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SEQUENCE COMPLEXITY OF MESSENGER RNA IN COTYLEDONS OF DEVELOPING PEA (*PISUM SATIVUM*) SEEDS

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Key Word Index—*Pisum sativum*; Leguminosae; poly(A)-containing mRNA; single-copy DNA; hybridization kinetics; storage proteins.

Abstract—The hybridization kinetics of poly(A)$^+$-RNA preparations from the cotyledons of developing pea (*Pisum sativum*) seeds to complementary DNAs have shown that the number of distinct sequences in poly(A)$^+$-RNA decreases from *ca* 20 000 at the early stage of cotyledon development to *ca* 200 at a late stage of cotyledon development. The decrease in sequences is accounted for entirely by the disappearance of 'rare' poly(A)$^+$-RNAs ($< 10^3$ copies/cell) as seed development proceeds. There is an increase (1–6) in very abundant poly(A)$^+$-RNA sequences ($> 5 \times 10^5$ copies/cell) from early- to mid-developmental stages, concomitantly with the increase in the synthesis of seed-specific storage protein polypeptides. In agreement with the continuing synthesis of most of these polypeptides to the end of seed development, the number of very abundant poly(A)$^+$-RNAs is maintained to the late cotyledon development stage. Abundant poly(A)$^+$-RNA sequences (*ca* 10^4 sequences/cell) increase from 80 to 180 during development, possibly corresponding to the polypeptides which are not storage proteins but are known to be accumulated in pea seeds. Hybridization of single-copy pea genomic DNA sequences to poly(A)$^+$-RNA from developing seeds showed that *ca* 5% of the single-copy sequences were present in mRNA from mid-development cotyledons. In addition, hybridization of cDNA prepared against poly(A)$^+$-RNA from nuclei of early development cotyledons to the corresponding cytoplasmic polysomal poly(A)$^+$-RNA showed that the cytoplasmic poly(A)$^+$-RNA contained *ca* 50% of the sequences present in the nuclei. These results are discussed and interpreted in the light of existing results from similar systems.

INTRODUCTION

The cotyledons of developing pea (*Pisum sativum* L.) seeds are an example of a differentiated eukaryotic cell system in which a relatively small number of proteins, the seed storage proteins, are synthesized to a very much greater extent than other proteins. In other similar developmental cell systems, such as HeLa cells [1] or guinea pig mammary gland [2], and more closely related systems, such as soybean seeds [3], mRNA species could be divided into a series of abundance classes by a study of the kinetics of their hybridization to homologous cDNAs. In these differentiated eukaryotic cells it has usually been assumed that the most abundant mRNA class consists of sequences coding for those polypeptides being predominantly synthesized [4]. We have previously shown [5–8] that poly(A)-containing messenger RNA isolated from cotyledons of developing pea seeds constitutes *ca* 1% of total RNA, exhibits an average size of 18S and encodes the precursors of the major storage proteins: legumin (60 000 M_r precursor), vicilin (50 000 M_r and 47 000 M_r precursors) and convicilin (71 000 M_r precursor). These polypeptides, constituting over 80% of total protein in the cotyledon, are major components of the total *in vitro* translation products and are presumed to be synthesized on abundant template mRNAs.

The present paper describes experiments intended to investigate the complexity of the mRNAs of developing pea cotyledons, the way in which they change during seed development and their relationship to protein synthesis. In addition, hybridization of mRNA to mainly non-repetitive pea genomic DNA sequences was carried out in order to estimate the number of distinct sequences transcribed into seed poly(A)$^+$-RNA and the proportion of total non-repetitive genomic DNA sequences. Since the pea haploid genome, which contains 4.8×10^9 nucleotide pairs [9], is much larger than many other plant genomes, it is of interest the estimate whether the number of transcribed sequences is similar to those in analogous cells in other plant systems. Limited hybridization of nuclear RNA to cotyledon mRNA was also carried out to assess the amount of post-transcriptional processing which occurred, i.e. to determine what proportion of the transcribed sequences were being exported to the cytoplasm as functional mRNA.

