The Stability, Polyadenylic Acid Content and Ribonucleoprotein Form of Nuclear Ribonucleic Acid in Artichoke

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A nuclear preparation, containing 60–80% of the total tissue DNA and less than 0.5% of the total rRNA, was used to characterize the nuclear RNA species synthesized in cultured artichoke explants. The half-lives of the nuclear RNA species were estimated from first-order-decay analyses to be: hnRNA (heterogeneous nuclear RNA) containing poly(A), 38 min; hnRNA lacking poly(A), 37 min; 2.5×10^6 -mol.wt. precursor rRNA, 24 min; 1.4×10^6 -mol.wt. precursor rRNA, 58 min; 1.0×10^6 -mol.wt. precursor rRNA, 52 min. The shorter half-lives are probably overestimates, owing to the time required for equilibration of the nucleotide-precursor pools. The pathway of rRNA synthesis is considered in terms of these kinetic measurements. The rate of accumulation of cytoplasmic polydisperse RNA suggested that as much as 40% of the hnRNA may be transported to the cytoplasm. The 14–25% of the hnRNA that contained a poly(A) tract had an average molecular size of 0.7×10^6 daltons. The poly(A) segment was 40–200 nucleotides long, consisted of at least 95% AMP and accounted for 8–10% of the [32 P]orthophosphate incorporated into the poly(A)-containing hnRNA. Ribonucleoprotein particles released from nuclei by sonication, lysis in EDTA or incubation in buffer were analysed by sedimentation through sucrose gradients and by isopycnic centrifugation in gradients of metrizamide and CsCl. More than 50% of the hnRNA remained bound to the chromatin after each treatment. The hnRNA was always associated with protein but the densities of isolated particles suggested that the ratio of protein to RNA was lower than that reported for mammalian cells. The particles separated from chromatin were not enriched for poly(A)-containing hnRNA.

hnRNA (heterogeneous nuclear RNA) has been extensively studied in various mammalian systems. It has a very broad size distribution extending up to 15×10^6 daltons, is rapidly labelled in radioactive-precursor-incorporation experiments and appears to be rapidly turned over within the nucleus (Weinberg, 1973; Lewin, 1975). Only a small part of the radioactivity incorporated into hnRNA ever reaches the cytoplasm (Scherrer *et al.*, 1970; Brandhorst & McConkey, 1974). Some hnRNA molecules possess a sequence of poly(A) at the 3' end (Greenberg & Perry, 1972; Nakazoto *et al.*, 1974). It is now generally accepted that at least some of the RNA molecules in the hnRNA fraction are precursors to cytoplasmic mRNA (Lewin, 1975). Fractions of rapidly labelled RNA heterogeneous in size and with base compositions resembling that of DNA have been separated from total plant RNA by chromatography on methylated albumin–kieselguhr columns (Ingle *et al.*, 1965). Further characterization of these fractions (Key *et al.*, 1972; Jackson & Ingle, 1973; Schmid *et al.*, 1975; Key & Siflow, 1975) has shown that they possess many of the properties expected of a combina-

tion of hnRNA and mRNA. Very little is known about the properties of hnRNA in plants.

In mammalian nuclei, at least a proportion of the hnRNA exists in the form of ribonucleoprotein particles. Material extracted from nuclei isolated from Ehrlich cells, rat liver and human cells sedimented uniformly in sucrose gradients as 30S particles containing a limited number of protein species (Samarina *et al.*, 1966; Lukanidin *et al.*, 1972). Ribonucleoprotein particles, released by the disruption of isolated HeLa-cell nuclei, sedimented over the range of 40–250S (Pederson, 1974). Most mammalian ribonucleoprotein particles containing hnRNA have a density in CsCl of 1.40–1.45 g/cm³.

In the present paper we have used nuclei isolated from cultured Jerusalem-artichoke explants to study some characteristics of nuclear RNA. Various properties of the hnRNA, including its stability, relationship to cytoplasmic mRNA, poly(A) content and its presence in ribonucleoprotein particles, have been studied. The labelling kinetics and stability of nuclear precursor rRNA molecules have also been determined, and the proposed pathway of rRNA

synthesis (Leaver & Key, 1970; Rogers *et al.*, 1970; Cox & Turnock, 1973; Grierson & Loening, 1974) has been re-examined in terms of these results.

Materials and Methods

Growth and incubation of artichoke explants

Cylindrical explants (2mm diam. $\times$ 2.4mm long) were isolated aseptically from surface-sterilized tubers of Jerusalem artichoke (*Helianthus tuberosus* L, var. Bunyards Round) and cultured in the presence of 2,4-dichlorophenoxyacetic acid (Yeoman *et al.*, 1965). These explants (70–80) were cultured in 7.5 ml of sterile medium in continuously revolving (2.5 rev./min) 250 ml bottles, and the tissue was used after 40–80 h, at which time cell division was asynchronous and rRNA per explant was still increasing (Gore & Ingle, 1974). To ensure a rapid uptake of the [32 P]-orthophosphate, no phosphate was added to the culture medium. Cultures were pulse-labelled, for the times indicated, by the injection of sterile [32 P]orthophosphate (The Radiochemical Centre, Amersham, Bucks., U.K.) into the culture bottles to a final concentration of 50 μ Ci/ml in 0.1 μ M-sodium phosphate, pH 7.0. In some experiments the explants were continuously labelled with [Me - 3 H]thymidine (The Radiochemical Centre; 3 μ Ci/ml in 10 μ M-thymidine) from the start of culture.

Isolation of nuclei

When assessing methods for the preparation of nuclei it was considered that the method of choice should be rapid and produce a high yield of nuclei with a minimum loss of nuclear components and a minimal contamination with cytoplasmic ribonucleoprotein. Contamination of the nuclei with cell-wall material was tolerated in preference to the decreased yields and nuclear damage which resulted from attempts to purify the nuclei further. Explants were normally frozen in solid CO₂ immediately after harvesting. The frozen tissue was ground to a powder with a pestle and mortar and the powder was transferred to a conical glass-in-glass homogenizer and suspended in 5 vol. of ice-cold buffer [0.3 M-sucrose, 5 mM-MgCl₂, 50 mM-KCl, 1% (v/v) 2-mercaptoethanol, 50 mM-Tris/HCl, pH 8.0] by a single plunge of the pestle. Triton X-100 was added to a final concentration of 2% (v/v) and the homogenate was filtered through two layers of Miracloth. The debris on the Miracloth was washed thoroughly three times with a total of 15 vol. of homogenization buffer. Nuclei were sedimented by centrifugation (6 min, 800g), and the pellet was washed twice by resuspension in 2 vol. of buffer and re-centrifugation (6 min, 500g). Examination by light microscopy indicated that most of the nuclei were intact, although a

limited amount of nuclear damage was suggested by the presence of some free nucleoli. A quantitative comparison of the nucleic acid prepared from such a nuclear preparation with that from total tissue showed that 60–80% of the DNA and 40–60% of the precursor rRNA were recovered. Less than 0.5% of the total tissue rRNA was present in the nuclear preparation. Nucleic acid prepared from nuclei isolated directly from fresh explants was indistinguishable from that prepared from frozen tissue, but freezing greatly facilitated the accurate timing of radioactive pulses, and the processing of many different samples of pulse-labelled material.

