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Regulation of Structural Gene Expression in Tobacco

Joseph C. Kamalay and Robert B. Goldberg*
Department of Biology
University of California
Los Angeles, California 90024

Summary

We have measured the extent to which structural gene expression is regulated in an entire tobacco plant. Using a sensitive RNA-excess/single-copy DNA hybridization strategy (Galau et al., 1976), our studies reveal that 3.0–3.4 × 10^7 nucleotides of single-copy transcript, or 24,000–27,000 average-sized mRNAs, are present in the polysomes of each organ system (leaf, root, stem, petal, anther and ovary). These mRNA sets are transcribed individually from 4.7–5.2% of the single-copy DNA. Approximately 1 × 10^7 nucleotides of single-copy transcript, or 8000 diverse mRNAs, were shown to be shared by the polysomes of all organs. The sequence composition of the remaining polysomal mRNAs was found to be highly regulated, each organ having at least 8 × 10^6 nucleotides of mRNA complexity, or 6000 diverse gene transcripts, which are not present in the polysomes of other organs. Hybridization experiments with nuclear RNA revealed that structural genes not utilized for the production of functional mRNAs in a given organ were nevertheless transcribed, and were represented by transcripts in the hnRNA. This suggests that post-transcriptional selection mechanisms may play an important role in the regulation of plant gene expression. Collectively, our measurements demonstrate that at least 11% of the single-copy DNA, or 60,000 diverse structural genes, are expressed in the entire plant during the dominant phase of its life cycle. This amount of genetic information constitutes only 4.6% of the tobacco genome.

Introduction

The total number of structural genes which are expressed during the entire life cycle of a complex eucaryote is not yet known. Although Galau et al. (1976) used DNA/RNA hybridization experiments to estimate that about 30,000 structural genes are required to program early sea urchin development, these measurements included only a fraction of the complete life cycle. Due to their morphological and ontogenetic complexity, a determination of the diverse structural gene sets which are expressed in an animal's life span would be nearly impossible. In contrast, higher plants offer a unique opportunity to make a limit estimate of the total genomic fraction of expressed structural genes. Plants have only three non-reproductive organ systems, less than 25 major tissue and cell types and relatively simple developmental processes in comparison to those of animals (Esau, 1977). Consistent with the molecular biology of multicellular eucaryotes, however, plants do possess large genomes (Hinegardner, 1976), intricate patterns of repetitive and single-copy DNA sequence organization (Walbot and Goldberg, 1979) and complex heterogenous nuclear RNA populations (Goldberg et al., 1978; Kiper et al., 1979).

The extent to which differential gene activity plays a role in the establishment and maintenance of specific developmental states in plants is not yet known, nor are the underlying molecular processes which regulate differential gene expression in plants understood. The activity of genes which code for abundant plant proteins has been shown to be qualitatively and quantitatively regulated (for example, carboxylase—Tobin, 1978; Link, Coen and Bogorad, 1978; Leghemaglobin—Auger, Baulcombe and Verma, 1979; α—amylase—Higgens, Zwar and Jacobsen, 1976; seed storage proteins—Sun et al., 1978; R. B. Goldberg et al., manuscript in preparation). There is little information, however, with regard to the differential expression of the large majority of plant structural genes, those which encode less abundant or rare cellular proteins (Goldberg et al., 1978; Silflow, Hammet and Key, 1979; Auger et al., 1979; Kiper et al., 1979).

In this paper we present the results of RNA-excess/single-copy DNA hybridization experiments (Gelderman, Rake and Britten, 1969; Hahn and Laird, 1971; Galau, Britten and Davidson, 1974) which compared the diverse mRNA sequence sets present in the polysomes of all vegetative and reproductive organ systems of a higher plant. Our measurements reveal that structural gene expression is strikingly regulated in plants, and that at least 11% of the single-copy DNA consists of coding sequences expressed throughout the dominant phase of the plant life cycle. Significantly, structural genes which were not expressed in a given organ system were found to be represented by transcripts in the nuclear RNA. Hence, as in animal cells (Wold et al., 1978; Davidson and Britten, 1979), post-transcriptional selection processes may play a major role in the regulation of gene expression.

Results

Characteristics of Leaf mDNA and Null mDNA

To compare mRNA sequence sets present in the polysomes of different organ systems, two ^{3}H-single-copy DNA fractions were isolated by hybridization with leaf polysomal RNA (see Experimental Procedures). One fraction, designated ^{3}H-mDNA, was en-

* To whom reprint requests should be addressed.

riched for single-copy sequences complementary to leaf mRNA. The other, referred to as ^{3}H-null mDNA, was depleted of these sequences. Hybridization of nonleaf polysomal RNAs with ^{3}H-mDNA measures the fraction of leaf mRNAs which are present in the cytoplasm of other organs, while hybridization with ^{3}H-null mDNA identifies mRNAs absent from leaf polysomes. The rationale for using this approach in structural gene expression studies has been discussed in detail by others (Galau et al., 1976; Hough-Evans et al., 1977, 1979; Wold et al., 1978; Ernst, Britten and Davidson, 1979). In this paper, we use the term "structural gene expression" to refer to genes represented in the cell by mRNAs which are releasable from polysomes by EDTA (Perry and Kelley, 1968; Penman, Vesco and Penman, 1968; Goldberg et al., 1973; Galau et al., 1974). We determined previously that >95% of the structural genes expressed in the leaf were single-copy sequences and that the complexity of the leaf mRNA set was 3.33×10^7 nucleotides (Goldberg et al., 1978). This value is used as a complexity reference for the ^{3}H-mDNA hybridization reactions presented here.

Characteristics of the parental ^{3}H-single-copy DNA, ^{3}H-mDNA and ^{3}H-null mDNA are presented in Table 1. These tracers reassociated to >80% with total leaf DNA, and with a rate expected for short tobacco single-copy sequences (Zimmerman and Goldberg, 1977; Goldberg et al., 1978). At Cot 100, <5% of the tracers reacted, demonstrating that there were no detectable repeated sequences in the parental or selected ^{3}H-single-copy DNAs.

The sequence complexity of leaf nuclear RNA exceeds that of mRNA by a factor of 3.6 (Goldberg et al., 1978). To prevent nuclear RNA contamination, all polysomal RNAs were isolated by an EDTA-release procedure (Goldberg et al., 1973, 1978). Approximately 10% of the reactive parental ^{3}H-single-copy DNA is expected to hybridize with nuclear RNA. Table 1 shows that the leaf polysomal RNA used to isolate ^{3}H-mDNA and ^{3}H-null mDNA saturated 2.8% of the reactive parental tracer, in excellent agreement with a value of 2.6% obtained in our more extensive earlier measurements (Goldberg et al., 1978). This result indicates that the rigorous polysome isolation procedure used effectively removed nuclear RNA contaminants.

