The expression of a plant genome in hnRNA and mRNA

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ABSTRACT

Representation of genomic kinetic sequence classes and sequence complexities were investigated in nuclear and polysomal RNA of the higher plant *Petroselinum sativum* (parsley). Two different methods indicated that most if not all polysomal poly(A)-RNA is transcribed from unique sequences.

As measured by saturation hybridization in root callus and young leaves 8.7% and 6.2%, respectively, of unique DNA were transcribed in mRNA corresponding to 13.700 and 10.000 average sized genes. Unique nuclear DNA hybridized with an excess of polysomal poly(A)mRNA to the same extent as with total polysomal RNA. ³H-cDNA - poly(A)mRNA hybridization kinetics revealed the presence of two abundance classes with 9.200 and about 30 different mRNAs in leaves and two abundance classes with 10.500 and 960 different mRNAs in callus cells.

The existence of plant poly(A)hnRNA was proven both by its fast kinetics of appearance, its length distribution larger than mRNA, and its sequence complexity a few times that of polysomal RNA.

INTRODUCTION

A special feature of higher plant genomes as compared with those of animals is their high content of repetitive DNA amounting to more than 60% of the whole genome (2). This additional large amount of repetitive DNA raises the question whether there is a substantial class of structural genes being repetitious in the genome. In order to answer this question labeled RNA transcripts of high specific activity or the corresponding labeled cDNAs can be hybridized with an excess of total nuclear DNA (25). We show here with two methods that in parsley it is only unique DNA which codes for mRNA.

Another intriguing problem in understanding plant DNA transcription hitherto was the failure to ascertain the existence of a class of nuclear mRNA precursors equivalent to the hnRNA of animal nuclei. Animal hnRNA is now well established by its kinetics of synthesis and decay, by its length distribution, by its sequence complexity and its sequence class representa-

tion (53, 47). On the contrary until now it was not possible to unequivocally demonstrate the existence of hnRNA in higher plants, in lower plants no nuclear confined RNA class was found which considerably differed in length and complexity from that of mRNA (26,5). Only recently Goldberg et al. (6) found in tobacco a nuclear RNA comparing well in its sequence complexity with animal hnRNA. We report here the existence of hnRNA in a higher plant which was characterized by its fast kinetics of labeling, its size distribution other than mRNA, and its sequence complexity greatly exceeding that of mRNA.

The haploid nuclear DNA content of flowering plants varies from less than 1pg to more than 100pg (7) the coding capacity for more than 10^6 different proteins. Genetic considerations suggest that in higher organisms a maximum of up to 50.000 structural genes may be expressed during life time (8), a value now established for higher animals by some direct measurements. As higher plants undergo sophisticated differentiation processes it is interesting to see which amount of genetic information they use for establishing the function of differentiated cells and tissues. In our experimental approach we used DNA/RNA hybridization techniques both with ^3H -cDNA and ^3H -unique DNA, respectively, and polysomal mRNA excess (9, 10). The kinetics of hybridization based on a suitable standard and the proportion of unique DNA saturated are a measure of different genes expressed in mRNA. An uncertainty, however, arises in determining the complexity of the unique DNA hybridized. We suggest to relate complexity measurements to the amount of unique DNA sequences in the genome resolved in renaturation kinetics of moderately long sheared nuclear DNA.

MATERIALS AND METHODS

Growth of cells

Freely suspended callus cells originating from root explants of *Petroselinum sativum* were cultured under sterile conditions in a synthetic medium as described by Seitz and Richter (11). Young leaves were harvested from whole plants grown under field conditions.

Labeling of RNAs

During the logarithmic growth phase $2\mu\text{Ci/ml}$ $5\text{-}^3\text{H}$ -uridine and $2\mu\text{Ci/ml}$ ^3H -adenosine were added directly to the cells suspended in culture medium for various length of time. To achieve high specific activities of the labeled polysomal RNAs cells were given $25\mu\text{Ci/ml}$ ^3H -uridine.

Preparation of poly(A)nuclear RNA

Nuclei from parsley were prepared according to a modified procedure from Tautvydas (12). Washed cells were suspended in GAS (4% gum arabic, 0.15 M sucrose, 4 mM Mgacetate, 5 mM 2-mercaptoethanol, 5 mM Mes buffer pH 6.1). The cell suspension was homogenized and filtered through a series of nylon-screens up to pore diameters of 10 μ . The filtrate was centrifuged for 10 min. at 1000 g to pellet the nuclei. For further purification the nuclei were centrifuged through a gum arabic gradient ranging from 12% GAS to 4% GAS.