Preparation of the cytoplasmic fraction

The cytoplasmic fraction was prepared from a sample of tissue directly comparable with that used for the nuclear preparation. The homogenization and filtration were essentially as described for nuclei isolation, except that 10 mM-dithiothreitol was used instead of 2-mercaptoethanol, the Tris/HCl concentration was increased to 200 mM, and 1 mg of polyvinylpyrrolidone/ml was included in the buffer. Nuclear material was removed by centrifugation at 15000g for 7 min and the cytoplasmic fraction was recovered from this supernatant by centrifugation through 1 ml of buffered 1 M-sucrose at 65000 rev./min in an MSE 10 $\times$ 10 Titanium rotor for 2 h at 0°C.

Preparation of nucleic acid

The nuclear pellet, nuclear subfractions or cytoplasmic pellet was suspended in detergent mixture and nucleic acid was prepared by the method of Parish & Kirby (1966) as previously described (Jackson & Ingle, 1973). For poly(A) analysis, nuclear RNA was further purified by salt precipitation. Total nucleic acid from nuclei was dissolved in 0.1 M-sodium acetate (0.2–1.0 mg of RNA/ml), and solid NaCl added to a final concentration of 3.0 M. After 15 h at 0°C the precipitate was collected by centrifugation (10 min, 2500g) and the pellet washed with 3.0 M-sodium acetate and subsequently with 0.2% (w/v) sodium acetate/75% (v/v) ethanol. About 90–95% of the hnRNA and precursor rRNA and less than 1% of the DNA were recovered in the final pellet.

Polyacrylamide-gel electrophoresis

Nucleic acid was fractionated on 2.3% (w/v) or 7.5% (w/v) gels (Loening, 1967) at 50 V (6 mA/tube). Gels were scanned at 265 nm, and the radioactivity in 1 mm slices was determined as described by Jackson & Ingle (1973).

Table 1. *Oligo(dT)-cellulose chromatography of nuclear RNA*

Nuclear RNA isolated from explants pulse-labelled for 1 h with [32 P]orthophosphate was purified by precipitation from 3.0M-NaCl, dissolved in 1.0ml of loading buffer and chromatographed on 200mg of oligo(dT)-cellulose. The wash fraction was rechromatographed on a second column (column 2). The fraction bound to column 1 was also rechromatographed (column 3). A portion of each fraction was precipitated with trichloroacetic acid (5%, w/v, final concentration), collected on a glass-fibre filter and the radioactivity determined. Portions of the wash fractions from columns 1 and 3 and the fraction bound to column 3 were also digested with ribonucleases A and T_1 to determine the poly(A) content.

	Percentage of radioactivity loaded on column		Percentage of radioactivity resistant to ribonucleases A and T_1	
	Wash	Bound	Wash	Bound
Column 1	90.2	9.8	0.2	—
Column 2 (wash from column 1)	99.3	0.7	—	—
Column 3 (bound from column 1)	30.7	69.3	0.15	8.4

Isolation of poly(A)-containing RNA

Poly(A)-containing RNA was isolated by chromatography on columns (200mg) of oligo(dT)-cellulose (Collaborative Research, Waltham, MA, U.S.A.). A sample of salt-precipitated RNA was dissolved in 1.0ml of loading buffer [400mM-NaCl, 0.1% (w/v) sodium dodecyl sulphate, 10mM-Tris/HCl, pH7.4] and loaded on to the column at a flow rate of 15ml/h. After the column had been washed with 6ml of buffer, the bound poly(A)-containing RNA was eluted with 1mM-Tris/HCl (pH7.4)/0.1% (w/v) sodium dodecyl sulphate. Fractions (1.0ml) were collected, 20 μ g of rRNA carrier was added to each fraction and the RNA was precipitated by the addition of an equal volume of 10% (w/v) trichloroacetic acid containing 10mM-orthophosphate, pH7.0. After 1 h at 0°C the precipitate was collected on to a 2.1 cm Whatman GF/C glass-fibre filter. Radioactivity was determined in an Intertechnique SL30 liquid-scintillation counter with a toluene-based scintillator {0.4% (w/v) butyl-PBD [5-(4-biphenyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole]}. Approx. 10% of the radioactivity was bound to the first column (Table 1). When the wash fraction from the first column was passed through a second column only 0.7% of the radioactivity bound (Table 1), indicating that most of the poly(A)-containing RNA was bound to the

first column. Recycling of the bound fraction from the first column resulted in about 30% of the radioactivity failing to bind, indicating that some RNA bound to the first column did not contain poly(A). The amount of RNA without poly(A) that bound to the first column was only slightly decreased when the column was washed with 150mM-NaCl before the normal elution in 1mM-Tris/HCl. The presence of DNA in the nucleic acid samples did not affect the binding of poly(A)-containing RNA to the column, but did interfere with subsequent nuclease digestion.

Nuclease digestion of RNA

Nucleic acid samples were washed twice with ethanol (to remove sodium dodecyl sulphate) and dissolved in 150mM-NaCl/10mM-EDTA/10mM-Tris/HCl (pH7.5) at less than 100 μ g/ml. The RNA was digested for 2 h at 37°C with 10 μ g of ribonuclease A/ml [Sigma (London) Chemical Co., London S.W.6, U.K.; type IIA] and 100 units of ribonuclease T_1 /ml (Boehringer, Mannheim, W. Germany). Non-RNA background radioactivity was determined by the complete digestion of RNA by the further addition of 10 units of ribonuclease T_2 /ml (Calbiochem, London W.1, U.K.). Undigested material was precipitated with trichloroacetic acid [5% (w/v) final concentration], collected on to glass-fibre filters and the radioactivity determined.

