central importance that the homogeneity of the purified cDNA be established, and for leghaemoglobin cDNA this was done by hybridisation and electrophoresis. The kinetics of hybridisation of the purified cDNA with 9S poly A(+) mRNA showed that this cDNA was homologous with the most abundant RNA component of the 9S poly A(+) mRNA. Assuming that cDNA accurately reflects the abundance of mRNA species and by comparing the rate of hybridisation of 9S-cDNA and 9S poly A(+) mRNA with that of globin cDNA and mRNA, the complexity of the abundant mRNA was shown to be approximately the length of one leghaemoglobin mRNA. This value would be an underestimate if the DNA sequences for LbF and LbS which have very similar primary protein sequences (13,14) could cross hybridise, but does indicate that the most abundant mRNA, and therefore cDNA, was a single molecular species. Electrophoresis in 98% formamide gels further evidenced the strong selection of a single component from the heterogenous 9S-cDNA by the low Rot hybridisations. The identification of this purified single species cDNA as leghaemoglobin cDNA is based on analysis of the in vitro translation products of the 9S poly A(+) mRNA, which showed that leghaemoglobin was the only major product. There is a discrepancy between the amount of leghaemoglobin in the in vitro translation products of 9S-poly A(+) mRNA as measured by immunoprecipitation (75-90% (12,27,34)) and the proportion of leghaemoglobin cDNA in the 9S-cDNA indicated by the hybridisation of 9S poly A(+) mRNA and 9S-cDNA (~30%). There are two likely explanations for this: either the leghaemoglobin mRNA was preferentially translated in vitro and leghaemoglobin was over represented in the products, or leghaemoglobin mRNA was reverse transcribed with low efficiency, resulting in under-representation of leghaemoglobin cDNA in the 9S cDNA. By titrating increasing amounts of pure leghaemoglobin cDNA to a fixed amount of 9S poly A(+) mRNA we have estimated that 9S poly A(+) mRNA is 40% leghaemoglobin sequences (data not shown). This value is near the kinetic hybridisation estimate of the concentration of leghaemoglobin sequences in the 9S poly A(+) fraction and suggests that the discrepancy is mostly due to the preferential translation of leghaemoglobin sequences in vitro. Sidloi Lumbroso et al. (36) described a "leghaemoglobin" cDNA which was prepared, without further purification, from 9S poly A(+) mRNA and used to probe the origin of leghaemoglobin genes. Based on translation analysis Sidloi Lumbroso et al. (36) conclude that their 9S poly A(+) mRNA was 80% pure leghaemoglobin mRNA and that the cDNA was similarly pure. They do note, however, that the cDNA would hybridise to the extent of no more than 40% with its template RNA.

In the light of our data it is likely that the cDNA used by Sidloi-Lumbroso et al. was extensively contaminated with non-leghaemoglobin sequences which would only hybridise at high Rot values.

The hybridisation of leghaemoglobin cDNA with soybean embryo DNA demonstrated directly that leghaemoglobin is a plant gene product. The previous, indirect evidence for this, based on correlation between electrophoretic properties of the leghaemoglobin and the plant rather than the bacterial component of the symbiosis, was open to other interpretations (15,16). The plant location of the leghaemoglobin genes is consistent with the polyadenylic acid content of leghaemoglobin mRNA (6) and its site of translation on 80S ribosomes in the plant cell cytoplasm (12). An unusual feature of the leghaemoglobin genes is the forty-fold repetition per constitutive genome. It is not known whether all 3 genes are repeated equally. With the exception of histone genes, identified structural gene sequences are not repeated more than a few times in eukaryotic genomes, although mammalian and insect cells do contain repeated sequence transcripts in the poly A(+) mRNA fraction (37). The pure leghaemoglobin cDNA will serve as a probe into the organisation of the leghaemoglobin genes.

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Abbreviations

poly A(+) mRNA, mRNA containing poly (adenylic acid); EDTA, ethylene diaminetetracetic acid; 9S-cDNA, complementary DNA for 9S poly A(+) mRNA; SDS, sodium dodecyl sulfate; Rot, the product of RNA concentration (mols l^{-1}) and time (sec); Cot, the product of DNA concentration (mols l^{-1}) and time (sec).

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