RNA was obtained according to a modified procedure of Glisin et al. (13) by lysing the nuclear fraction in RM medium: 0.1 M Tris-HCl pH 8.0, 1% (w/v) sarcosyl, 0.01 M EDTA, 0.1 M NaCl, 10 μ g/ml Proteinase K and 1 g/ml CsCl. The solution was layered onto a cushion of 2.4 ml 5.7 M CsCl with 0.1 M EDTA (density 1.707) in a cellulosenitrate tube. 0.5 ml of RM medium was then layered on top. The gradient was centrifuged in a Beckman SW 40 rotor at 28 000 rpm for 14 h at 20°C. The supernatant with the banded polysaccharides, DNA and proteins was removed, and RNA pellet resuspended in 0.5 ml NETSP buffer (0.5 M NaCl, 10 mM Tris-HCl pH 7.5, 0.5% sodium dodecyl sulfate, 10 μ g/ml Proteinase K). The RNA was incubated for 3 min at 65°C and applied to an oligo-dT-cellulose column equilibrated with NETSP. The bound poly(A)RNA was eluted with ETSP (10 mM EDTA, 10 mM Tris-HCl pH 7.5, 0.5% Sodium dodecyl sulfate, 10 μ g/ml Proteinase K). The pooled RNA was precipitated by the addition of absolute ethanol.

Preparation of polysomal RNA

Polysomes were prepared as described by Pfisterer and Kloppstech (14). RNA was isolated as described for nuclear RNA from nuclei. Poly(A)mRNA was obtained by repeated oligo-dT-cellulose chromatography.

Size estimation of RNA in denaturing gradients

Size estimation of heat denatured RNA was performed by layering onto a 5 - 20% (w/v) sucrose gradient containing 50% DMSO as described by Bantle et al. (15). Centrifugation at 40.000 rpm for 24 h at 20°C in a SW rotor (Beckman) gave optimal results in sedimenting of RNA.

Preparation of total nuclear DNA and of ³H-single-copy DNA

Petroselinum root callus DNA was prepared as previously described (16); the DNA used for hybridization assays with RNA was further purified by incubation for 20 h at 37°C in 0.4 M NaOH. Single-copy DNA was isolated by two cycles of renaturation of 300 N long DNA to a C_0t of 1000 and isolation of the single-stranded fraction by HAP chromatography. The single-copy DNA

was incubated to a C_{0t} of 4×10^4 and labeled with $^3\text{HdTTP}$ in vitro using E.coli DNA polymerase I as described by Galau et al. (17). The single-copy DNA prepared in this manner had a specific activity of 5×10^6 cpm/ μg and a mean length of 260 N. In RNA driven reactions this tracer DNA displayed a self reactivity of up to 0.7% at the highest C_{0t} values used (50.000).

Preparation of ^3H -cDNA

^3H -cDNA was synthesized from polysomal poly(A)RNA according to the procedure of Friedman and Rosbash (18).

Chain length determination of DNA

Hind III restriction fragments of SV 40 DNA and λ -DNA were used to calibrate the electrophoresis for molecular weight determinations. Digestions of SV 40 and λ -DNA by Hind III enzyme were performed according to Maniatis et al. (19). The molecular weights of the resulting fragments were derived from the data of Danna et al. (20) for SV 40 DNA and from those of the Boehringer Catalogue 78/79 for λ DNA.

DNA samples were denatured as described by Mc Master and Carmichael (21). Polyacrylamide (2.4%) - agarose (0.5%) composite disc-gels of a length of 12 cm were used (22). Electrophoresis was carried out at 5°C room temperature for 4 h with a maximum of 5 mAmp per gel.

DNA containing bands were stained by acridine orange according to Mc Master and Carmichael (21).

The size distribution of sheared unlabeled DNAs was determined by using a Joyce Loebel D8 MK 2 gel scanner. Gels with labeled DNA were sliced in 1 mm segments, and the radioactivity was measured.