Poly(A) size determination

For the determination of poly(A) chain length, the nuclease digestion was terminated by saturation with diethyl pyrocarbonate. After the addition of detergent mixture (Parish & Kirby, 1966) and unlabelled poly(A) (Boehringer, Mannheim) plus 4 S tRNA and 5 S rRNA as carrier and size markers, the mixture was extracted with phenol and the poly(A) size determined by electrophoresis on 7.5% (w/v) polyacrylamide gels. The average size of the poly(A) tract was calculated after adjusting the radioactivity profile for molecular size.

Base composition

RNA, or nuclease-resistant material, which had been precipitated and collected on a glass-fibre filter, was hydrolysed together with 50 μ g of carrier RNA in 1-2ml of 10% (w/v) piperidine/10mM-EDTA for 48 h at 60°C. The piperidine was removed by evaporation, and the residue dissolved in 0.2ml of 7.5% (v/v) acetic acid/0.75% (v/v) pyridine. The nucleotides were separated by high-voltage paper electrophoresis (1200 V for 2 h) in the acetic acid/pyridine buffer, and the chromatograms were then dried, cut into 1.0cm strips, and the radioactivity was determined.

Disruption of nuclei

(a) *Sonication.* Nuclear pellets, resuspended in homogenizing buffer including 0.2% (v/v) diethyl pyrocarbonate, were sonicated in plastic tubes cooled in ice. Disruption was for a total of 10–40 s in 10 s bursts separated by 1 min for cooling. Some nuclei were disrupted after 10 s, but 40 s was required for complete disruption.

(b) *Lysis in EDTA.* Nuclear pellets were suspended in 50 mM-Tris/HCl (pH 7.5)/5 mM-EDTA/0.2% (v/v) diethyl pyrocarbonate and shaken vigorously. All nuclei were rapidly lysed in this buffer.

(c) *Extraction of ribonucleoprotein particles.* Following the method of Georgiev (Samarina *et al.*, 1966; Lukanidin *et al.*, 1972), nuclear pellets were resuspended in 100 mM-NaCl/1 mM-MgCl₂/2% (v/v) Triton X-100/0.2% (v/v) diethyl pyrocarbonate/50 mM-Tris/HCl (pH 8.0) and incubated for 15 min at 25°C. Nuclei were then separated from the extracted ribonucleoprotein particles by sedimentation at 2500g for 5 min. Some nuclei were lysed during this extraction.

Analysis of subnuclear fractions

(a) *Sucrose-gradient centrifugation.* Sucrose gradients [7.5–35% in 2 mM-MgCl₂/100 mM-NaCl/0.2% (v/v) diethyl pyrocarbonate/50 mM-Tris/HCl (pH 7.5)] were layered over a 2 ml cushion of 60% (w/v) sucrose in 25 ml centrifuge tubes (MSE; 3 × 25 swing-out rotor). Samples of up to 2 ml of disrupted nuclei were layered on the gradients and centrifuged for 17 h at 16000 or 20000 rev./min. at 2°C. Gradients were then displaced by pumping a saturated sucrose solution into the tube and fractions were collected from the top. *E*₂₅₈ was monitored with an LKB Uvicord II detector connected to a Vitatron recorder.

(b) *Isopycnic centrifugation in density gradients of metrizamide.* Samples to be analysed were either mixed with, or layered on to, 50% (w/v) metrizamide (Nyegaard and Co., Oslo, Norway) in 2 mM-EDTA/0.2% (v/v) diethyl pyrocarbonate/50 mM-Tris/HCl (pH 8.0) to give a total volume of 5.0 or 7.5 ml with average metrizamide concentration of 36% (1.20 g/cm³). Tubes were filled with liquid paraffin and centrifuged in the MSE 10 × 10 aluminium angle rotor for 65 h at either 30000 or 35000 rev./min at 2°C. Gradients were pumped out by displacement with Fluorinert FC48 (MSE, London S.W.1, U.K.), and fractions were collected from the top. The metrizamide gradient was determined by measuring the refractive index of the fractions.

(c) *Isopycnic centrifugation in density gradients of CsCl.* Nucleoprotein particles suspended in 50 mM-KCl/10 mM-MgCl₂/1.0% (w/v) Brij-35/20 mM-triethanolamine hydrochloride/NaOH (pH 7.7) were fixed for 24 h at 0°C in 6% (w/v) formaldehyde

(neutralized with bicarbonate). The sample (0.5 ml) was layered on 4.0 ml of CsCl in the same buffer (1.55 g/cm³), and, after the interphase was gently mixed to form a rough gradient, the gradient was equilibrated by centrifuging for 42 h at 35000 rev./min in the 6 × 5.5 MSE swing-out rotor at 2°C. Gradients were displaced with Fluorinert FC48, fractions were collected, and the CsCl gradient was determined by measuring the refractive index of the fractions. Fractions were then precipitated with trichloroacetic acid [5% (w/v) final concentration], collected on glass-fibre filters, and the radioactivity was determined.

Results

Nuclear nucleic acid species

The fractionation of nucleic acid prepared from nuclei isolated from explants labelled for 1 h with [³²P]orthophosphate is shown in Fig. 1(a). The absorbance profile shows the major peak of DNA with only traces of the mature rRNA species with mol.wt. 1.3 × 10⁶ and 0.7 × 10⁶. The radioactivity profile shows, in addition to the DNA, major peaks of the 2.5 × 10⁶- and 1.4 × 10⁶-mol.wt. precursor rRNA molecules and minor peaks of 1.0 × 10⁶-mol.wt. precursor rRNA and the 0.7 × 10⁶-mol.wt. rRNA, overlying a polydisperse distribution of hnRNA. Although the 1.4 × 10⁶-mol.wt. precursor rRNA and the 1.3 × 10⁶-mol.wt. rRNA, which in *H. tuberosus* are actually 1.39 × 10⁶ and 1.33 × 10⁶ daltons respectively (Hepburn & Ingle, 1975), are not clearly resolved, the radioactive component from the nuclear preparation migrated more slowly than the absorbance peak and showed very little evidence of a shoulder on the faster side of the peak, indicating that 1.3 × 10⁶-mol.wt. mature rRNA was only a very minor component of this radioactive peak. When the nucleic acid sample is heated at 60°C for 5 min, the 7S (5 × 10⁴ daltons) rRNA is removed from the 1.3 × 10⁶-mol.wt. rRNA but not from the 1.4 × 10⁶-mol.wt. precursor rRNA, and these two species are then separated by polyacrylamide-gel electrophoresis (Hepburn & Ingle, 1975). This procedure demonstrated that after a 1 h pulse in [³²P]orthophosphate the nuclear pellet contained very little radioactive 1.3 × 10⁶-mol.wt. rRNA (the ratio of 0.7 × 10⁶- to 1.3 × 10⁶-mol.wt. rRNA species was similar to that observed in the cytoplasm), confirming that the 1.4 × 10⁶-mol.wt. precursor rRNA is the major labelled component of the heavy rRNA peak. The comparable cytoplasmic fraction (Fig. 1b) shows the 1.3 × 10⁶- and 0.7 × 10⁶-mol.wt. rRNA species on both absorbance and radioactivity profiles, together with a polydisperse distribution of radioactivity. The bulk of the polydisperse RNA in this cytoplasmic fraction has been shown to be associated with polyribosomes