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RESULTS

Sequence complexity of poly(A)$^+$-RNAs

The hybridization curves for each poly(A)$^+$-RNA with its homologous [^{3}H]cDNA are shown in Fig. 1, with standard curve obtained for the hybridization of globin mRNA with its [^{3}H]cDNA included for comparison.

Globin mRNA hybridized to its [^{3}H]cDNA with the expected kinetics for a single sequence, with the single biphasic hybridization curve extending over two log units of R_0t and giving an $R_0t_{1/2}$ value of 4×10^{-4} mol/l. This figure, which was used in all subsequent calculations, is comparable with the reported values of $R_0t_{1/2}$ between

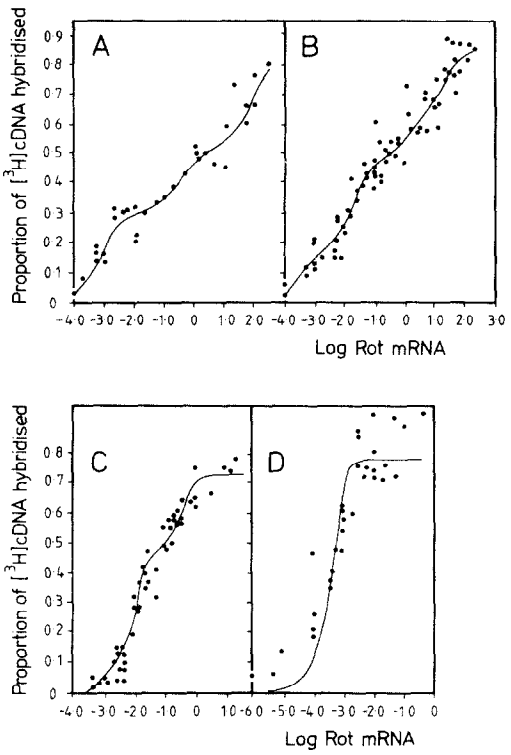

Fig. 1. Hybridization of [^{3}H]cDNAs to homologous poly(A)$^+$-RNA templates. Poly(A)$^+$-RNAs were isolated from pea cotyledons at different stages of development: (A) 9 days; (B) 14 days; (C) 19 days after flowering; (D) hybridization curve for rabbit globin mRNA and its [^{3}H]cDNA (standard curve). Each curve drawn is a best fit computed from the least squares analysis of the data. The proportion of [^{3}H]cDNA hybridized was corrected for self-annealing of the [^{3}H]cDNA, which was between 0.005 and 0.02.

globin mRNA and its cDNA. (Hastie and Bishop [10] reported that a pure mRNA species of 6.5×10^5 M_r will hybridize a cDNA copy with a $R_0t_{1/2}$ at 8×10^{-4}.) The hybridization curves for the poly(A)$^+$-RNA preparations with their cDNAs extended over 4–6 log units of R_0t and contained 2–4 distinct transitions. These curves were thus analysed as a series of single transitions, each transition corresponding to an mRNA abundance class, as described by Bishop *et al.* [1]. The numerical analysis of these hybridization curves is shown in Table 1.