Reassociation and hybridization

Annealing of DNA was performed as described previously (16). DNA/RNA hybridization reactions were carried out in 0.12 phosphate buffer/0.5% SDS at 60°C or 0.4 phosphate buffer/0.5% SDS at 64°C in sealed capillaries. The resulting C_{0t} or R_{0t} values were corrected to 0.18 M Na^+ as suggested by Britten et al (23). Maximum DNA and RNA concentrations used were 8 mg/ml. Tracer reactions were driven by an at least 1000 fold excess of the other component.

DNA reassociation and single-copy DNA/RNA excess hybridization reactions were analyzed by HAP chromatography as described by Kiper (24) with the modification that phosphate buffer was made 0.5% in SDS.

cDNA/RNA excess hybridization reactions were analyzed by S1 nuclease according to Hereford and Rosbash (25). RNA/DNA excess hybridization reactions were analyzed according to Melli et al. (26).

Computer analysis

First and second order reactions were analyzed by a BMD-least square computer program.

RESULTS

Characterization of polysomes and poly(A)mRNA

Plant cell cultures of parsley growing heterotrophically were chosen for this study, because large quantities of metabolically active cells can easily be obtained, under well defined and sterile conditions. Polysomes obtained from the postmitochondrial supernatant were sedimented in a sucrose density gradient (14), and poly(A)mRNA was isolated as described in methods.

After incubation of the cells for 45' with ^3H -Uridine 18% of the polysomal RNA carrying label were poly(A)RNA. By monitoring the absorbance at 260 nm the portion of poly(A)RNA turned out to be 1.05% of the total polysomal RNA. These results show that the poly(A)RNA has a higher rate of turnover than the ribosomal RNA. The poly(A)mRNA was active in a cell-free translation system of wheat germ extract which suggests its biological function.

The poly(A)mRNA sedimented as a broad peak ranging from 8 to 14 S (Figure 1). The number average size of the polysomal poly(A)RNA as determined by isokinetic gradient centrifugation turned out to be 1400 nucleotides (results not shown).

DNA sequence representation in polysomal poly(A)RNA

To answer the question which DNA sequence classes are represented in poly(A)mRNA hybridization of an excess of DNA with labeled RNA of high specific activity (26) as well as with labeled cDNA prepared from poly(A)-mRNA (9, 3) can be employed.

Trace amounts of ^3H -polysomal poly(A)mRNA were hybridized to an excess of sheared homologous nuclear DNA (700 N long). The results of this experiment are shown in Figure 2a. For comparison the reassociation kinetics of total nuclear DNA are also shown (in Figure 3). All the RNA hybridized with kinetics ($K = 0.9 \times 10^{-3} \text{ M}^{-1} \text{ sec}^{-1}$) indicating that it was complementary to and hence transcribed from single-copy DNA.

In another assay the kinetics of reassociation of cDNA from poly(A)mRNA with an excess of nuclear DNA was determined (Figure 2b). The cDNA reassociated as a single second order reaction with a K of $9.07 \times 10^{-4} \text{ M}^{-1} \text{ sec}^{-1}$. As the reassociation kinetics of ^3H -single-copy-DNA (about 300 N long) with the

Figure 1. Size distribution of mRNA and hnRNA. o represents ^{14}C -poly(A)mRNA and x represents ^3H -poly(A)hnRNA. The positions of unlabeled molecular weight markers are indicated by arrows.

total nuclear DNA (700 N long) has a $K = 5.6 \times 10^{-4} \text{ M}^{-1} \text{ sec}^{-1}$ and since the ratio of fragment lengths of cDNA to single-copy DNA is about 2, the result

Figure 2a. Hybridization of ^3H -poly(A)mRNA to an excess of nuclear DNA.
Figure 2b. Reassociation of cDNA with nuclear DNA

of the hybridization kinetics clearly indicates that all of the polysomal poly(A)RNA is transcribed from single-copy DNA.

Sequence complexity in callus polysomal mRNA

Sequence complexity of total callus polysomal mRNA was determined by measuring the percentage of unique nuclear DNA which can be rendered double-stranded when annealed to total polysomal RNA in RNA excess (10). The reassociation of the purified parsley single-copy ^3H -DNA in the presence of an excess of unfractionated 700 N nuclear DNA is shown in Figure 3. The dashed line described the reassociation of the driver DNA, which has been quantitatively described elsewhere (16). Figure 3 shows that the rate constant for reassociation of the single-copy tracer with excess whole 700 nucleotides long nuclear DNA fragments was about $0.56 \times 10^{-3} \text{ M}^{-1} \text{ sec}^{-1}$. By C_0t 50.000 78.9% of the ^3H -DNA reacted. Figure 3 indicates that the single-copy ^3H -DNA contains no detectable contamination with repetitive sequences. In the absence of driver DNA less than 0.4% of the ^3H -DNA binds to hydroxyapatite at C_0t below 1; up to 0.7% binds at the highest C_0t s measured in the presence of excess yeast tRNA (50.000). These values routinely are subtracted from the hybridization data with excess RNA.