Figure 1A shows the hybridization of ^{3}H-mDNA with two different leaf polysomal RNA preparations. At saturation, 63% of the reactive ^{3}H-mDNA hybridized. Analysis of the hybrids with S1 nuclease (Maxwell, Van Ness and Hahn, 1978) produced a similar hybridization value, indicating the absence of unhybridized noncoding sequences on the ^{3}H-DNA contained in ^{3}H-mDNA/polysomal RNA hybrids. Since no measurable amount of ^{3}H-mDNA bound to hydroxyapatite after low salt RNAase treatment (Figure 1A), all the observed ^{3}H-mDNA hybridization was with leaf polysomal RNA. It is possible to enrich 36 fold (100/2.8) for single-copy sequences complementary to leaf mRNA. Figure 1A shows that we achieved a 23 fold enrichment (63.1/2.8), or about two thirds the maximum. The rate at which ^{3}H-mDNA hybridized with leaf polysomal RNA (1.35×10^{-4} M^{-1} sec^{-1}) was similar to that obtained with the parental tracer (8.19×10^{-5} M^{-1} sec^{-1}; Goldberg et al., 1978), indicating that the same fraction of polysomal RNA was driving both ^{3}H-single-copy DNAs. Using these rates and the leaf mRNA sequence complexity, we estimate that about 20% of the mRNA drives the reaction. This mRNA fraction consists of rare class messages (Galau et al., 1976; Davidson and Britten, 1979) which collectively

Table 1. Characteristics of Labeled Single-Copy DNAs[a]

Tracer	Size (Nucleotides)[b]	% Reassociation with Leaf DNA[c]	% Hybridization with Leaf Polysomal RNA[d]
^{3}H-single copy (parental tracer)[e]	210	84.2	2.8
^{3}H-mDNA[f]	210	88.1	63.1
^{3}H-null mDNA	210	86.1	<0.03

[a] Tracer characteristics of time of preparation. Reactivities decreased approximately 2% per month. The sizes, however, remained the same. The reassociation kinetics of tobacco single-copy DNA, including the preparation used here, have been described extensively elsewhere (Zimmerman and Goldberg, 1977; Goldberg et al., 1978).

[b] Modal single-stranded fragment length measured by alkaline sucrose density centrifugation.

[c] Extent of reassociation with a >100,000 fold mass excess of unlabeled leaf DNA at DNA Cot 50,000. At this Cot >95% of the unlabeled DNA reacted. No detectable amounts of zero-time binding (Cot $<10^{-5}$) were observed.

[d] Percent hybridization with a >5000 fold mass excess of leaf polysomal RNA at RNA Cot 35,000. Values were corrected to 100% tracer reactivity as described in Experimental Procedures. The 37% of ^{3}H-mDNA which does not react with any RNA (Table 2, Figure 4) probably represents random ^{3}H-single-copy DNA contaminants (Galau et al., 1976; Wold et al., 1978). The maximum extent that these contaminants could contribute to the ^{3}H-mDNA hybridization with a heterologous polysomal RNA is 1% (37% × 0.028).

[e] Tracer from which the mDNA and null mDNA were prepared.

[f] Goldberg et al. (1978) demonstrated that leaf ^{3}H-mDNA and ^{3}H-single-copy DNA displayed identical reassociation kinetics in the presence of excess, unlabeled leaf DNA. These tracers reassociated as a pure second-order reaction, having a K of 4.8×10^{-4} M^{-1} sec^{-1}. No repeated sequences were observed within the detection limits of the experiment.

comprise >95% of the leaf mRNA sequence diversity, but individually only 10^{-3}% of the mass (approximately 10 molecules cell^{-1} sequence^{-1}; Goldberg et al., 1978).

Hybridization of ^{3}H-null mDNA with leaf polysomal RNA is shown in Figure 1B. Within the limits of the technique there was no significant reaction. Since 86% of the ^{3}H-null mDNA reassociated with total leaf DNA (Table 1), single-copy sequences complementary to leaf mRNA were effectively removed. The ^{3}H-null mDNA/leaf polysomal RNA hybridization reaction allows an upper limit to be placed on the number of diverse mRNA species which would go undetected in experiments with nonleaf polysomal RNAs. The scatter (one standard deviation = ± 0.03%) represents approximately ± 300 different structural gene transcripts (Table 2). Hence if mRNA sequence sets of two organs differed by <300 transcripts, this difference would not be reliably detected.

Hybridization of Leaf mDNA and Null mDNA with Stem and Root Polysomal RNAs

To measure structural gene expression in other vegetative organs, ^{3}H-mDNA and ^{3}H-null mDNA were hybridized with stem and root polysomal RNAs. These reactions are shown in Figure 1, and the results are summarized in Table 2. Figure 1A demonstrates that a large fraction of leaf mRNAs are present in stem and root polysomes. From the extents of the ^{3}H-mDNA reactions, we calculate that 75 and 65% of the leaf mRNA sequence set are shared with those of stem and root, respectively. These amounts of overlap represent 2.5×10^7 and 2.2×10^7 nucleotides of diverse gene transcripts, and indicate that between 17,000 and 20,000 structural genes expressed in the leaf are also expressed in other vegetative organs.

The ^{3}H-null mDNA hybridization reactions with stem and root polysomal RNAs are presented in Figure 1B. In contrast to the result with leaf polysomal RNA, approximately 0.65% of the reactive ^{3}H-null mDNA hybridized with stem and root polysomal RNAs. This amount of hybridization is equivalent to approximately 7.8×10^6 nucleotides of diverse mRNA sequences or about 6000 different transcripts. Thus both stem and root possess a set of mRNAs which are undetectable in leaf polysomes.

Hybridization of Reproductive Organ Polysomal RNAs with Leaf mDNA and Null mDNA

While a large number of mRNAs were shared by vegetative organs, it was possible that the reproductive organs of the flower expressed very different sets of structural genes. To test this possibility, petal, ovary and anther polysomal RNAs were isolated, and each was reacted with ^{3}H-mDNA and ^{3}H-null mDNA. The results of these experiments are presented in Figure 2 and summarized in Table 2.

We included petal as a distinct organ type and as a

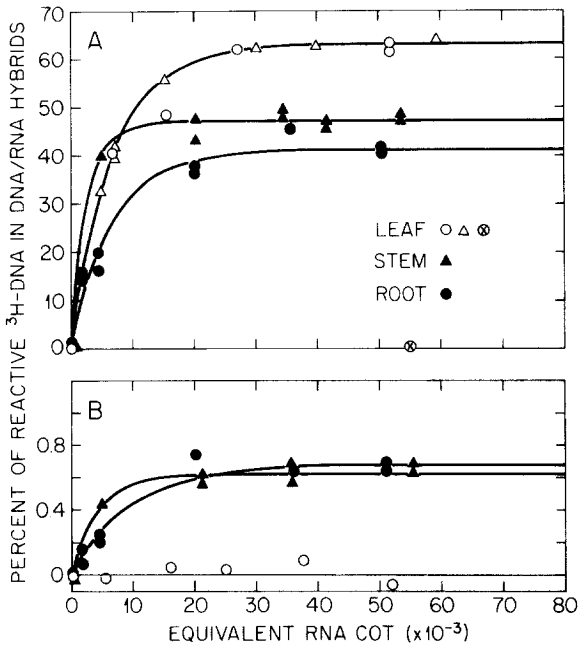

Figure 1. Hybridization of Leaf mDNA and Null mDNA to Polysomal RNAs of Vegetative Organ Systems

Trace amounts of ^{3}H-mDNA or ^{3}H-null mDNA were hybridized separately to a >2000 fold sequence excess of leaf, root and stem polysomal RNAs. The extent of hybridization was assayed by hydroxyapatite chromatography as described in Experimental Procedures. The curves through the data points represent the best least-squares solutions for one pseudo first-order component. Attempts to fit the data to more than one component were unsuccessful. The terminal values and rate constants obtained from these solutions are listed in Table 2. These data have been normalized to 100% tracer reactivity as indicated in Experimental Procedures.