Poly(A)$^+$-RNA isolated from cotyledons 9 days after flowering (d.a.f.) (early in cotyledon development at the onset of storage protein synthesis) showed a hybridization curve with three transitions (Fig. 1A). The hybridization was not brought extensively to a completion due to a shortage of poly(A)$^+$-RNA from these early cotyledons. The three transitions corresponded to a very abundant mRNA class, apparently a single sequence, present at *ca* 3×10^6 copies/cell, an abundant mRNA class (80 sequences, at *ca* 8×10^3 copies/cell) and a rare mRNA class (19 000 sequences, at *ca* 40 copies/cell). The mid-development poly(A)$^+$-RNA, 14 d.a.f., gave a hybridization curve containing four transitions. The lowest transition had a very low calculated sequence complexity (85 nucleotides), was present in small proportion and was possibly due to mRNA degradation; it was, therefore, disregarded. The remaining three transitions corresponded to the very abundant, abundant, and rare mRNA classes of 9 d.a.f., with six sequences at *ca* 7×10^5 copies/cell, 120 sequences at *ca* 2×10^4 copies/cell, and 4800 sequences at *ca* 7.1×10^2 copies/cell, respectively. The late development poly(A)$^+$-RNA, 19 d.a.f. (towards the end of the period of storage protein synthesis) gave a hybridization curve cotaining two transitions only, and contained very abundant (six sequences at *ca* 6×10^5 copies/cell) and abundant (170 sequences at *ca* 2×10^4 copies/cell) mRNA classes only.

Characterization of single-copy DNA

Total pea DNA of an average single strand length > 50 000 bases melted at $T_m = 88.3°$, whereas fragmented pea DNA melted at $T_m = 83°$; these values are in a reasonable agreement with literature data [11]. The preparations showed 22.4% and 18.2% hyperchromicity, respectively.

The established conditions for sonication produced DNA fragments of average size of 350 nucleotides, with size distribution ranging from 150–1000 nucleotides, as determined by dissociating gel electrophoresis [12]. After re-association of fragmented DNA to $C_0t = 200$, the fraction of DNA not bound to hydroxylapatite (i.e. single-stranded) constituted *ca* 33% of the total DNA input. This fraction was isolated, concentrated and re-associated to $C_0t = 1000$. The final isolated, single-stranded, non-repetitive DNA constituted *ca* 12% of total pea DNA, in reasonable agreement with the data of Murray *et al.* [9]. ^{32}P-5′-end labelling of single-copy DNA produced fragments of average size 180 nucleotides, labelled to the specific activity of 7×10^6 cpm/μg. The reactivity of this single-copy DNA ([^{32}P]scDNA) was determined by hybridization to a vast mass excess of fragmented leaf DNA (driver DNA) to $C_0t = 50\,000$ [13]. The duplexes were eluted at 98° with 0.12 M phosphate buffer from a hydroxylapatite column and the reactivity determined as 70%. When molecular hybridization was carried out to $C_0t = 20$ and $C_0t = 1.5$, 22% and 9%, respectively, hybridized indicating the presence of some repeated sequences in the single-copy DNA. The latter hybridizations were carried out at 50° instead of 68°, so that the fraction of repeats is probably $\leqslant 0.1$.

Hybridization of [^{32}P]-single copy DNA to poly(A)$^+$-RNA

The hybridization data for [^{32}P]scDNA with poly(A)$^+$-RNA isolated from 14-day-old cotyledons, are plotted in Fig. 2. At the highest R_0t value of 10^5 *ca* 5% of the scDNA formed duplexes with poly (A)$^+$-RNA. This R_0t value should be high enough to account for complete hybridization, including rare sequences, as the homologous annealing of these sequences in 14 day poly (A)$^+$-RNA to cDNA was complete at $\log R_0t = 2$. Clearly, the hybridization reactions occurred over R_0t values for rare sequences of poly(A)$^+$-RNA [14]. A small but significant amount of hybridization, *ca* 0.7%, was observed below $\log R_0t = 0$. The self-reassociation was estimated as *ca* 0.75% and the results were corrected using this factor. However, the results obtained were somewhat underestimated since the self-reassociation of scDNA would be lower in the presence of poly(A)$^+$-RNA.