Figure 3. Reassociation of unique DNA. The x represent the reassociation kinetics of 700 N total nuclear DNA with trace amounts of ^3H -unique DNA (250-300 N long). The curve is a least squares fit to the data with an RMS of 0.1% and a plateau value of reactivity of 78.9%. The rate constant K was $0.56 \times 10^{-3} \text{ M}^{-1} \text{ sec}^{-1}$. Included are the reassociation kinetics of total 300 N long nuclear DNA (dashed line) quantitatively described elsewhere (16). The open circles represent the hybridization of unique ^3H -DNA originally hybridized to polysomal poly(A)mRNA and rehybridized to an excess of 700 N long total nuclear DNA.

The actual reactivity was determined for individual experiments. For reasons of comparability all unique ^3H -DNA saturation data are presented as reflecting a 78.9% reactivity of the tracer.

It must be pointed out here that according to more conventional conceptions this unique DNA preparation would be thought of as to be a representative probe of the total unique DNA fraction of the parsley genome, i.e. 30% of the 1.9×10^9 Np haploid genome. This only holds true if the assumption is valid that unique sequences are at least a few times longer than the median length of sheared fragments, as found for some animals (27, 28, 29). However, in plants it appears that the vast majority of all unique sequences can be rather short, about 300 N long (30). This must be true, too, in parsley where we showed that of the 30% genomic unique fraction only 12% may be revealed in reassociation kinetics of 300 N long total nuclear DNA (16).

As is argued in length elsewhere (54) we thus take our unique DNA preparation to be a representative probe of $0.12 \times 1.9 \times 10^9$ Np = 2.3×10^8 Np.

At C_0t 50.000 apparently saturation of ^3H -unique DNA with total polysomal RNA is reached (Figure 4a). The plateau level is 3.43%. The rate constant is $0.55 \times 10^{-3} \text{M}^{-1} \text{sec}^{-1}$ equaling that of total DNA excess hybridization of the same tracer. As it has been shown that the rate of RNA/DNA hybridization is very close to that predicted from DNA/DNA reassociation (32) this reflects a mRNA fragment length of about 700 N during incubation. After correcting the amount of ^3H -unique DNA which hybridized to the polysomal RNA for asymmetric transcription and tracer reactivity we calculate that 8.7% of the single-copy DNA in the parsley genome is represented in callus cell polysomes (Table 1). Taking the single-copy fraction a representative probe of 2.3×10^8 Np nuclear DNA this corresponds to 1.98×10^7 nucleotides of mRNA transcripts or approximately 13.700 different 1400 N sized genes. An additional feature of the data presented in Figure 4a is the fact that the single-copy DNA tracer is rendered double-stranded with kinetics which are well approximated by a theoretical pseudo first-order reaction. These kinetics indicate that most of the 13.700 different RNA species are present at about the same intracellular concentration.

Sequence complexity in callus polysomal poly(A)⁺mRNA

In parsley cells only 20-40% of the total polysomal mRNA is polyadenylated (33). To test whether poly(A)⁺mRNA and poly(A)⁻mRNA are different pools with respect to their complexity poly(A)⁺mRNA was brought to reaction in excess with the ^3H -single-copy tracer DNA (Figure 4a). At C_0t 3000 apparent-

Figure 4a. Hybridization of unique ^3H -DNA with root callus polysomal mRNA. The curves represent the hybridization of unique ^3H -DNA with an excess of polysomal poly(A)mRNA (X) and an excess of total polysomal RNA (O). Saturation values were 3.35 and 3.43% respectively, rate constants were $0.054 \text{ M}^{-1}\text{sec}^{-1}$ and $0.0016 \text{ M}^{-1}\text{sec}^{-1}$.