Fig. 1. Polyacrylamide-gel electrophoresis of nuclear and cytoplasmic RNA

Nuclear (a) and cytoplasmic (b) fractions were prepared from explants labelled for 1 h with [32 P]orthophosphate. Total nucleic acid was prepared and fractionated by polyacrylamide-gel electrophoresis (2.3%; 50 V; 3 h). —, E_{265} ; the histogram is c.p.m./1 mm gel slice. The molecular weights ($\times 10^{-6}$) of the rRNA and precursor rRNA species are indicated. ----, Polydisperse RNA component.

(D. Baulcombe, personal communication), and is termed mRNA on this basis. The polydisperse RNA in the nuclear preparation (hnRNA) is larger than that in the cytoplasm, with radioactivity distribution means of 1.5×10^6 and 1.0×10^6 daltons respectively. Correction of the radioactivity distribution for molecular size indicates average sizes of 0.8×10^6 and 0.55×10^6 for hnRNA and cytoplasmic mRNA.

Kinetics of labelling of the nuclear and cytoplasmic RNA species

The approach to steady-state labelling of the various RNA species was studied by gel fractionation of RNA prepared from nuclear and cytoplasmic fractions isolated from explants cultured in [32 P]-orthophosphate for increasing periods of time. The kinetics of short-term labelling (20–60 min) of the nuclear RNA species is shown in Fig. 2(a). The hnRNA and the 2.5×10^6 -mol.wt. precursor rRNA are the most rapidly labelled species and hnRNA is the major labelled component throughout the time-course. After 20 min labelling, the 2.5×10^6 -mol.wt. component is the major precursor rRNA, but, with increasing labelling time, more radioactivity appears in the 1.4×10^6 -mol.wt. precursor rRNA. For a kinetic analysis of the nuclear RNA species, continuous labelling periods of up to 6.5 h were used (Fig. 2b). By this time all the nuclear RNA species were labelled to equilibrium. The comparable data for the cytoplasmic RNA species are presented in Fig. 3, and show that mRNA is approaching equi-

librium, whereas the accumulation of radioactivity into mature rRNA species (1.3×10^6 and 0.7×10^6) increases linearly after a lag of approx. 1 h.

If the specific radioactivity of the nucleotide-precursor pool is constant over the period of labelling, a class of RNA synthesized after a specific time $t = 0$ will accumulate according to the equation (Greenberg, 1972; Brandhorst & McConkey, 1974):

$$S_t = S_{\infty} [1 - e^{-\ln 2 (\frac{1}{t_D} + \frac{1}{t_{\frac{1}{2}}}) t}]$$

where S_t is the amount of RNA of that class per cell accumulated at time t , S_{∞} is the steady-state amount of that class of RNA per cell, $t_{\frac{1}{2}}$ is the half-life of that class of RNA, and t_D is the cell doubling time (15 h for *H. tuberosus* in this culture system). The equation describes an accumulation curve in which it is assumed that RNA decays with first-order kinetics and is synthesized with zero-order kinetics. S_t can only be measured accurately by radioactive-precursor-incorporation studies if the specific radioactivity of the nucleotide-precursor pool reaches a constant value immediately on addition of the radioactivity. This is impossible to achieve. However, as the accumulation of radioactivity into DNA is approximately linear after 60 min (Fig. 2), as would be expected for a stable molecule during a labelling period much shorter than the doubling time, an approximation to constant precursor specific radioactivity must be achieved by this time.

The equation may be rewritten:

$$\ln(S_{\infty} - S_t) = -\ln 2 \left(\frac{1}{t_D} + \frac{1}{t_{\frac{1}{2}}} \right) t + \ln S_{\infty}$$

Fig. 2. Radioactivity-accumulation curves for nuclear RNA species

Artichoke explants were labelled with [32 P]orthophosphate for the times indicated, nuclei were isolated and total nucleic acid was prepared. The RNA was separated from DNA by salt precipitation and fractionated by gel electrophoresis. The incorporation of radioactivity into each species was determined and expressed as c.p.m./ μ g of DNA in the nuclear preparation. The specific radioactivity of the DNA was determined after ethanol precipitation of the salt-soluble fraction and analysis by gel electrophoresis. A correction was made for 1.3×10^{-6} -mol.wt. rRNA contamination in the 1.4×10^{-6} -mol.wt. precursor rRNA peak by subtracting twice the number of 0.7×10^{-6} -mol.wt. rRNA counts present in the nuclear preparation. (a) Short-term labelling; (b) labelling up to 6.5 h. ●, DNA; ○, hnRNA; □, 1.4×10^{-6} -mol.wt. precursor rRNA; ■, 2.5×10^{-6} -mol.wt. precursor rRNA; ▲, 1.0×10^{-6} -mol.wt. precursor rRNA; ▼, poly(A)-containing hnRNA; ▽, 0.7×10^{-6} -mol.wt. rRNA.

so that, if $\ln(S_{\infty} - S_t)$ is plotted against time, a straight line with a slope of $-\ln 2 \left(\frac{1}{t_D} + \frac{1}{t_h} \right)$ results. This first-order-decay analysis for the nuclear RNA species and the cytoplasmic RNA is shown in Fig. 4. From the slopes of the straight lines, the half-lives of the RNA species have been calculated (Table 2).