(A) Hybridization with ^{3}H-mDNA. The observed terminal value, ^{3}H-mDNA reactivity and RMS error for each curve were: leaf-1 (△), 55.7%, 88.2%, 3.1%; leaf-2 (○), 54.5%, 85.9%, 3.1%; stem (▲), 40.4%, 85.9%, 3.2%; root (●), 35.4%, 85.9%, 5.5%. The data point (⊗) represents the percentage of ^{3}H-mDNA bound to hydroxyapatite after treatment of leaf polysomal RNA/^{3}H-mDNA hybrids with RNAase under low salt conditions (see Experimental Procedures).

(B) Hybridization with ^{3}H-null mDNA. The observed terminal value, ^{3}H-null mDNA reactivity and RMS error for each curve were: leaf (○), <0.025%, 83%; stem (▲), 0.50%, 79.9%, 7%; root (●), 0.54%, 79.9%, 8.2%.

biological control, since leaf and petal are morphologically homologous organ systems (Esau, 1977). Figure 2A demonstrates that virtually all leaf mRNAs are present in petal polysomes, since ^{3}H-mDNA hybridized to the same extent with petal polysomal RNA as with that of leaf (Table 2). Strikingly, there was no measurable ^{3}H-null mDNA reaction (Figure 2B), indicating that within detection limits there is virtual identity in the structural genes expressed in petal and leaf. If the leaf and petal mRNA sequence sets differ at all, such differences must be either quantitative (that is, prevalence differences) or involve <2% of the structural gene transcripts represented in leaf and petal polysomes (see footnote g, Table 2).

Hybridization of ^{3}H-mDNA with anther and ovary polysomal RNAs produced very different results (Fig-

Table 2. Summary of mDNA and Null mDNA Hybridization Reactions

	mDNA Reactions					Null mDNA Reactions					
RNA	% of Reactive ^{3}H-mDNA Hybridized[a]	K (M^{-1} sec^{-1})[b]	% of Leaf Polysomal RNA Reaction	Complexity (Nucleotides)[c]	Number of mRNAs Shared with Leaf[d]	% of Reactive ^{3}H–Null mDNA Hybridized[a]	K (M^{-1} sec^{-1})[b]	Complexity (Nucleotides)[e]	Number of mRNAs Not Shared with Leaf[d]	Total mRNA Complexity (Nucleotides)[f]	Number of Diverse mRNAs[d]
Leaf	63.1 ± 0.8	1.3×10^{-4}	100	3.33×10^7	27,000	<0.03				3.33×10^7	27,000
Stem	47.0 ± 1.9	3.8×10^{-4}	75 ± 3	2.48×10^7	20,000	0.63 ± 0.06	2.3×10^{-4}	7.6×10^6	6,000	3.24×10^7	26,000
Root	41.2 ± 2.4	1.4×10^{-4}	65 ± 4	2.18×10^7	17,500	0.68 ± 0.07	1.1×10^{-4}	8.2×10^6	6,500	3.00×10^7	24,000
Petal	61.5 ± 0.7	3.0×10^{-4}	99 ± 1	3.30×10^7	27,000	<0.03		$<3.7 \times 10^{5}$[q]	<300[q]	3.30×10^7	27,000
Anther	36.8 ± 2.3	1.4×10^{-4}	58 ± 4	1.94×10^7	15,500	1.06 ± 0.06	1.3×10^{-4}	1.29×10^7	10,500	3.23×10^7	26,000
Ovary	36.0 ± 2.3	8.4×10^{-4}	57 ± 4	1.90×10^7	15,000	1.00 ± 0.10	9.4×10^{-5}	1.21×10^7	10,000	3.11×10^7	25,000

[a] Represents the terminal values obtained from the least-squares solution to the hybridization data presented in Figures 1 and 2. These values were normalized to 100% tracer reactivity as indicated in Experimental Procedures. In no case was the tracer reactivity less than 80%. Standard deviations were calculated from hybridization values at RNA Cot >35,000.

[b] Pseudo first-order rate constant. Rates represent least squares solutions with the lowest RMS errors (see Experimental Procedures). These solutions are probably not unique, since most rates can be varied by ≤25% with little or no change in RMS error. Due to insufficient low Cot data, rate constants for the stem and ovary ^{3}H-mDNA reactions are more uncertain.

[c] The leaf mRNA sequence set has a complexity of 3.33×10^7 nucleotides (Goldberg et al., 1978). Since 63.1% of the reactive ^{3}H-mDNA hybridized to leaf polysomal RNA:

$$C_L = \frac{(\%\text{ of reactive }^3\text{H–mDNA hybridized}) \times (3.33 \times 10^7)}{63.1\%}$$

[d] The number-average size of poly(A) mRNA in leaf polysomes is 1340 nucleotides. This includes a 100 nucleotide stretch of poly(A) (Goldberg et al., 1978). Poly(A) mRNAs of other organ systems were found to be similar in size. Thus number of mRNAs $= \frac{C}{1240}$. This calculation assumes that abundant and rare class messages have the same size distribution.

[e] Tobacco single-copy DNA has a complexity of 6.4×10^8 nucleotide pairs (Zimmerman and Goldberg, 1977). The null mDNA complexity equals that of total single-copy DNA (6.4×10^8) less the complexity of leaf mDNA (3.33×10^7; Goldberg et al., 1978), or 6.07×10^8 nucleotide pairs. Hence C_{NL} = (% of reactive ^{3}H–null mDNA hybridized) × (6.07×10^8) × (2). The latter value corrects for asymmetric transcription.

[f] $C_T = C_L + C_{NL}$.

[q] Represents the upper limit of diverse mRNA sequences which would not be reliably detected by the methods used here.

ure 2A). Approximately 58% of the leaf mRNAs were found in the polysomes of these organs (Table 2). This degree of overlap represents approximately 1.9×10^7 nucleotides of diverse mRNA sequences, or the equivalent of 15,000 diverse gene transcripts. Figure 2B shows that anther and ovary polysomal RNAs each saturate approximately 1% of the reactive ^{3}H-null mDNA. This amount of hybridization equates to approximately 1.2×10^7 nucleotides of mRNA sequence complexity, or 10,000 diverse gene transcripts, and shows that almost half of the structural genes expressed in reproductive organs are not expressed in the leaf, and vice versa.

Hybridization of Polysomal RNA Mixtures with Leaf Null mDNA

With the exception of petal, each organ system contains a mRNA set which is absent from leaf polysomes (Figures 1B and 2B). To test whether there was a unique collection of nonleaf structural gene transcripts in the polysomes of each organ, equimolar mixtures of polysomal RNAs were reacted with ^{3}H-null mDNA. If nonleaf mRNAs of two organs differ, then the extent of ^{3}H-null mDNA hybridization with the mixture should equal the sum of that obtained with each polysomal RNA alone. On the other hand, if there is complete overlap of the nonleaf mRNAs in the polysomes of two organs, no change in the individual ^{3}H-mDNA saturation values should be observed. The results of these experiments are presented in Figure 3 and Table 3.