Table 1. mRNA complexity calculated from hybridization data (Fig. 1)

Poly(A)$^+$-RNA source*	'Abundance' class	Fraction of labelled cDNA† (P)	Fraction of mRNA (mass)‡	$R_0t_{1/2}$§ (A) (M. sec)	k_{obs}‖ (M^{-1}/sec)	k_{pure}¶ (M^{-1}/sec)	Complexity** (No. of nucleotides)	No. of diverse mRNAs††	Copies of mRNA per cell‡‡
9-day	1 (v. ab.)	0.29	0.37	0.0007	985.70	264.9	6.68×10^2	1	2.6×10^6
	2 (ab.)	0.20	0.26	0.232	2.97	11.38	1.56×10^5	78	7.7×10^3
	3 (rare)	0.29	0.37	41.480	0.02	0.05	3.85×10^7	19,300	4.4×10^1
14-day	1 (s. ab.)	0.14	0.17	0.0002	3,450	20,671	85	1	4.5×10^7
	2 (v. ab.)	0.31	0.37	0.013	53.10	142.9	1.24×10^4	6	6.9×10^5
	3 (ab.)	0.13	0.16	0.600	1.15	7.16	2.47×10^5	124	1.5×10^4
	4 (rare)	0.25	0.30	12.622	0.05	0.18	9.65×10^6	4,825	7.1×10^2
19-day	1 (v. ab.)	0.43	0.59	0.0075	92	156.3	1.13×10^4	6	6.4×10^5
	2 (ab.)	0.30	0.41	0.327	2.11	5.12	3.45×10^5	173	1.5×10^4

Calculations are based on data from Fig. 1 using the methods of Goldberg *et al.* [3, 4]. s. ab., Super abundant; v. ab., very abundant; ab., abundant.

*Age of cotyledons from which the poly(A)$^+$-RNA was isolated.

†The fraction of [^{3}H]cDNA hybridized in each class. This is the value P from the computed curve.

‡The fraction of poly(A)$^+$-RNA mass hybridized, i.e. †normalized to 100% cDNA reactivity.

§$R_0t_{1/2}$ for each class—a value computed from the kinetic curve.

‖$k_{observed}$. The pseudo-first-order rate constant, where $k = \dfrac{0.69}{R_0t_{1/2}}$ M^{-1}/sec.

¶k_{pure}. The pseudo-first-order rate constant, if the abundance class was 100% pure, $k_{pure} = \dfrac{k_{obs}}{\ddagger}$.

**Complexity $= \left(\dfrac{k_{globin} \times C_{globin}}{k_{pure}}\right) \times \left(\dfrac{L}{600}\right)^{1/2} \times \left(\dfrac{L}{M}\right)^{1/2}$. The standard curve obtained for rabbit globin mRNA had a $R_0t_{1/2}$ of 0.0004 M. sec. The pseudo-first-order rate constant, k, was: 1725 M^{-1}/sec. The complexity of rabbit globin mRNA (C_{globin}) is 1178 nucleotides [33]. The last two terms of the equation correct for differences in lengths of cDNA and poly(A)$^+$-RNA, which affects reaction rates. (Globin cDNA was assumed to have a complexity of 600 nucleotides [28]) L, the modal cDNA size was 3.1×10^5 MW (*ca* 954 nucleotides); M, the number-average poly(A)$^+$-RNA size at the beginning of hybridization, was 6.5×10^5 MW (*ca* 2000 nucleotides) [25].

††No. of diverse poly(A)$^+$-RNAs $= \dfrac{\text{Complexity of the abundance class}}{N}$, where N is the number-average polysomal poly(A)$^+$-RNA size, *ca* 2000 nucleotides [25].

‡‡No. of molecules per cell per sequence is the No. of nucleotides in an abundance class per cell, divided by the complexity of that abundance class:

i.e. No. molecules/cell·sequence $= \dfrac{(F_n) \times [\text{g poly(A)}^+\text{-RNA/cell}] \times (6 \times 10^{23} \text{ nucleotides/mol})}{(C_n) \times (325 \text{ g/mol nucleotides})}$. The prefix n refers to the abundance class; F is the fraction of poly(A)$^+$-RNA mass‡; C is the complexity of the class expressed in nucleotides**; g poly(A)$^+$-RNA/cell was 2.5 pg for 9-day-old cotyledons, 12.4 pg for 14-day-old cotyledons and 6.7 pg for 19-day-old cotyledons [18]. Avogadro's No. divided by the MW of approximate MW of nucleotides gives No. of nucleotides/g.