Poly(A)-containing hnRNA

When nuclear RNA prepared from tissue labelled for 2 h with [32 P]orthophosphate was fractionated on an oligo(dT)-cellulose column, only hnRNA was bound (Fig. 5b). The radioactivity was distributed over a molecular-weight range of 0.5×10^6 – 2.5×10^6 , and when the radioactivity profile was corrected for molecular size the average molecular weight was 0.7×10^6 . The RNA fraction not bound to the column (wash) contained all of the precursor rRNA species and a major proportion of the hnRNA (Fig. 5a).

The RNA which did not bind to oligo(dT)-cellulose (wash) had a base composition (Table 3) typical of rRNA (Rogers *et al.*, 1970; Jackson & Ingle, 1973). RNA bound to the oligo(dT)-cellulose (column 3) was highly enriched in AMP, as would be expected. However, even after subtracting the 8.4% of the radioactivity which was found by nuclease

digestion to be in the poly(A) tract (Table 1), the AMP content of the remainder of the molecule was still high. RNA bound to column 3, and resistant to ribonuclease A and T_1 digestion, consisted of at least 95% AMP (Table 3) with most of the remaining counts migrating slightly slower than GMP. Radioactivity in the oligo(dT)-cellulose column wash fractions which was resistant to nuclease digestion (Table 1) either remained at the origin during electrophoresis of the hydrolysed nucleotides or was distributed throughout all four bases. Only a minor proportion of these resistant counts could therefore be in poly(A).

The proportion and stability of poly(A)-containing hnRNA relative to hnRNA without poly(A) was studied by oligo(dT)-cellulose fractionation of nuclear RNA prepared from tissue labelled for increasing periods with [32 P]orthophosphate (Table 4). The first set of data was obtained from slowly growing explants, prepared from tubers stored for 9 months, and the second was from rapidly growing explants prepared from freshly harvested tubers of the subsequent year's crop. With the stored material there was a slight decrease, from 17 to 14%, in the proportion of poly(A)-containing hnRNA with increasing labelling time, suggesting that this fraction may be turning over slightly more rapidly than the remainder of the hnRNA. With freshly harvested

Fig. 3. Radioactivity-accumulation curves for cytoplasmic RNA species

Cytoplasmic extracts were prepared from the same bottles of explants used in Fig. 2(b) (half of the explants in each bottle were used for cytoplasmic and half of them for nuclear preparations). After the removal of nuclear material by sedimentation at 10000*g* for 10 min, nucleic acid was extracted from the cytoplasmic fraction and analysed by gel electrophoresis. The incorporation of radioactivity into each species was determined and expressed as c.p.m./μg of DNA in the comparable nuclear preparation. ▽, 1.3×10^6 -mol.wt. rRNA; ▾, 0.7×10^6 -mol.wt. rRNA; ●, mRNA.

material 20–25% of the hnRNA contained poly(A), but there was no consistent change in the proportion with time.

The size of the nuclease-resistant poly(A) tract was determined by electrophoresis in 7.5% (w/v) polyacrylamide gels (Fig. 6). The poly(A) segment in hnRNA prepared from tissue labelled for 1 h showed a peak of radioactivity smaller than 4 S RNA with a tail extending up to 7 S RNA. Correction of the radioactivity profile for molecular size indicated the average size of poly(A) to be 40–50 nucleotides. After 2 h labelling the radioactivity profile was more symmetrical, with a peak at 5–7 S. Correction of this profile for molecular size gave an average poly(A) tract of 70–80 nucleotides. After labelling for 1½ or 3 h the radioactivity profiles and average sizes were similar to those obtained after labelling for 2 h.

Fractionation of nuclei

(a) *Sonication.* Nuclei disrupted by sonication for 30 s were fractionated by centrifugation through 7.5–60% (w/v) sucrose gradients. Most of the DNA

Fig. 4. First-order-decay analysis of nuclear RNA species and cytoplasmic mRNA

The results shown in Figs. 2(b) and 3 were analysed as described in the text and $\ln(S_\infty - S_t)$ was plotted against time of incubation. (a) ●, Cytoplasmic mRNA; Δ, total nuclear RNA; ○, hnRNA; (b) □, 1.4×10^6 -mol.wt. precursor rRNA; ■, 2.5×10^6 -mol.wt. precursor rRNA; ▲, 1.0×10^6 -mol.wt. precursor rRNA.

Table 2. Stability of nuclear RNA species and cytoplasmic mRNA

From the slopes of the straight lines in Fig. 4 the half-lives of the species were calculated from the equation:

$$\ln(S_\infty - S_t) = -\ln 2 \left(\frac{1}{t_D} + \frac{1}{t_{\frac{1}{2}}} \right) t + \ln S_\infty$$

where the slope = $-\ln 2 \left(\frac{1}{t_D} + \frac{1}{t_{\frac{1}{2}}} \right)$ and $t_D = 15$ h.

Species	Half-life (min)
Total nuclear RNA	42
hnRNA	38
Poly(A)-containing hnRNA	37
2.5×10^6 -mol.wt. precursor rRNA	24
1.4×10^6 -mol.wt. precursor rRNA	58
1.0×10^6 -mol.wt. precursor rRNA	52
Cytoplasmic mRNA	127

(89%) and much of the ^{32}P -labelled nucleic acid (73%) was sedimented. Since 89% of the DNA, which accounted for 18% of the ^{32}P incorporation, was in the pellet, the proportion of the RNA in the

Fig. 5. Gel electrophoresis of nuclear RNA fractions separated by oligo(dT)-cellulose chromatography

Salt-precipitated nuclear RNA was fractionated on oligo(dT)-cellulose columns as described in the Materials and Methods section (Table 1). (a) RNA not bound to the first column (wash) was precipitated with 2 vol. of ethanol and analysed by gel electrophoresis (2.3%; 50V; 3h); (b) 50 μ g of carrier rRNA was added to the RNA eluted from column 3 [poly(A)-containing hnRNA] and, after precipitation with 2 vol. of ethanol, the RNA was analysed by gel electrophoresis (2.3%; 50V; 3h). —, E_{265} ; the histogram is the c.p.m./1 mm gel slice.

Table 3. Base composition of RNA fractions separated by chromatography on oligo(dT)-cellulose

RNA was prepared, fractionated and precipitated with trichloroacetic acid as described in Table 1. After piperidine hydrolysis of the RNA on the glass-fibre filters, the nucleotides were separated by high-voltage paper electrophoresis in acetic acid/pyridine buffer, and the radioactivity was determined in 1 cm segments of the electrophoresis paper.