The reactions shown in Figure 3 display kinetics similar to those observed in the individual polysomal RNA/^{3}H-null mDNA reactions (Figures 1B and 2B). These results show that the concentration of nonleaf

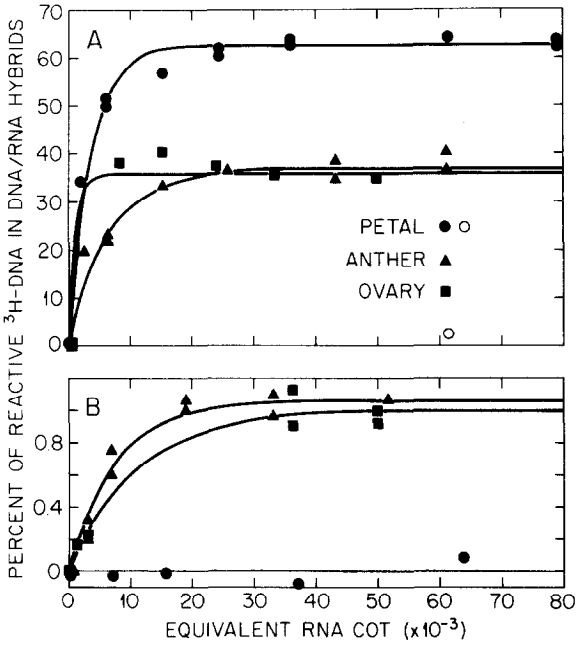

Figure 2. Hybridization of Leaf mDNA and Null mDNA to Polysomal RNAs of Reproductive Organ Systems

Trace amounts of ^{3}H-mDNA and ^{3}H-null mDNA were hybridized separately to a >2000 fold sequence excess of petal, anther and ovary polysomal RNAs. The percent hybridization was determined by hydroxyapatite chromatography as described in Experimental Procedures. The curves through the data points represent the best least-squares solutions for one pseudo first-order component. The kinetic parameters and terminal values for these curves are listed in Table 2. These data have been normalized to 100% tracer reactivity.

(A) Hybridization with ^{3}H-mDNA. The observed terminal value, ^{3}H-mDNA reactivity and RMS error for each curve were: petal (●), 53%, 84.8%, 4.9%; anther (▲), 31.6%, 85.9%, 8.6%; ovary (■) 31.7%, 88.2%, 4.6%. (○) ^{3}H-mDNA/petal polysomal RNA hybrids which were treated with RNAase under low salt conditions.

(B) Hybridization with ^{3}H-null mDNA. The observed terminal value, ^{3}H-null mDNA reactivity and RMS error for each curve were: petal (●), <0.025%, 78.4%; anther (▲), 0.85%, 80%, 6.9%; ovary (■) 0.80%, 80%, 6.3%.

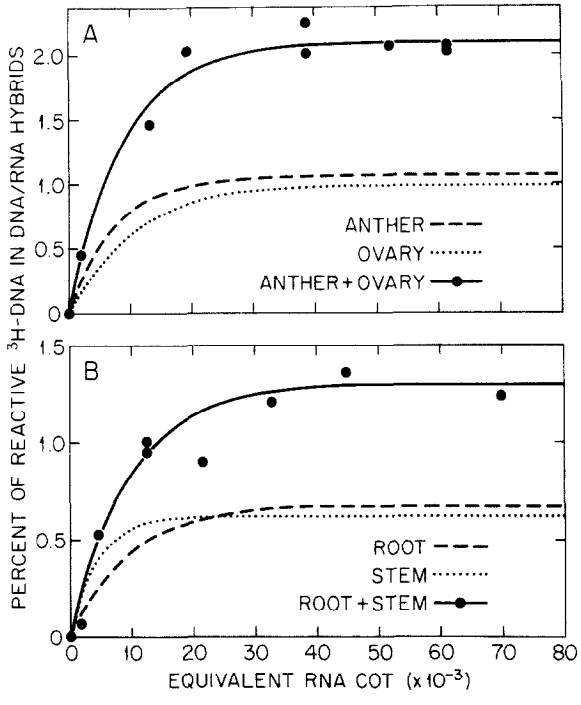

Figure 3. Hybridization of Leaf Null mDNA to Polysomal RNA Mixtures

Trace amounts of ^{3}H-null mDNA were hybridized to equimolar mixtures of polysomal RNAs. The RNA/DNA sequence ratio for each polysomal RNA in these mixtures was >2000. The percentage of hybridization was determined by hydroxyapatite chromatography using the "two-column" procedure described in Experimental Procedures. Hybridization reactions with individual polysomal RNAs are portrayed by the dashed curves (data of Figures 1B and 2B). The solid curves through the data points represent the best least-squares solutions for one pseudo first-order component. The rate constants and terminal values for these curves are listed in Table 3. These data have been normalized to 100% tracer reactivity as described in Experimental Procedures.

(A) Hybridization with a mixture of reproductive organ polysomal RNAs. The observed terminal value, ^{3}H-null mDNA reactivity and RMS error were 1.58%, 74.8% and 4.5%, respectively.

(B) Hybridization with a mixture of vegetative organ polysomal RNAs. The observed terminal value, ^{3}H-null mDNA reactivity and RMS error were 1.02%, 78.4% and 8.2%, respectively.

Table 3. Hybridization of Null mDNA to Polysomal RNA Mixtures

Polysomal RNAs in Mixtures	% of Reactive ^{3}H-Null mDNA in DNA/RNA Hybrids		
	Expected[a]	Observed	K (M^{-1} sec^{-1})
Anther Ovary	2.06 ± 0.16	2.11 ± 0.09[b]	1.13×10^{-4}
Root Stem	1.31 ± 0.13	1.30 ± 0.13[b]	1.06×10^{-4}
Anther Ovary Root Stem	3.37 ± 0.29	3.84 ± 0.52[c]	

[a] Predicted hybridization value if mRNAs not present in leaf polysomes are different in each organ system. Calculated from the sum of the terminal values obtained from the least-squares solutions to the hybridization data presented in Figures 1B and 2B. Terminal values for individual reactions are listed in Table 2, column 7.

[b] Terminal values obtained from the least-squares solution of the hybridization data presented in Figure 3. The observed values and tracer reactivities are presented in the figure legend. Standard deviations were computed from the plateau hybridization values (RNA Cot >35,000).

[c] ^{3}H-Null mDNA was hybridized with an equimolar mixture of anther, ovary, root and stem polysomal RNAs to RNA Cot 54,000. The extent of hybridization was assayed as described in Experimental Procedures. Each RNA was present in >2000 fold sequence excess over the labeled DNA. Listed value represents the average of three hybridization reactions. The average observed percent hybridization was 2.87% and the ^{3}H-null mDNA reactivity was 74.8%.

mRNAs in the polysomal RNA combinations was equivalent to that in each polysomal RNA alone. The extents of these reactions (Table 3) demonstrate that the nonleaf mRNA sets of ovary and anther, as well as those of stem and root, are distinct. For example, 2.1% of the reactive ^{3}H-null mDNA hybridized to the reproductive polysomal RNA mixture (Figure 3A), exactly the sum of that obtained in the individual ovary and anther polysomal RNA reactions (Figure 2B, Table 2). Similarly, 1.3% of the reactive ^{3}H-null mDNA hybridized to the vegetative polysomal RNA mixture (Figure 3B); again, the sum of the separate hybridization values (Figure 1B, Table 2).

To determine the degree of overlap in the vegetative and reproductive nonleaf mRNA sets, a combination of anther, ovary, root and stem polysomal RNAs was hybridized with ^{3}H-null mDNA to RNA Cot 54,000. Figures 1–3 show that this Cot exceeds the termination point for ^{3}H-null mDNA reactions. The measurements listed in Table 3 demonstrate that the extent of ^{3}H-null mDNA reaction (3.84%) was about the same as that expected (3.37%) from the summation of the four individual polysomal RNA reactions (Figures 1B and 2B, Table 2). This observation indicates that a large fraction of the total polysomal mRNA sequence complexity of each organ (25–45% or $0.8–1.4 \times 10^7$ nucleotides; Table 2) is unique to that system.