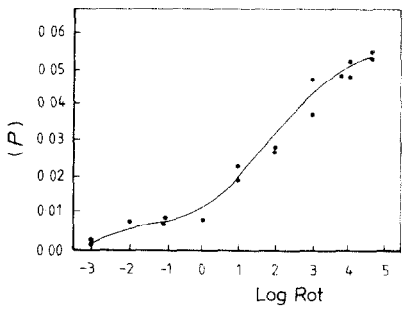

Fig. 2. Hybridization of single-copy DNA to poly(A)$^+$-RNA. P, Proportion of [^{32}P] labelled single copy DNA which hybridized to 14-day poly(A)$^+$-RNA. The line was visually fitted.

Hybridization of nuclear [^{3}H]*cDNA to polysomal poly(A)$^+$-RNA*

A partial kinetic curve was also obtained (data not shown) for molecular hybridization of the nuclear [^{3}H]cDNA to the polysomal poly(A)$^+$-RNA, both isolated from cotyledons harvested 9 days after flowering, i.e. at the early stage of cotyledon development. At the highest value used, log $R_0t = 2$, a maximum of 50% of nuclear [^{3}H]cDNA hybridized to polysomal poly(A)$^+$-RNA, indicating that 50% of the sequences in nuclear poly(A)$^+$-RNA were present in 9 d.a.f. cotyledon poly(A)$^+$-RNA.

DISCUSSION

Although there was considerable scatter in the results particularly at 9 d.a.f. (see Fig. 1A) it was quite clear from Fig. 1 that relatively rare sequences with log $R_0t > 0$ do not occur in 19 d.a.f. cotyledons (see Fig. 1C). Since the general validity of the method was clearly established from the control hybridization of globin mRNA with its cDNA, it was concluded that a major change in the complexity of poly(A)$^+$-RNA during cotyledon development was a decrease in the number of distinct sequences present over that period. This trend was also shown by the intermediate situation as depicted by results in Fig. 1(B). Since the cotyledon cells were still dividing and growing at 9 d.a.f., they required a large number of diverse enzymes and structural proteins; as development proceeded, cell division ceased and synthesis was concentrated on storage reserves and, thus, the number of mRNAs required decreased as observed. Since transcription decreases after the cessation of cell division [15–17], and mRNA production also decreases [18], the disappearance of the rare sequences of poly(A)$^+$-RNA may be taken as a result of a 'switch off' in their production. The total number of poly(A)$^+$ sequences in 9 d.a.f. cotyledons, *ca* 20 000, is of the same order as the number of sequences (25 000) calculated to be present in cotton seed [19] and in mid-embryo soyabeans (32 000; [3]). The data of Fig. 1 were used to evaluate the abundance classes (see Table 1), but since at 9 and 14 d.a.f. the curves were not taken to completion, due to lack of material, additional classes may have been present. However, more than three abundance classes have not been found in other systems and other evidence suggests that the very abundant class of poly(A)$^+$ sequences may be equated with the mRNAs encoding the storage protein polypeptide precursors since these are known to be major translation products of poly(A)$^+$-RNA *in vitro* [5–8]. The increase in sequences in this class, 9–14 d.a.f., thus parallels the increase in storage protein polypeptides synthesized in this interval, since at 9 d.a.f. only vicilin is undergoing appreciable synthesis, whereas at 14 d.a.f. legumin, vicilin and convicilin are all being synthesized rapidly [8, 18]. The six very abundant mRNA species at 14 d.a.f. may thus be equated with the vicilin 50 000 M_r and 47 000 M_r, the legumin 60 000 M_r and the convicilin 71 000 M_r polypeptide precursors. The method is not sufficiently exact to allow an exact correspondence to the drawn; it is possible that mRNAs for other seed proteins (e.g. the major albumin or the lectin) also fall into this class. Synthesis of legumin, convicilin and some of the vicilin polypeptides continues until the end of development, i.e. 19 d.a.f., in agreement with the maintenance of the very abundant poly(A)$^+$ sequences over the interval 14–19 d.a.f. Levels of legumin and vicilin mRNA are known to be maintained over this development interval [18]. If a more advanced developmental stage had been studied (e.g. 22 d.a.f.), a decrease in very abundant poly(A)$^+$-RNA, as seen by Düre and Galau [19] in cotton seed, may have been observed corresponding to the cessation of storage protein synthesis. Both Beevers and Poulson [20] and Bollini and Chrispeels [21] reported that a decrease in protein synthesis at maturity in pea and bean (*Phaseolus vulgaris*) seeds was due to the limited availability of mRNA. We have shown [18] that the amount of polysomes decreases in pea cotyledons after 18 d.a.f. and that specific mRNAs decrease in relative amount.