	Composition (mol/100 mol)			
	CMP	AMP	GMP	UMP
Wash, column 1	21.0	27.0	31.9	20.1
Wash, column 3	20.9	26.7	29.3	23.1
Bound, column 3	17.7	39.0	21.1	22.2
Bound, column 3 and resistant to ribonucleases and A and T ₁	—	95.2	4.2	0.6

gradient was approx. 30%. A large proportion of this 32 P-labelled RNA in the gradient sedimented slowly at the top of the gradient and the remainder was spread uniformly throughout. When the nucleic acid from sonicated nuclei was analysed by gel electrophoresis, much of the DNA migrated as a polydisperse profile, and in many experiments some RNA had also been degraded. The sonicated nuclei were also fractionated by isopycnic centrifugation in metrizamide gradients. The results of two experiments are shown in Fig. 7. Under these gradient

conditions the banding densities of DNA, chromatin, RNA and ribosomes were 1.12, 1.18–1.20, 1.17 and 1.24 g/cm³ respectively. When nuclei were extensively sonicated (40s) a clear separation of DNA and RNA components was achieved (Fig. 7a), but the densities suggest that both deoxyribonucleoprotein and ribonucleoprotein had been deproteinized. Gel electrophoresis of the nucleic acids extracted from a sample of the sonicated nuclei used in this experiment revealed that DNA had been sheared to sizes migrating more rapidly than rRNA, and much of the precursor rRNA and hnRNA had also been degraded. In an experiment in which nuclei were sonicated for only 20s, most of the DNA still migrated as a high-molecular-weight DNA peak on a polyacrylamide gel and there was no sign of gross RNA degradation. This preparation showed very little separation of DNA and RNA components in metrizamide (Fig. 7b), with 50% of the DNA banding with the RNA at the density of chromatin (1.20 g/cm³) and the remainder banding as deproteinized DNA. In each experiment there was a tail of labelled material at higher densities of 1.23–1.28 g/cm³, suggesting that there may be some protein-rich ribonucleoprotein particles. The deproteinization of deoxyribonucleoprotein and probably also of ribonucleoprotein which results from sonication of nuclei invalidates the use of this procedure for the investigation of ribonucleoprotein particles.

(b) *Lysed nuclei.* After lysing nuclei in EDTA, 50% of the 32 P-labelled nucleic acid was separated from [3 H]thymidine-labelled chromatin by centrifugation through a 7.5–60% (w/v) sucrose gradient (Fig. 8).

Table 4. Proportion of hnRNA containing poly(A)

Explants isolated from tubers that had been stored for 9 months (old) or freshly harvested (new) were pulse-labelled with [³²P]orthophosphate for the times indicated. Nuclear nucleic acid was prepared, salt-precipitated and the poly(A)-containing RNA[poly(A)⁺] separated by oligo(dT)-cellulose chromatography. The wash and bound fractions were then analysed by polyacrylamide-gel electrophoresis to determine the hnRNA radioactivity.

Labelling time	Old tissue			New tissue		
	Radioactivity in hnRNA (c.p.m./μg of DNA)		Poly(A) ⁺ (%)	Radioactivity in hnRNA (c.p.m./μg of DNA)		Poly(A) ⁺ (%)
	Poly(A) ⁺	Poly(A) ⁻		Poly(A) ⁺	Poly(A) ⁻	
40 min	131	628	17.2	24	94	20.3
60 min	201	1060	15.9	80	295	21.3
90 min	—	—	—	171	494	25.7
120 min	359	2108	14.6	—	—	—
150 min	—	—	—	179	631	22.1
3½ h	408	2390	14.6	—	—	—
4 h	—	—	—	341	999	25.4
6½ h	391	2444	13.7	—	—	—
8 h	—	—	—	290	1135	20.4
13 h	—	—	—	340	1225	21.7

Fig. 6. Size of the poly(A) tract in hnRNA

Poly(A)-containing hnRNA eluted from oligo(dT)-cellulose was digested for 2 h at 37°C with ribonucleases A and T₁, and the digestion was terminated with diethyl pyrocarbonate. Carrier and size markers of unlabelled poly(A), 4S tRNA and 5S and 7S rRNA species were added, the RNA was recovered by phenol extraction and ethanol precipitation, and analysed by electrophoresis on 7.5% (w/v) polyacrylamide gels. —, E₂₆₅; the two histograms show the radioactivity per 2mm gel slice for tissue labelled for 1 and 2h.

The distribution of ³²P-labelled nucleic acid species along the gradient was determined by dividing the gradient into the three sections indicated, and analysing the nucleic acid by gel electrophoresis and oligo(dT)-cellulose chromatography (Table 5). Most of the labelled DNA (96.3%) was in the pellet as chromatin, but only about 50% of the hnRNA was associated with the chromatin pellet, the remainder being distributed along the gradient. The distribu-

tions of poly(A)-containing hnRNA and hnRNA without poly(A) were similar. Most of the 1.4 × 10⁶-mol.wt. precursor rRNA was in a band corresponding approximately to the large ribosomal subunit (fraction 2). The presence of 31% of this precursor in the pellet may indicate that some nucleoli are still intact, or that aggregation leads to the sedimentation of some non-chromatin material. Much of the 2.5 × 10⁶-mol.wt. precursor rRNA was also in the pellet, but this is consistent with the high sedimentation coefficient of this large ribonucleoprotein molecule. The size distribution of the hnRNA increased down the gradient, with the mean size within each gradient section corresponding approximately to the rRNA or precursor rRNA species present in that section.

Lysed nuclei were also analysed by isopycnic centrifugation in metrizamide gradients (Fig. 9). Deoxyribonucleoprotein (chromatin) banded as a single component at 1.17g/cm³, whereas ribonucleoprotein had a much broader distribution with a shoulder at 1.21g/cm³. The separation of ribonucleoprotein from chromatin was not complete, but at the higher densities the fractions were enriched for RNA (³²P-labelled) over DNA (³H-labelled). The gradient fractions were combined as indicated in Fig. 9, and the nucleic acid was extracted and analysed by gel electrophoresis and oligo(dT)-cellulose chromatography. No clear separation of components was obtained (Table 6). The main chromatin band (sections 1 and 2) contained 87% of the DNA but only 55% of the precursor rRNA and 69% of the hnRNA. The precursor rRNA species were concentrated into sections 2 and 3, particularly section 3, where the ratios of precursor rRNA species to

Fig. 7. Isopycnic density centrifugation of sonicated nuclei in metrizamide gradients

Nuclei isolated from explants continuously labelled with [^3H]thymidine and pulse-labelled for 1 h with [^{32}P]orthophosphate were disrupted by sonication for 40 s (a) or 20 s (b). The nuclear sonicate was centrifuged in a metrizamide gradient for 65 h at 35000 rev./min. Fractions were collected, precipitated with trichloroacetic acid on to glass-fibre filters, and the ^3H and ^{32}P radioactivities determined. $\circ$, ^3H c.p.m.; $\bullet$, ^{32}P c.p.m.; ----, metrizamide density.