Hybridization of Leaf mDNA to Polysomal RNA Mixtures

Although the polysomes of each organ contain a large fraction of leaf structural gene transcripts (55%–75%; Figures 1A and 2A, Table 2), the composition of leaf mRNA subsets in various organ systems may be dissimilar. To determine whether this was the case and to obtain an estimate of the number of ubiquitous polysomal mRNAs in tobacco, all possible double-mixture combinations of stem, root, ovary and anther polysomal RNAs were hybridized with ^{3}H-mDNA. The rationale of this approach is similar to that used in the ^{3}H-null mDNA reactions with polysomal mRNA mixtures. That is, the presence of different leaf messages in the polysomes of two organs will be reflected by an increase in the extent of ^{3}H-mDNA reaction. The average hybridization values for 3–5 separate reactions per mixture to RNA Cot 60,000 are listed in Table 4.

The measurements support the proposition that distinct leaf mRNA subsets are present in the polysomes of vegetative and reproductive organs. These subsets may be distinguished by an incongruous number of leaf mRNAs (for example, stem and anther), by the presence of different leaf mRNA species (for example, anther and ovary) or by both (for example, stem and ovary). Minimally, only about 1×10^7 nucleotides of mRNA sequence complexity, or approximately 8000 diverse mRNAs, are shared by the polysomes of all organs.

Hybridization of Leaf mDNA with Nuclear RNAs

The experiments described above demonstrate that the expression of genes which encode leaf and nonleaf mRNAs is highly regulated. They do not, however, address the issue of whether the sequence composition of each mRNA set is regulated at the transcriptional or the post-transcriptional level. To distinguish between these alternatives, nuclear RNA was isolated from leaf and stem (see Experimental Procedures) and each was then hybridized with ^{3}H-mDNA (Wold et al., 1978). If the principal mode of regulation is transcriptional, the amount of ^{3}H-mDNA which hybridizes with stem nuclear RNA should be identical to that obtained with stem polysomal RNA (that is, nonutilized structural genes are repressed). On the other hand, if control is primarily post-transcriptional, all reactive ^{3}H-mDNA should hybridize to stem nuclear RNA, irrespective of the results obtained with polysomal RNA (that is, structural genes are transcribed constitutively). The experimental findings are shown in Figure 4.

Approximately 62% of the reactive ^{3}H-mDNA hybridized with both stem and leaf nuclear RNAs, an amount equal to the maximum reaction obtained with leaf (Figure 1A) or petal (Figure 2A) polysomal RNAs. After low salt RNAase treatment, no detectable ^{3}H-mDNA bound to hydroxyapatite (open symbols, Figure

Table 4. Hybridization of Leaf mDNA to Polysomal RNA Mixtures[a]

Polysomal RNAs in Mixture	% of Reactive ^{3}H-mDNA in DNA/RNA Hybrids		% of Leaf mRNA Set Shared by Polysomal RNAs in Mixture[e]	Complexity of Shared Leaf mRNA Set (Nucleotides)[f]	% of Each Leaf mRNA Subset Represented in the Polysomes of the Other Organ[g]
	Expected[b]	Observed[c]			
Stem	47.0 ± 1.9	46.4 ± 3.0	59	1.97×10^7	79
Anther					100
Stem	47.0 ± 1.9	58.4 ± 4.2	49	1.63×10^7	65
Root					75
Stem	47.0 ± 1.9	65.2 ± 2.1	28	0.93×10^7	37
Ovary					49
Root	41.2 ± 2.4	46.9 ± 4.5	49	1.63×10^7	75
Anther					85
Root	41.2 ± 2.4	56.1 ± 2.1	34	1.13×10^7	52
Ovary					60
Anther	36.8 ± 2.3	50.8 ± 3.3	35	1.17×10^7	60
Ovary					61
Leaf[d]					
Root					
Stem	63.1 ± 0.8	64.5 ± 1.9[d]			
Ovary					
Anther					

[a] Trace amounts of ^{3}H-mDNA were hybridized to equimolar mixtures of polysomal RNAs as described in Experimental Procedures. At RNA Cot 60,000 the reactions were terminated and the extent of hybridization assayed by hydroxyapatite chromatography. For each polysomal RNA in a mixture the RNA/DNA sequence ratio was >2000. Figures 1A and 2A show that RNA Cot 60,000 is beyond the termination point for reactions with ^{3}H-mDNA.

[b] The hybridization of ^{3}H-mDNA with polysomal RNA mixtures takes the form of a union of two sets. If the leaf mRNAs of two organs are the same (that is, their leaf mRNA sets are equal) or if the leaf mRNAs of one organ are a subset of those in the other, then the amount of ^{3}H-mDNA reaction should equal the maximum obtained with either polysomal RNA alone (Table 2, column 2). We refer to the maximum as the expected value. If the ^{3}H-mDNA reaction with the mixture (observed value) is greater than expected, then different leaf messages are present in the polysomes of the two organs (that is, their leaf mRNA sets are unequal).

[c] These values are the union of leaf mRNA sets in both organs (that is, $X \cup Y$) and represent the average percent hybridization from 3–5 individual reactions. The observed average hybridization values were: 37, 46.5, 52, 37.4, 44.7, 40.5 and 51%, respectively (in the order listed in table). The ^{3}H-mDNA reactivity was 79.7%.

[d] Figures 1A and 2A showed that the maximum amount of ^{3}H-mDNA hybridization was 63%. A control hybridization reaction was carried out with ^{3}H-mDNA and a mixture of five different polysomal RNAs, including that of leaf. The results presented in this table also demonstrate that, within 1–2%, the maximum possible reactive ^{3}H-mDNA hybridization was 63%.

[e] The percentage of the leaf mRNA set which is shared by each polysomal RNA in the mixture was estimated using the following relationships. Let X equal the set of leaf mRNAs in organ X. This set possesses leaf mRNAs unique to organ X (A), as well as those shared with organ Y (S), or X = [S, A]. Let Y equal the set of leaf mRNAs in organ Y. This set possesses leaf mRNAs unique to organ Y (B) as well as those shared with organ X (S), or Y = [S, B]. The intersection of both mRNA sets ($X \cap Y$) defines those messages shared between them (S). A straightforward calculation enables S to be solved. That is: $S = (X \cap Y) = (X) + (Y) - (X \cup Y)$. Values for X and Y are presented in Table 2, column 2. Values for $X \cup Y$ are presented in this table, column 3. Thus the percentage of shared leaf mRNAs is computed from the relationship:

$$S = \left[\frac{(\sum \text{individual hybridization values}) - (\text{mixture hybridization value})}{63.1}\right] \times 100$$

[f] Complexity = ($S \times 3.33 \times 10^7$ nucleotides). The number of diverse leaf mRNAs shared is simply the quotient of the complexity and the number-average size of leaf mRNA (1240 nucleotides; see footnote d, Table 2).

[g] The percentage of each organ's leaf mRNA subset which is represented in the polysomes of the other organ was calculated by taking the quotient of S and the percentage of total leaf mRNAs present in the polysomes of each organ (Table 2, third column).

4), demonstrating that all measured duplexes were ^{3}H-mDNA/nuclear RNA hybrids. Figure 1A shows that 47% of the reactive ^{3}H-mDNA hybridized with stem polysomal RNA, indicating that 75% of the leaf mRNA set is present in stem polysomes (Table 2). Figure 4, however, unequivocally demonstrates that the entire leaf mRNA set is contained within the stem nuclear RNA population. This significant observation is consistent with the post-transcriptional model, and shows that there are leaf mRNAs present in stem nuclei which are absent from stem polysomes. The leaf mRNA subset which is not selected for cytoplasmic transport has a sequence complexity of approximately 8.3×10^6 nucleotides.