The class of mRNAs designated 'abundant' in this study increases from *ca* 80 sequences at 9 d.a.f. to 180 sequences at 19 d.a.f. and thus represents, at least in part, a further set of cotyledon-specific proteins and mRNAs. These proteins may include the 'non-functional' enzymes and other proteins which accumulate in cotyledons (e.g. amylase [Gatehouse, J. A., unpublished results]). However, this set of mRNAs probably also contains protein components of the protein synthesizing and energy generating systems which are needed in relatively large amounts but are not tissue specific. The overall sequence complexity classes of the mRNA from developing peas are very similar to those of soyabean [3, 4] with the difference that the numbers of mRNA sequences per cell in pea are consistently an order of magnitude greater. This is due to a higher measured RNA content in pea cotyledons compared to soyabeans.

The results of Fig. 2 indicate that the sequences responsible for hybridization at log $R_0t < 0$ are due to abundant and very abundant mRNAs hybridizing rapidly to single or a few copy genes and not to many copy genes. If the latter occurred these sequences would represent a much greater proportion than < 0.01 of the single-copy DNA. This argument holds even if single-copy DNA was contaminated with a fraction of repeated sequences, which in this case was estimated as $\leqslant 0.1$. Legumin and vicilin mRNAs (i.e. very abundant mRNAs) are known to be the products of genes present in a low number of copies [22].

Ca 5% of single-copy DNA of the pea genome was estimated to be present as transcripts which were being translated in the pea cotyledon at 14 d.a.f.; this compares with 2% for soyabean [3], 1.4% for sea urchin [14], 4.7–5.2% for tobacco cells [13] and 1.05% for HeLa cells [1].

Ca 50% of nuclear poly(A)$^+$-RNA appeared in polysomal poly(A)$^+$-RNA at an early stage in cotyledon

development. This result may be compared with other estimates of the proportion of nuclear poly(A)$^+$-RNAs being exported to the cytoplasm in eukaryotic systems, which range from 40% or greater [23] or *ca* 40% in guinea-pig mammary gland [2] to 10–25% in mammalian liver [24]. These authors have concluded that significant regulation of genetic expression occurs at the post-transcriptional level, and this view is supported by the results of Kamalay and Goldberg [13] who have suggested that the majority of tobacco genes (those responsible for 'rare' poly (A)$^+$-RNAs) are transcribed in all the tissues of the plant. On the other hand, some at least of the nuclear poly(A)$^+$-RNA sequences, which do not reach the cytoplasm, may be accounted for by intron sequences in the mRNA precursors.

In conclusion, it is suggested that the primary means of regulation of genes coding for very abundant and abundant mRNAs in peas is transcriptional control, but that post-transcriptional processing cannot be ruled out as a less important control mechanism.