Fig. 8. Sucrose-gradient sedimentation of lysed nuclei

Nuclei isolated from explants continuously labelled with [^3H]thymidine and pulse-labelled for 1 h with [^{32}P]orthophosphate were lysed in EDTA and centrifuged on a 7.5–60% (w/v) sucrose gradient for 17 h at 20000 rev./min. Samples (100 μl) were removed from each fraction, precipitated with trichloroacetic acid, collected on to glass-fibre filters and the ^3H and ^{32}P radioactivities determined. —, E_{258} ; the broken-line and solid-line histograms show ^3H and ^{32}P radioactivity respectively. Direction of sedimentation was from right to left. The bars indicate the regions of the gradient taken for further analysis.

hnRNA and DNA were much higher than in any other section. The hnRNA was distributed across the gradient, but predominantly in the denser region of the chromatin band (section 2). The distribution of poly(A)-containing hnRNA was similar to that of hnRNA without poly(A). A limited amount of RNA degradation was observed after recovery of the RNA from the gradient, so the estimates of the proportions of poly(A)-containing hnRNA will be minimum values.

(c) *Extraction of ribonucleoprotein particles.* During 15 min incubation of nuclei at 25°C, 20–30% of the total ^{32}P -labelled nucleic acid was released and recovered in the supernatant after sedimentation of the nuclei. Gel electrophoresis showed that about 50% of the released ^{32}P radioactivity was in hnRNA and the remainder in 0.7×10^6 -mol.wt. rRNA and precursor rRNA molecules. Some 12% of the hnRNA released contained poly(A). Sucrose-gradient sedimentation indicated that most of the ribonucleoprotein particles in the extract sedimented more slowly than the small ribosomal subunit. As the RNA was mostly within the size range 0.5×10^6 – 1.0×10^6 daltons, this would suggest that the ribonucleo-proteins have a low protein content. Analysis on metrizamide density gradients identified two density components (Fig. 10b), a major band in the density range 1.21–1.24 g/cm 3 and a broad band of

Table 5. *Distribution of ^{32}P -labelled nucleic acid species of lysed nuclei in the sucrose gradient*

Sections of the sucrose gradient (Fig. 8) were combined as indicated, and nucleic acid was extracted and analysed by electrophoresis on 2.3% (w/v) polyacrylamide gels and chromatography on oligo(dT)-cellulose columns. The distribution of each nucleic acid species between the gradient sections (% of total radioactivity in that species) is shown across the Table. The values in parentheses are the proportions of the different nucleic acid species within each gradient section (% of total radioactivity in that section).

Gradient section ... Nucleic acid species	Percentage of total radioactivity			
	1	2	3	Pellet
DNA	2.6 (5.9)	0.9 (2.2)	0.2 (1.1)	96.3 (54.5)
hnRNA lacking poly(A)	28.8 (85.9)	15.7 (51.5)	8.9 (65.8)	47.1 (34.8)
Poly(A)-containing hnRNA	27.9 (8.2)	11.9 (3.7)	7.8 (5.6)	52.4 (3.8)
1.4×10^6 -mol.wt. precursor rRNA	—	59.1 (41.5)	9.7 (15.6)	31.2 (5.1)
2.5×10^6 -mol.wt. precursor rRNA	—	8.0 (1.1)	38.2 (11.9)	53.8 (1.8)

Fig. 9. *Isopycnic density centrifugation of lysed nuclei in a metrizamide gradient*

Nuclei isolated from explants continuously labelled with [^3H]thymidine and pulse-labelled for 1 h with [^{32}P]orthophosphate were lysed in EDTA and centrifuged in a metrizamide gradient for 65 h at 30000 rev./min. Fractions were collected, and portions (100 μl) were precipitated with trichloroacetic acid on to glass-fibre filters and the ^3H and ^{32}P radioactivity determined. $\circ$, ^3H c.p.m.; $\bullet$, ^{32}P c.p.m.; ----, Metrizamide density. The bars indicate the regions of the gradient used for further analysis.

1.24–1.33 g/cm 3 . The density of the major band corresponds to that of ribosomes, and at least part of the band is therefore probably due to ribosomal subunits and ribosomal precursors present in the extract. A small peak of [^3H]thymidine-labelled DNA was also present at 1.14 g/cm 3 (Fig. 10b), suggesting that some chromatin was partially deproteinized and released during the incubation. Analysis of the nuclear remains (Fig. 10a) showed that most of the ^{32}P -labelled RNA was associated with the chromatin band, with only small amounts at higher densities. The double DNA peak again indicated that some

deproteinization of chromatin occurred during the incubation.

The extract was also analysed by isopycnic centrifugation in a CsCl density gradient. The material released from the incubated nuclei was recovered by centrifugation at 200000g for 16 h, resuspended in triethanolamine hydrochloride/NaOH buffer (pH 7.7), fixed in formaldehyde and centrifuged to equilibrium in CsCl. The band at 1.53 g/cm 3 (Fig. 11) corresponds to the density of ribosomes and is therefore probably due to ribosomal subunits or ribosomal precursors. Much of the ^{32}P -labelled RNA banded at higher densities of 1.6–1.7 g/cm 3 , indicating a protein/RNA ratio lower than that of ribosomes. Less than 20% of the radioactivity was in the pellet, showing that most of the RNA was associated with protein.