Figure 4b. Hybridization between unique ^3H -DNA and leaf polysomal mRNA and nuclear RNA. The $\square$ represent the hybridization kinetics of unique ^3H -DNA with an excess of leaf polysomal mRNA. Saturation value was 2.45%. The X show the hybridization of unique ^3H -DNA with an excess of total nuclear RNA. The saturation value was 9.79% the rate constant $0.00012 \text{ M}^{-1}\text{sec}^{-1}$. The O points represent the hybridization of poly(A)hnRNA with the tracer ^3H -unique DNA. Included is the hybridization of root callus polysomal mRNA with ^3H -unique DNA (dashed line).

ly saturation of ^3H -unique DNA with polysomal poly(A)⁺mRNA is reached. The plateau level is 3.35%, the rate constant $0.018 \text{ M}^{-1}\text{sec}^{-1}$. Compared with the rate constant of total polysomal RNA this means a 33fold acceleration. As poly(A)⁺mRNA makes up only one third of the total polysomal mRNA this means an about 100fold purification of poly(A)⁺mRNA. After correcting the amount of hybridized ^3H -unique DNA for asymmetric transcription and tracer reactivity, we calculate that 8.5% of the single-copy DNA in the parsley genome is represented in callus poly(A)⁺mRNA (Table 1) which corresponds to 1.94×10^7 nucleotides of mRNA transcripts or approximately 13.300 different 1400 N sized genes. This value does not differ significantly from that for total polysomal mRNA indicating that functional poly(A)⁻mRNAs carry no information different from that of poly(A)⁺mRNA. The similar shape of

Table 1. Sequence complexity of nuclear and polysomal RNA as measured by hybridization to ^3H -unique DNA.

RNA	saturation value (%)	corrected saturation value (%) ^a	complexity (nucleotides) ^b	number of 1400 N sequences ^c
root callus polysomal poly(A)mRNA	3.35	4.25	1.9×10^7	13.300
root callus polysomal mRNA	3.43	4.35	2.0×10^7	13.700
leaf polysomal mRNA	2.45	3.11	1.4×10^7	10.000
root callus total nuclear RNA	9.79	12.4	5.7×10^7	
root callus poly(A) hnRNA	7.30	9.25	4.3×10^7	

^acorrected for 78.9% reactivity of ^3H -unique DNA tracer^breferring to a unique DNA sequence complexity of 2.3×10^8 Np/haploid genome^cassuming asymmetric transcription

both DNA/RNA hybridization curves indicates that the complex class driving the reaction has about the same frequency distribution in both fractions.

To ascertain the gene number by an independent method and to provide an estimate of the range of mRNA abundance classes in parsley callus cells poly(A)mRNA was hybridized to complementary ^3H -cDNA (9). The results are presented in Figure 5a. The hybridization of rabbit globin mRNA with its labeled cDNA as a kinetic standard with which to compare more complex hybridization systems is included in Figure 5b. Rabbit globin mRNA which consists of α and β globin sequences with a total complexity of 4×10^5 daltons (34) hybridized with a rate constant $K = 260 \text{ M}^{-1}\text{sec}^{-1}$.

Computational analysis showed that the data for the reaction of parsley poly(A)mRNA with its homologous cDNA are best described by two components representing different abundance classes.

The data could be resolved into a larger number of components but this did not significantly improve the degree of fit. Furthermore, their division into abundance classes is not quantitatively exact in the sense, that, within a given class, not all sequences will have exactly the same abundance. As is listed in Table 2 the greatest contribution to the total base sequence

Figure 5a and 5b. Hybridization between parsley root callus (a) and leaf (b) polysomal poly(A)mRNA with its complementary ^3H -cDNA. The line through the data represents a computer least square fit to the data using two components (dashed lines). The dashed pointed line represents the kinetics of hybridization of globin mRNA with its complementary cDNA. The parameters are listed in Table 2.

Table 2. Abundance classes in parsley polysomal poly(A)mRNA.

poly(A)mRNA source	fraction of polysomal poly(A)RNA mass ^a	K ($\text{M}^{-1}\text{sec}^{-1}$)	K_{pure} ($\text{M}^{-1}\text{sec}^{-1}$)	Number of 1400 N sequences ^c	Number of molecules per cell per sequence ^d
root callus	0.56	0.1328	0.235	960	60
	0.44	0.0094	0.022	10.500	4
leaf	0.29	2.34	8.07	28	1.060
	0.71	0.020	0.028	9.200	8

^a the fraction of polysomal poly(A)RNA mass is calculated as the individual component to the total reacting ^3H -cDNA

^b K_{pure} is calculated dividing K with the fraction of polysomal poly(A)RNA

^c the number of molecules represented by each transition was calculated referring to the complexity of globin mRNA (34) and to the rate constant of hybridization of rabbit globin ^3H -cDNA (600 N) with its complementary poly(A)mRNA found to be $260 \text{ M}^{-1}\text{sec}^{-1}$ under our hybridization conditions. As the parsley cDNA preparations were of equal length we directly compared its K_{pures} with that of globin ^3H -cDNA/poly(A)mRNA hybridization.