EXPERIMENTAL

Materials. Seeds of *Pisum sativum* L., var. 'Feltham First' were grown as previously described [5]. Cotyledons were excised from freshly harvested seeds under sterile conditions and immediately frozen in liquid N_2 for storage. Pea leaves were harvested in a cold room from plants grown from seeds germinated first for 4 days in a spray room and then transferred to a growth cabinet for 9 days. Rabbit globin mRNA was purchased from Uniscience Ltd., Cambridge, U.K. Whatman glass fibre discs (GF/C), DEAE-cellulose paper and Drummond microcaps were from A. & J. Beveridge Ltd., Edinburgh, U.K. Chelex 100 (200–400 mesh), analytical grade chelating resin, and hydroxylapatite (Bio-Gel HTP) lot No. 17115 were from Bio-Rad Laboratories. All buffers and DNA preparations used for the isolation of non-repetitive DNA were treated with Chelex 100 before use. All glassware was siliconized and sterilized. All other chemicals, radiochemical compounds and other materials were as described previously [5, 25].

Preparation of poly(A)$^+$-RNA and cDNA. These were made from cotyledons harvested 9, 14 and 19 d.a.f. [16, 18] as described previously [5, 18, 25]. The sp. act. of each [^{3}H]cDNA preparation was *ca* 1.4×10^7 cpm/μg, and they ranged in size from 600 to 1100 nucleotides, as determined by 7 M urea-polyacrylamide gel electrophoresis [25].

Isolation of total DNA and single-copy DNA. Total DNA, from 9-day-old pea leaves, was prepared essentially as described by Graham [26]. Final purification was by banding in $CsCl_2$–ethidium bromide gradients. Usually 2.5 mg DNA was obtained from 10 g pea leaves, as determined by UV *A*, assuming $A_{260}^{1\,mg/ml} = 20$. The ratio of A_{260}/A_{280} was on average 1.9. The final DNA preparation was stored over $CHCl_3$ on ice at 4°.

Single-copy DNA was prepared from fragmented total DNA. A sample of total DNA (10 mg) was dissolved in 10 mM Tris–HCl, pH 7.4, 0.3 M NaCl and sonicated (9 × 1 min pulse; 3 min cooling in ice after each pulse) using a Soniprep 150 (MSE) at about maximum amplitude. An aliquot of fragmented DNA was then removed for analysis by gel electrophoresis and the rest was pptated with EtOH. Fragmented DNA was then dissolved in 50 mM Tris–HCl, pH 7.4 and 0.3 M NaCl, passed through a column of Chelex 100 in the same buffer and pptated with EtOH overnight at −20°. The final preparation of fragmented DNA was stored in 0.12 M NaPi buffer (PB; equimolar mixture of sodium mono- and dibasic phosphate), pH 6.8, over $CHCl_3$ on ice at 4°.

The sizes of the total and fragmented DNAs were obtained by gel electrophoresis of glyoxylated samples [12], using digests of lambda DNA with Hind III and pBR 322 plasmid with Hpa II, as size markers.

Total and fragmented pea DNA aliquots in 0.12 M PB were melted in a SP8-150 spectrophotometer (Pye-Unicam) using Thermomix 1460 (B. Braun) bath for heating. The conditions for complete separation of single- and double-stranded DNA, on a hydroxylapatite column, were established before the batch of hydroxylapapite was used for analysis of re-associated pea DNA. For this purpose we analysed native calf-thymus DNA, a mixture of it with single-stranded [^{3}H]cDNA and a mixture of it with double-stranded (resistant to nuclease S1 digestion) [^{3}H]cDNA. The recovery of input DNA from hydroxylapatite, as monitored at A_{260} was 90% ± 2%, and the recovery of input radioactivity was 92% ± 6%.