We showed previously that the parental ^{3}H-single-copy DNA hybridized to leaf nuclear RNA with a pseudo first-order rate constant of 1.5×10^{-4} M^{-1} sec^{-1} (Goldberg et al., 1978). Using this rate and the sequence complexity of leaf nuclear RNA (1.2×10^8 nucleotides), we calculated that each complex single-copy transcript is present an average of twice per nucleus. Figure 4 shows that ^{3}H-mDNA hybridized with identical kinetics to leaf and stem nuclear RNAs, the pseudo first-order rate constant being 1.4×10^{-4} M^{-1} sec^{-1} in each case. This rate is the same as that observed with the unselected parental tracer, indicating that the steady-state concentration of leaf mRNAs in the nuclei of stem and leaf is similar, and is equal to that of the entire complex nuclear RNA population. We infer from these observations that all complex single-copy transcripts are present in the nucleus at the same steady-state levels, whether or not they are destined to become functional mRNAs.

Discussion

Polysomal mRNA Sequence Complexity of Diverse Organ Systems

Figure 5 summarizes the ^{3}H-mDNA and ^{3}H-null mDNA measurements with various polysomal RNAs.

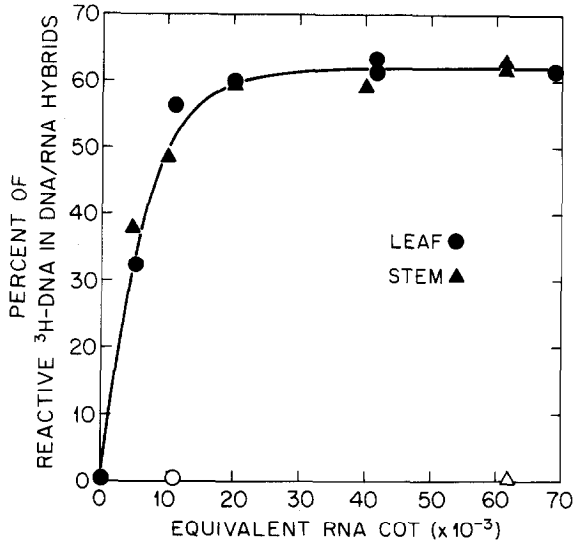

Figure 4. Hybridization of Leaf mDNA to Leaf and Stem Nuclear RNAs

Trace amounts of ^{3}H-mDNA were separately reacted with a >5000 fold mass excess of leaf and stem nuclear RNAs, and the extent of hybridization was measured by hydroxyapatite chromatography. Open symbols represent the amount of ^{3}H-mDNA bound to hydroxyapatite after treatment of ^{3}H-mDNA/nuclear RNA hybrids with RNAase under low salt conditions (see Experimental Procedures). The line drawn through the data points represents the best least-squares solution to the combined leaf and stem nuclear RNA data for one pseudo first-order component. The rate constant and observed terminal value for this solution were 1.4×10^{-4} M^{-1} sec^{-1} and 37%, respectively. Since the tracer was over 1 year old, its reactivity with leaf DNA was only 60%. Individual solutions to the leaf and stem data were not significantly different from that of the pooled data.

The length of each bar is proportional to the polysomal mRNA complexity of the corresponding organ. Complexities are portrayed in two ways: in nucleotides of single-copy sequence, and as the number of diverse mRNA species. One fascinating observation from Figure 5 is that neither the morphological form nor the physiological role of an organ affects the total number of genes expressed. Approximately $3.0–3.4 \times 10^7$ nucleotides of diverse single-copy transcript are present in the polysomes of each organ. The narrow range of complexities is probably not biologically meaningful, but is simply a consequence of the precision obtainable in RNA-excess/single-copy hybridization reactions (see standard deviation values listed in Tables 2–4). Zimmerman and Goldberg (1977) showed that tobacco single-copy DNA has a complexity of 6.4×10^8 nucleotide pairs. Thus 4.7–5.2% of the single-copy DNA, or 25,000–27,000 structural genes, are expressed in any given organ system. With the exception of brain, these amounts of single-copy sequence expression are similar to those observed in more

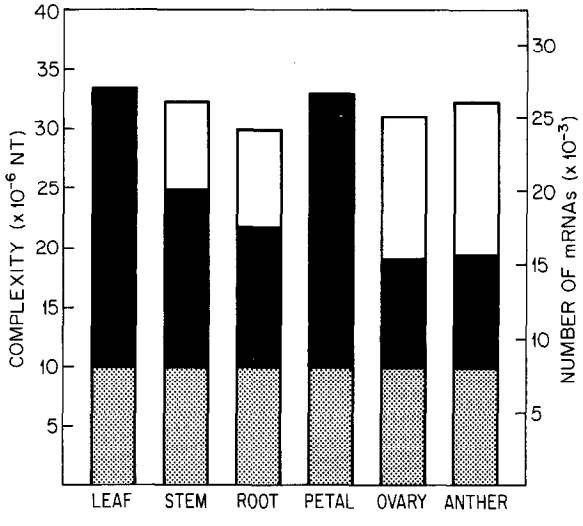

Figure 5. Polysomal mRNA Sequence Sets of Tobacco Organ Systems

Bar lengths represent the total mRNA sequence complexity of each organ expressed in nucleotides or in numbers of diverse 1240 nucleotide gene transcripts (Table 2). The complexity of the leaf mRNA set was determined previously to be 3.33×10^7 nucleotides (Goldberg et al., 1978). Dark and stippled portions of the bars summarize the results of the ^{3}H-mDNA/polysomal RNA reactions (Figures 1A and 2A, Table 2) and represent leaf mRNAs present in the polysomes of each organ. Stippled portions depict a common set of mRNAs present in the polysomes of all organs (Table 4). Bar open areas summarize the results of the ^{3}H-null mDNA/polysomal RNA reactions (Figures 1B, 2B and 3, Table 2) and depict polysomal mRNAs unique to each organ but absent from leaf polysomes. Since each organ system comprises several cell and tissue types (Esau, 1977), we cannot exclude the possibility that mRNAs scored as "absent" are actually present in polysomes, but at undetectable levels. Using least-squares fitting procedures (Hough et al., 1975; Pearson et al., 1977) and the data of Figures 1B and 2B, we estimate that organ-specific mRNAs would have a prevalence of only 0.01–0.001 molecules $cell^{-1}$ $sequence^{-1}$ in the polysomes of a heterologous organ.

complex animal organs, such as liver, kidney and oviduct (reviewed by Davidson and Britten, 1979).

Striking Differences in Rare Class Polysomal mRNA Sequence Sets

One of the major findings of this study is the remarkable extent to which rare class polysomal mRNA sequence sets are regulated in plant cells. Using sensitive hybridization procedures (Galau et al., 1976) capable of resolving as few as 300 transcripts (see footnote g, Table 2), our measurements demonstrate that each organ system has thousands of unique mRNAs which are absent from the polysomes of other organs (Figure 5, open portion of bars). Especially significant is the observation that homologous organs (leaf and petal), which differ in time and origin of development (Esau, 1977), have indistinguishable rare class mRNA sequence sets. Thus the collection of structural genes expressed in any given organ system is highly correlated with a unique morphogenetic state.