^d in parsley root cells we determined the ratio of poly(A)mRNA to DNA to be $0.08 \text{ pg}/4.0 \text{ pg} = 0.02$. This amounts to about 100.000 mRNA sequences of length 1400 N per cell. Copies per sequence per cell then was calculated as fraction of polysomal poly(A)RNA $\times 10^5$ / number of 1400 N sequences

complexity is from the least abundant resolvable class which is the most slowly hybridizing component. Comparing the $R_{ot_{1/2}}$ of both components with that of globin we conclude that there are two different abundant classes in parsley root callus suspension cells one with 56% of all mRNAs representing about 960 different sequences of average length 1400 N and the other comprising 44% and representing about 10,500 different species. Frequencies are 60/cell for the more abundant and 4/cell for the less abundant class.

Polysomal mRNA sequence complexity in leaves

Up to this point only mRNA complexities of root callus cells were regarded representing one special plant cell type. To get a deeper insight into the genetic information needed for a whole tissue we examined the mRNA complexity of young leaves of parsley, too. As is shown in Figure 4b, ^3H -single-copy DNA hybridized to about 2.45% with total polysomal leaf RNA at a rate constant of $6.6 \times 10^{-4} \text{ M}^{-1} \text{ sec}^{-1}$ representing a complexity of 1.42×10^7 N or 10,000 genes of average length 1400 N. This is a somewhat lower value than the 13,700 active genes found in callus cells.

To verify the gene number found in leaves by an independent method we hybridized poly(A)mRNA with its complementary cDNA. As is displayed in Figure 5 the reaction is best described by two components. The more abundant class represents 29% of all mRNAs and comprises 28 different mRNAs each of which being present about 1060 times per cell. The more complex class representing 71% of the mRNA population comprises 9,200 different structures of average length 1400 N each being present about 8 times per cell. Results obtained according to both methods match rather well as they did with callus cells. It seems that both in root callus cells and in leaf tissue there is about the same number of different expressed genes. A dramatic change, however, occurs in what concerns the existence of a highly abundant class. While in the fast proliferating root callus cells a broad class of abundant mRNAs exists, in leaf only a class of highly abundant mRNAs is revealed.

Preparation and size of poly(A)hnRNA

To characterize primary transcripts nuclei were rapidly isolated, and a nuclear poly(A)RNA fraction was extracted.

Comparing with poly(A)mRNA label in nuclear poly(A)RNA appeared much faster than in poly(A)mRNA, as can be seen in Table 3.

To characterize poly(A)nuclear RNA with respect to its relationship with poly(A)mRNA the size distribution of both species was determined by cosedimenting both RNAs in a denaturing sucrose gradient which contained 50%DMSO. Previously, cells of the same sample were either labeled with ^{14}C -uridine

Table 3. RNA labeling kinetics.

Time of incubation with ³ H-uridine and ³ H-adenosine (min)	specific activity	(cpm/100 μg RNA)
	poly (A) hnRNA	poly (A) mRNA
30	22.500	10.250
60	37.500	17.500

for 120 min, and their polysomes prepared or they were labeled for 20 min with ^3H -uridine and ^3H -adenosine, and nuclei isolated.

Nuclei and polysomes were put together in RM medium with CsCl and then poly(A)nuclear RNA and poly(A)mRNA were coisolated and cosedimented in a denaturing sucrose gradient. The results presented in Figure 1 reveal that a fraction of poly(A)-nuclear RNA sediments faster than the ^{14}C -labeled poly(A)mRNA. This indicates that a fraction of nuclear RNA differs from mRNA with respect to size distribution.

Sequence complexity of poly(A)hnRNA and of total nuclear RNA

To further characterize this hnRNA which has a somewhat larger size distribution than that of mRNA it is of interest to see whether it displays a sequence complexity vastly in excess of that of mRNA as was reported for most animal hnRNAs investigated so far (38, 39).