Fragmented DNA was dissolved in 0.12 M PB at a concn of at least 5 mg/ml in a sealed capillary tube, denatured for 6 min at 100°, transferred immediately to 60° and incubated for an appropriate time to $C_0t = 200$. Subsequently, the sample was diluted to 1 ml with 0.12 M PB and loaded on a 400 mg hydroxylapatite column at 60°. The unbound and bound DNAs were separated, as described by Murray *et al.* [9], and collected in 0.5 ml aliquots. The unbound DNA fraction was exhaustively dialysed against 10 mM Tris–HCl, pH 7.5, pptated with EtOH and incubated in 0.12 M PB at 60° to $C_0t = 1000$. To accumulate enough DNA for this reassociation reaction, samples were quickly frozen in liquid air and stored at −20° until processing. Separation was performed on a hydroxylapatite column, as previously described, and the unbound fraction isolated. This single-stranded, largely non-repetitive DNA fraction was 5'-end labelled [27] with [γ-^{32}P]ATP and used for hybridization with poly(A)$^+$-RNA in excess.

Hybridization of [^{3}H]cDNA to poly(A)$^+$-RNA. The proportion of [^{3}H]cDNA which hybridized to poly(A)$^+$-RNA at specific R_0t values was estimated as described by Goldberg *et al.* [28]. The R_0t value is a product of initial poly(A)$^+$-RNA concn expressed in mol nucleotides/l. and hybridization time in sec. Poly(A)$^+$-RNA was usually used in at least a 1000 mol excess over [^{3}H]cDNA, so that the reaction was assumed of a pseudo-first-order. For very low R_0t values, the excess was 100, but this did not affect the reaction rate. The hybridization rate is directly proportional to the salt concn [29] and inversely proportional to the sequence complexity of the poly(A)$^+$-RNA population [30]. Hybridization reactions were carried out in buffer [30 mM Pipes, piperazine-*N*,*N*'-bis-(2-ethanesulphonic acid), pH 6.7, 0.1 mM EDTA, 0.1% SDS, (sodium dodecyl sulphate)] containing three different concns of NaCl: 0.045, 0.18 and 0.75 M, which altered the hybridization rates by factors of 0.0133, 1.0 and 5.82, respectively [29]. Before hybridization the samples were denatured at 100° for 5 min. The reactions (total vol. 2–5 μl) were carried out in sealed, siliconized microcaps at 60° for the lower salt concns, but at 68° for 0.75 M NaCl. At required time intervals reactions were quenched by freezing the microcap in liquid air, and the contents were expelled into 110 μl cold S1 nuclease buffer (0.25 M NaCl, 1 mM ZnOAc, 30 mM NaOAc, pH 4.5). Denatured calf-thymus DNA (to a concn of 20 μg/ml) was added as a carrier and the sample was halved. To one half was added 3 μl S1 nuclease (750 units) and to the other, 3 μl S1 nuclease buffer. Both samples were incubated at 37° for 80 min and the amount of radioactivity retained in a hybrid form after digestion with S1 nuclease relative to total radioactivity was determined using the DEAE-81 filter disc assay [31]. The final results were corrected for self-hybridization of [^{3}H]cDNA by carrying out the control hybridization procedures in the absence of poly(A)$^+$-RNA.

Hybridization curves, expressing the proportion of [^{3}H]cDNA hybridized vs R_0t values, for homologous hybridiz-

ations with poly(A)$^+$-RNA from 9-, 14- and 19-day-old cotyledons, were accumulated. The best fit curves were determined using a least squares fit computer programme, kindly supplied by Dr. J. O. Bishop, Department of Genetics, Edinburgh, and based on one described by Pearson *et al.* [32]. The numerical analysis of these fitted data was carried out according to the methods of Goldberg *et al.* [3].

Hybridization of [^{32}P]*-labelled non-repetitive DNA to poly(A)$^+$-RNA.* [^{32}P]-5′-end labelled non-repetitive DNA from pea leaves was hybridized to an excess of poly(A)$^+$-RNA extracted from 14-day-old cotyledons at different R_0t values, as described above, and the hybridization curve was visually fitted. At low R_0t values a mass excess of poly(A)$^+$-RNA to single-copy DNA of 750–1000 was used, while at higher R_0t values it was possible to attain an excess of 6000–7000.

The nuclear [^{3}H]cDNA, originating from 9-day-old cotyledons, was also hybridized to an excess of poly(A)$^+$-RNA.

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