Proteins encoded by rare class messages have yet to be identified experimentally. Galau et al. (1976, 1977) and Davidson and Britten (1979) discussed this issue at great length, and presented convincing indirect evidence for the occurrence of organ-specific proteins coded by rare class mRNAs. Since rare class mRNA sequence sets have been shown to be highly regulated in all major eucaryotic taxons (Firtel, 1972; Hastie and Bishop, 1976; Axel, Fiegelson and Schutz, 1976; Galau et al., 1976; Davidson and Britten, 1979; Timberlake, 1980), our presumption is that they are translated into developmentally important proteins.

Ubiquitous Polysomal mRNA Sequence Sets

Even though a significant portion of each polysomal mRNA set is organ-specific, a large amount of sequence overlap does occur. Our measurements reveal that approximately 15,000–20,000 leaf mRNAs (55–75% of the entire set; Table 2) are present in the polysomes of heterologous organs. The dark and stippled portions of each bar in Figure 5 depict these shared leaf mRNA sets. Large extents of rare class mRNA sequence overlap were not unexpected, since common maintenance or "housekeeping" activities must be carried out by all cells. Similar observations have been made by others using animal systems (Ryffel and McCarthy, 1975; Hastie and Bishop, 1976; Young, Birnie and Paul, 1976; Axel et al., 1976; Galau et al., 1976). Quite surprising, however, was our finding that each heterologous organ has a distinct leaf mRNA subset (Figure 5, dark portion of each bar). Only a minor fraction (25–30%) of leaf messages appear to be universally present in the polysomes of all organs (Figure 5, stippled portion of each bar). These ubiquitous mRNAs are transcribed from 1.5% of the single-copy DNA, suggesting that approximately 8000 structural genes code for proteins common to all cells.

An Estimate of the Number of Structural Genes Expressed in a Plant

Our measurements enable us to approximate the extent of gene expression in the entire plant. This is achieved by simply summing the leaf mRNA complexity (Figure 5, complete length of leaf bar) and those of all unique heterologous organ mRNA subsets (Figure 5, length of each open bar). The calculation indicates that 7×10^7 nucleotides of diverse messages are present collectively in the polysomes of all plant organ systems. This amount of mRNA complexity is transcribed from 11% of the single-copy DNA, or 4.6% of the genome (Zimmerman and Goldberg, 1977), and indicates that approximately 60,000 structural genes are required to maintain and program the dominant or spore-producing phase of the tobacco life cycle.

Post-transcriptional Selection of Rare Class Polysomal mRNA Sequence Sets

Figure 4 shows that leaf mRNAs, undetectable in stem polysomes, are represented in stem nuclear RNA. This surprising result indicates that structural genes not utilized for the production of functional mRNAs in a given organ are transcriptionally active. This form of indiscriminate transcription appears to be a common occurrence in multicellular eucaryotes, since similar observations have been made in sea urchin (Wold et al., 1978) and mouse (Table 2 in Davidson and Britten, 1979). At present we do not know whether leaf mRNAs are contained within similar transcription units in stem and leaf nuclei (see Wold et al., 1978; Davidson and Britten, 1979). Nevertheless, our finding that stem nuclear RNA contains the same steady-state concentration of cytoplasmically exported and nonexported leaf mRNA subsets (approximately 2 transcripts nucleus^{-1} sequence^{-1}) strongly suggests that structural genes encoding rare class mRNAs are constitutively transcribed in plant cells. While still tentative, this conclusion implies that post-transcriptional processing and/or selection mechanisms operate to regulate the sequence composition of each specific rare class polysomal mRNA sequence set (Tobin, 1979; Davidson and Britten, 1979).

Structural Gene Regulation in Relation to Plant Differentiation

Experiments with higher plants have provided spectacular evidence for the persistence of developmental potentialities in eucaryotic cells (see Street, 1973). Differentiated tobacco cells, in particular, retain their ability to undergo "directed" organogenesis (for example, root, stem, leaf, flower) as well as embryogeny in culture (Raghavan, 1976). Perhaps simple alterations in environmental effectors (for example, hormones) modulate and select specific sets of preexisting, nuclear-restricted polysomal mRNAs, enabling single cells to develop into mature plants. Whatever the exact mechanisms prove to be, our data indicate that regulation of rare class polysomal mRNA se-

quence sets is an integral aspect of the differentiation process in higher plants.

Experimental Procedures

Growth of Plants

Tobacco plants were grown as outlined by Goldberg et al. (1978). With the exception of roots, all tissues were harvested from plants grown in a vermiculite-soil mixture. Roots were harvested from plants grown hydroponically, eliminating the difficult and tedious task of soil removal. Developmental consistency was strictly adhered to by using the following growth indicators: leaves, 1–3 cm long, from 15–20 cm tall plants; roots, 3–4 cm long, including apex, from 15–20 cm plants; petals, pigmented, from fully opened flowers which showed no signs of senescence; stems, 10 cm long, from 12–15 cm tall plants, leaves removed from all nodes and apex; ovaries and anthers, from unopened flowers in developmental stage 3 of Nitsch (1969).

Preparation of Polysomes and Polysomal RNA

Tissues were ground to a fine powder in liquid nitrogen and polysomes were then isolated according to a modification (Jackson and Larkins, 1976) of the procedure of Goldberg et al. (1978). This technique yielded undegraded polysomes, containing >80% of the cellular RNA, from all organ systems. To ensure that all polysomal RNAs were free of nuclear RNA contamination, the EDTA-release procedure of Goldberg et al. (1973) was used to isolate <80S ribonucleoprotein particles from polysomes which sedimented at >100S (see Figure 1 of Goldberg et al., 1978). We previously demonstrated that this procedure prevents nuclear RNA contamination of tobacco polysomal RNA preparations.

Polysomal RNAs were isolated and purified by standard procedures used in our laboratory (Timberlake, Shumard and Goldberg, 1977; Goldberg et al., 1978). Prior to use in hybridization reactions, all polysomal RNAs were passed over a Sephadex G-100 column, and the exclusion peak, which contained >80% of the mass, was harvested. The number-average poly(A) mRNA sizes of all RNA preparations exceeded 600 nucleotides.

Preparation of Nuclear RNA

Leaf and stem nuclear RNAs were isolated as described previously, except that the CsCl centrifugation step was omitted (Goldberg et al., 1978). Approximately 5% of the cellular RNA was localized in the nucleus. Both RNAs were passed over a Sephadex G-100 column before use in hybridization reactions.

Preparation of Leaf mDNA and Null mDNA

Single-copy sequences were isolated from total leaf DNA (Zimmerman and Goldberg, 1977; Goldberg et al., 1978) and labeled in vitro with ^{3}H-dTTP (New England Nuclear, 55 Ci/mmole) using the "gap-translation" procedure of Galau et al. (1976). Foldback regions were removed by two cycles of annealing to Cot $<10^{-5}$ and hydroxyapatite fractionation at 50°C. The specific activity was 7×10^6 cpm/μg. The size and reactivity of the ^{3}H-single-copy DNA are presented in Table 1.