Accordingly, we hybridized ^3H -unique DNA with an excess of root callus poly(A)hnRNA and of callus total nuclear RNA. As is seen in Figure 4b poly(A)hnRNA hybridized with single-copy DNA to 7.3%, total nuclear RNA to 9.79%. Assuming asymmetric transcription and correcting for tracer reactivity this accounts for 18.5 and 24.8%, respectively of unique DNA, i.e. 2.2 and 3.0% of the whole nuclear genome are represented in nucleus confined RNA. Compared with 1.0% genomic DNA transcribed into functional mRNA this amounts to a two and threefold higher complexity in nuclear poly(A)hnRNA and total hnRNA.

DISCUSSION

DNA sequence representation in mRNA

Highly labeled poly(A)mRNA and ^3H -cDNA transcribed from poly(A)mRNA were hybridized with an excess of total nuclear DNA. Both experiments revealed that poly(A)mRNA annealed mainly or exclusively to nonrepetitive DNA (Figures 2a, b), i.e. that only single-copy DNA codes for poly(A)mRNA. As we found that poly(A)mRNA is a representative probe of all mRNA as to what

concerns complexity this result extends to all polysomal mRNAs. However, a small population of mRNA lacking poly(A) like histone mRNAs would have stayed undetected. Concerning the sequence representation of poly(A)mRNA in plants similar experiments have been performed with tobacco plants (6). The results obtained are in fair agreement with that found for parsley.

In contrast, findings of similar studies with animals and fungi showed that beside the single-copy DNA also a small repetitive component was coding for poly(A)mRNA (40, 41, 42, 43, 5). Most of the plant genome is made up of repetitive DNA. Surprisingly, it appears that all that repetitive DNA does not play any role in coding for mRNA. Preliminary results even show that most hnRNA is not coded for by repetitive DNA.

Sequence complexities of root callus poly(A)⁺ and poly(A)⁻mRNA

Sequence complexities of mRNAs were determined by saturation of ³H-labeled single-copy DNA. As is displayed in Figure 3 the unique DNA preparation used in these experiments seems to be free of repetitive DNA, thus errors due to the latter remaining small. Another argument, suggesting that the results are not biased for higher gene numbers due to hybridization to repetitive DNA comes from our finding that it is only unique DNA which codes for mRNA. From our finding that the parsley genome contains 2880 rRNA genes (4), when assuming a coding length of 6×10^3 N for all three rRNAs follows that a total of 0.9% of the genome codes for rRNAs. Taking into account that only this minor fraction of repetitive DNA if present in our incubation mixture, might hybridize to mRNA and that the genome fraction measured coding for mRNAs exceeds that coding for rRNAs by a factor of three we are sure that we are really measuring mRNA complexities under our conditions. This has been confirmed, too, by an experiment, given in Figure 3, where at least 80% of the unique DNA previously hybridized to mRNA and rehybridized to total nuclear DNA reacted with the kinetics of single-copy DNA.

As shown in Figure 4a sequence complexities measured for poly(A)⁺ and poly(A)⁻mRNA do not differ significantly. This can not be an effect of contamination of poly(A)⁺mRNA with poly(A)⁻mRNA. As was pointed out earlier our poly(A)⁺mRNA preparation is purified about 100fold compared to total polysomal mRNA preparations. As the hybridization reactions are driven by the least abundant and most complex class of mRNA any large contamination of poly(A)⁺mRNA by a complex poly(A)⁻mRNA population must give rise to a retarded fraction of the hybridization kinetics. As we have not obtained evidence for this, we conclude that poly(A)⁻mRNA is not a class of mRNA differing in sequence complexity from that of poly(A)⁺mRNA.

This extends the finding of Ragg et al. (44) that in parsley root callus suspension cells both the poly(A)⁺ and the poly(A)⁻ polysomal RNA added to a reticulocyte cell free translation system stimulated the synthesis of proteins with similar size distribution.