Leaf mDNA and null mDNA were isolated from ^{3}H-single-copy DNA according to methods developed by the Britten-Davidson group (Galau et al., 1976; Hough-Evans et al., 1977, 1979; Wold et al., 1978; Ernst et al., 1979), with modifications. Briefly, ^{3}H-single-copy DNA was hybridized with a 1000 fold mass excess (300 fold sequence excess) of leaf polysomal RNA to RNA Cot 35,000. Goldberg et al. (1978) showed that this Cot is sufficient to hybridize >98% of the reactive ^{3}H-single-copy sequences complementary to leaf mRNA, and is sensitive enough to detect mRNAs represented by three molecules cell^{-1} sequence^{-1}. The reaction mixture was then adjusted to 0.25 M PB and the unhybridized RNA was digested with 5 μg/ml of RNAase A for 1 hr at room temperature. RNAase was removed by treatment with 500 μg/ml proteinase K, the mixture was deproteinized with 24:1 chloroform-isoamyl alcohol and the nucleic acids were then passed over hydroxyapatite at 60°C in 0.12 M PB,0.2% SDS. The use of ≥500 μg/ml Proteinase K was required to remove all traces of RNAase activity from tracer preparations. ^{3}H-single-copy DNA which did not bind to hydroxyapatite was used to prepare ^{3}H-null mDNA. The bound ^{3}H-single-copy DNA, containing leaf mRNA/DNA hybrids and DNA/DNA duplexes, was eluted from hydroxyapatite with 0.5 M PB and sedimented from solution by ultracentrifugation to a size >4S. The ^{3}H-single-copy DNA pellet was resuspended in 0.05 M PB and incubated for 15 hr at 37°C with 10 μg/ml RNAase A to destroy the RNA in DNA/RNA hybrids (Galau et al., 1974). Following treatment with Proteinase K and chloroform-isoamyl alcohol, the ^{3}H-single-copy DNA was passed over hydroxyapatite in 0.12 M PB,0.2% SDS at 60°C. The ^{3}H-single-copy DNA which bound to hydroxyapatite (containing DNA/DNA duplexes) was discarded, while the single-stranded ^{3}H-single-copy DNA (derived from mRNA/DNA hybrids) was concentrated and hybridized a second time with a 300 fold sequence excess of leaf polysomal RNA to Cot 35,000. The reaction mixture was then incubated with RNAase A under high salt conditions, treated with Proteinase K and chloroform-isoamyl alcohol and finally passed over hydroxyapatite. The ^{3}H-single-copy DNA which bound, containing only DNA/RNA hybrids, was eluted with 0.5 M PB, concentrated by sedimentation and then incubated for 45 min at 37°C in 0.3 M NaOH to hydrolyze residual RNA. This ^{3}H-single-copy DNA, designated leaf ^{3}H-mDNA, was neutralized and stored at −20°C in 0.01 M Tris (pH 7.6), 0.1 mM EDTA, 0.1% SDS. Approximately 15% of the maximum amount of leaf ^{3}H-mDNA was recovered, corresponding to >90% recovery at each step in the isolation procedure.

Null mDNA was obtained from the ^{3}H-single-copy DNA which failed to bind to hydroxyapatite in the first hybridization reaction described above. This fraction of ^{3}H-single-copy DNA was sedimented out of solution by ultracentrifugation to a size >4S and then hybridized with a 3000 fold mass excess of leaf polysomal RNA to RNA Cot 35,000. The reaction mixture was then adjusted to 0.25 M PB and the unhybridized RNA digested with 10 μg/ml of RNAase A for 1 hr at room temperature. After incubation with Proteinase K and extraction with chloroform-isoamyl alcohol, the mixture was passed over hydroxyapatite in 0.12 M PB,0.2% SDS at 60°C. The ^{3}H-single-copy DNA which did not bind to hydroxyapatite, designated leaf ^{3}H-null mDNA, was concentrated, treated with 0.3 M NaOH and stored as described for the ^{3}H-mDNA. The yield was 30% of the theoretical maximum, corresponding to >90% recovery at each step in the procedure. Characteristics of the ^{3}H-mDNA and ^{3}H-null mDNA are listed in Table 1.

DNA/RNA Hybridization

DNA/RNA hybridization reactions were carried out in 0.5 M PB, 0.1 mM EDTA, 0.2% SDS at 68°C as described previously (Timberlake et al., 1977; Goldberg et al., 1978). Cot values were corrected to equivalent RNA Cot by multiplying by a factor of 5.82 (Britten, Graham and Neufeld, 1974). All reactions contained >2000 fold sequence excess of RNA and enough ^{3}H-mDNA or ^{3}H-null mDNA to ensure that >100 cpm was present in DNA/RNA hybrids.

To analyze the fraction of ^{3}H-mDNA in DNA/RNA hybrids, a "one-column" hydroxyapatite procedure was used. Reaction mixtures were diluted to 0.25 M PB, and unhybridized RNA was digested with 5 μg/ml of RNAase A for 1 hr at room temperature. The mixtures were then deproteinized with chloroform-isoamyl alcohol and passed over hydroxyapatite in 0.12 M PB,0.2% SDS at 60°C. The ^{3}H-mDNA which bound to hydroxyapatite represented that which was present in DNA/RNA hybrids. No low salt RNAase step was required in this analysis, since ^{3}H-single-copy DNA self-reassociation did not occur (see Figures 1A and 2A). Analysis of the reaction mixtures with S1 nuclease (Maxwell et al., 1978) yielded identical results.

The fraction of ^{3}H-null mDNA in DNA/RNA hybrids was determined by using a "two-column" hydroxyapatite procedure as described by Goldberg et al. (1978). Briefly, reaction mixtures were split into two aliquots. One aliquot (sample T) was processed exactly as that outlined for the ^{3}H-mDNA reactions. The fraction of ^{3}H-null mDNA which bound to hydroxyapatite in sample T contained DNA/DNA duplexes as well as DNA/RNA hybrids. The second aliquot (sample D) was treated with 10 μg/ml RNAase A in 0.05 M PB for 15 hr at 37°C, deproteinized with chloroform-isoamyl alcohol, adjusted

to 0.12 M PB, 0.2% SDS and passed over hydroxyapatite at 60°C. Only ^{3}H-null mDNA which was present in DNA/DNA duplexes bound to hydroxyapatite. The fraction of ^{3}H-null mDNA in DNA/RNA hybrids was calculated by subtracting the fraction of ^{3}H-null mDNA which bound to hydroxyapatite in sample D from that of sample T. In all hybridization reactions >95% of the input cpm was recovered.

Normalization to 100% ^{3}H-DNA Reactivity

Since the ^{3}H-single-copy DNAs lost reactivity at the rate of 1–2% per month, the reactivities of ^{3}H-mDNA and ^{3}H-null mDNA with total leaf DNA were assayed each time a series of hybridization reactions was set up. To do this, trace amounts of ^{3}H-DNA were reassociated with a >100,000 fold excess of unlabeled leaf DNA to DNA Cot 50,000 and the fraction of ^{3}H-single-copy DNA in DNA/DNA duplexes was measured by hydroxyapatite chromatography (Zimmerman and Goldberg, 1977; Goldberg, 1978; Goldberg et al., 1978). Reactivity values presented in the figure legends and tables are the average of ⩾3 individual reassociation reactions. To compare the results of separate mDNA and null mDNA hybridization experiments, all DNA/RNA hybridization terminal values were normalized to 100% tracer reactivity by dividing the observed terminal value by the most recently measured fraction of ^{3}H-mDNA or ^{3}H-null mDNA which bound to hydroxyapatite at DNA Cot 50,000. The range of reactivities was 70–90%.

Computer Analysis

Pseudo first-order rate constants and terminal values of DNA/RNA hybridization curves were obtained by using the computer program described by Pearson, Davidson and Britten (1977). Least-squares fitting procedures for RNA-excess/single-copy DNA hybridization experiments have been described by Galau et al. (1974, 1976).

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