Sequence complexities of root callus and leaf cells

We conclude from the ³H-DNA saturation hybridization data presented in Figure 5a and Table 1 that in parsley root callus 8.7% and leaves 6.2% of unique DNA codes for mRNA. Assuming a 30% fraction of the genome to be unique (16) and assuming the single-copy tracer to be a representative probe of it, we would estimate that in root callus cells there are 35,000 active genes and in leaves 25,000. This diverges greatly with gene number estimates obtained from the hybridization of poly(A)mRNA with its complementary cDNA. Since poly(A)⁺ and poly(A)⁻ mRNA are mRNA subsets of equal complexity as demonstrated this large divergence is not attributable to a difference measured between both pools. The excessive difference of 1 : 3 observed here was also found for gene numbers in tobacco (6). Since the relative proportion of gene numbers obtained according to both methods are constant $9200/25000 = 36.8\%$; $11500/35000 = 32.9\%$ and since these large discrepancies up to now are confined to higher plants (in lower plants and in animals rather consistent results may be achieved (25, 46) we suppose that the difference reflects a special feature of plant genomes as compared to animal genomes. As derived in length elsewhere (54), it will be more valid to base gene number estimates on the amount of unique DNA revealed in reassociation kinetics of moderately long sheared DNA.

Based on this 12% genomic unique DNA content (16) we estimate that in parsley root callus and leaves there are 13,700 and 10,000 different active genes according to saturation experiments. This compares reasonably well to the 11,500 and 9,200 found according to the cDNA method. This estimate lies well within gene numbers determined for higher animals (47) and proves that higher plants, too, have at their disposal a mass of information allowing them to build up as intricate differentiated cells and tissues as do animals.

mRNA abundancies in root callus and leaves

Taken together the results described above imply that gene numbers in root callus and in leaf differ by about 30% ($11,500/9,200 = 1.25$; $13,700/10,000 = 1.37$) as measured by both methods. However, there is a major difference in what concerns abundancies of individual mRNAs. Whereas in root callus cells a broad spectrum of about 960 mRNA species make up the

most abundant mRNA class, in leaf there exists a rather narrow class of about 30 different species making up 30% of all mRNAs and each being present more than 1000 times per cell as contrasted to the other 70% being present only 8 times per cell on an average. These differences between root callus cells and leaf cells may really reflect two different states of differentiation as it can be correlated with different spectra of proteins, too. Whereas in leaf cells a minor number of prominent protein bands can be distinguished (37) in root callus no prominent bands are formed but rather a continuum of many different proteins (Schröder, personal communication). Since root callus cells are fast proliferating we assume that these numerous abundant proteins are confined to growth and division processes, whereas in leaves the few most abundant mRNAs are involved in manufacturing proteins for establishing photosynthetic active structures (37).

Evidence for hnRNA in a higher plant

The data presented here show that a fraction of poly(A) nuclear RNA sediments more rapidly than does poly(A)mRNA, i.e. there exists a fraction of nuclear poly(A)RNA which is larger than poly(A)mRNA.

The poly(A)mRNA from parsley sedimented with a similar size distribution as that found in other plant tissues (6, 44, 48, 49). Whereas hnRNA isolated from most animal tissues is much larger in size than mRNA (47), we could not find any poly(A)-hnRNA in parsley sedimenting more rapidly than 21 S, though a posttranscriptional degradation cannot be excluded. In lower eucaryotic plants the size difference between hnRNA and mRNA is much less or not detectable (50, 5). Up to now little is known about hnRNA in higher plants, but a nuclear poly(A)RNA has been described (51, 52).

The isolation of hnRNA is difficult for several reasons. Because of the massive cell wall a rapid and careful preparation of nuclei is complicated. Sometimes plant cells contain a rather high concentration of RNases which will prevent the isolation of high molecular weight RNAs. In animal cells the synthesis of ribosomal RNA can be depressed by low doses of actinomycin, which are ineffective in plants; thus in plants only the poly(A)hnRNA fraction can be isolated. As evidence is lacking for the involvement of polyadenylation in the transcription of poly(A)hnRNA the isolated product may not simply be considered to represent the primary transcript, indeed.

However, measurements of sequence complexity with single-copy DNA are not significantly affected by degradation of RNAs. It is hence of special interest to determine sequence complexities of nuclear RNA. The results

reported in Figure 5a and Table 1 demonstrate that a hnRNA exists in plant cells like in those of numerous animals which exhibits a sequence complexity of three times that of mRNA as well as a poly(A)hnRNA which exhibits still more than two times a complexity that of mRNA. This is consistent with recent findings (6) showing that in tobacco, too, such a hnRNA exists. These results provide strong evidence for the existence of hnRNA in higher plants. The feature of this hnRNA is its fast kinetics of labeling, its sequence complexity being several times that of mRNA and its size larger than that of mRNA.

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