Discussion

Stability and labelling kinetics of nuclear RNA

The equation used to calculate the half-lives of the unstable RNA species should be applied to molar accumulation curves (Greenberg, 1972; Brandhorst & McConkey, 1974). These can only be calculated if either the specific radioactivity of the nucleotide precursor or the steady-state amounts of the components are known. The amounts of precursor rRNA molecules and hnRNA present in the artichoke explants were too small to be directly measured and we have not determined the specific radioactivity of the nucleotide-precursor pool. We have instead assumed that the amounts of hnRNA, precursor rRNA species and DNA per cell remain constant over the period of the experiment (3½ h). This assumption appears to be valid because the incorporation of radioactivity into DNA was linear, and the hnRNA and precursor rRNA radioactivity accumulation curves reached steady-state plateau values. Expressing

Table 6. *Distribution of the ^{32}P -labelled nucleic acid species of lysed nuclei in the metrizamide gradient*

Sections of the metrizamide gradient (Fig. 9) were combined as indicated, and nucleic acid was extracted and analysed on 2.3% (w/v) polyacrylamide gels and oligo(dT)-cellulose columns. The distribution of each nucleic acid species between the gradient sections (% of total radioactivity in that species) is shown across the Table. The values in parentheses are the proportions of the different nucleic acid species within each gradient section (% of total radioactivity in that section).

Gradient section ... Nucleic acid species	Percentage of total radioactivity			
	1	2	3	4
DNA	44.8 (27.8)	42.6 (18.9)	9.3 (8.2)	3.3 (6.4)
hnRNA lacking poly(A)	26.6 (58.2)	42.3 (67.9)	21.0 (64.2)	10.0 (78.3)
Poly(A)-containing hnRNA	29.8 (6.2)	34.6 (5.1)	30.0 (8.8)	5.6 (4.3)
Precursor rRNA species	22.1 (7.8)	32.7 (8.2)	36.5 (18.8)	8.7 (11.0)

Fig. 10. *Isopycnic density centrifugation of nuclear fractions in metrizamide gradients*

Nuclei isolated from explants continuously labelled with ^3H thymidine and pulse-labelled for 1 h with ^{32}P orthophosphate were incubated for 15 min at 25°C in 100 mM-NaCl/1 mM-MgCl₂/2% (v/v) Triton X-100/0.2% (v/v) diethyl pyrocarbonate/50 mM-Tris/HCl (pH 8.0). The nuclei were separated from the extract by centrifugation at 2500g for 5 min, and then resuspended in 2 mM-EDTA/0.2% (v/v) diethyl pyrocarbonate/50 mM-Tris/HCl (pH 8.0). Fractions of the nuclear remains (a) and the extract (b) were centrifuged in metrizamide gradients for 65 h at 35000 rev./min. Fractions were collected, precipitated with trichloroacetic acid, collected on to glass-fibre filters and the ^3H and ^{32}P radioactivities determined. $\circ$, ^3H c.p.m.; $\bullet$, ^{32}P c.p.m.; —, metrizamide density.

the accumulation of radioactivity on a DNA basis will therefore produce accumulation curves of shape identical with molar accumulation curves, and the plateau values will be related directly to the molar amounts by the specific radioactivity of the nucleotide precursor.

The equation also requires that the nucleotide precursor pools should reach a constant specific radioactivity immediately on addition of the radioactive label. The kinetics of ^3H adenosine conversion into ATP in the explant culture, by the procedure of Brandhorst & Humphreys (1971), indicated that more than 3 h was required for equilibration of the nucleotide pool (D. Melanson, personal communication). With ^{32}P orthophosphate, which precludes the direct measurement of the nucleotide specific

radioactivity, the equilibration time was less. Approx. 60 min was required before the accumulation of radioactivity into DNA was linear (Fig. 2). Until this is achieved, the accumulation of radioactivity into the nucleotide precursors will follow a curve similar to that of an unstable RNA. Thus the RNA-accumulation curves will contain a component due to increasing precursor specific radioactivity. If this was a significant component it would be seen as non-linearity on the first-order-decay analysis. Some of the plots in Fig. 4 do show evidence of a slightly decreased slope (longer half-life) between the 40 and 60 min points, but, as this is only small, and the remainder of the plot is linear, any influence of the delay in reaching constant nucleotide-precursor specific radioactivity on the calculated half-lives must be

small. It does, however, mean that there must be some overestimation of the half-lives, and the error would be greatest in the most rapidly turning over species, the 2.5×10^6 -mol.wt. precursor rRNA. The linearity of the log plot for total nuclear RNA (Fig. 4), which contains various proportions (Fig. 2) of components with half-lives varying from 24 to 58min (Table 1), clearly illustrates the limitations of this method of analysis. The limits of the range of possible deviations from the stability of the 'average' molecule, suggested by Brandhorst & McConkey (1974), were calculated on the basis of one additional component, a situation not really relevant to the analysis of nuclear RNA, which must contain at least four different unstable components.

The rates of accumulation of radioactivity into 1.3×10^6 - and 0.7×10^6 -mol.wt. rRNA species were 3900 and 1750 c.p.m./h per μg of DNA respectively (Fig. 3). This would be equivalent to a total incorporation of 1130 c.p.m./h per explant ($0.2 \mu\text{g}$ of DNA/explant) and, since the rate of accumulation of RNA averaged $0.062 \mu\text{g/h}$ per explant over this period, the specific radioactivity of the accumulated rRNA is 18200 c.p.m./ μg . By using this as the value for the specific radioactivity of the nucleotide-precursor pool, the plateau values for accumulated radioactivity of the various RNA components (Figs. 2 and 3) may be converted into amounts, and hence expressed as percentages of the total RNA (Table 7). Thus the cytoplasmic mRNA fraction accounts for about 3% of total RNA, and the hnRNA rather less than 1%. The amount of the large precursor rRNA, 0.4% of the total, is consistent with its detection as a 260nm peak on over-loaded gel fractionations (Rogers *et al.*, 1970; Cox & Turnock, 1973).

It has been proposed (Leaver & Key, 1970; Rogers *et al.*, 1970; Cox & Turnock, 1973; Grierson & Loening, 1974) that the pathway of synthesis of plant rRNA is:

From the half-lives (Table 2) and the molecular weights of the species, we can predict that the amounts of the 2.5×10^6 -, 1.4×10^6 - and 1.0×10^6 -mol.wt. precursor rRNA species should be in the proportions 1.00:1.35:0.87. The values in Table 7, calculated from the radioactivity-accumulation plateaus, are 1.00:2.08:0.15. The overestimation of the half-life of the 2.5×10^6 -mol.wt. precursor rRNA, due to the time required for the nucleotide-precursor pool to reach a constant specific radioactivity, will be greater than that for the more stable precursor rRNA species,

Fig. 11. Isopycnic density centrifugation of nuclear particles in a CsCl gradient

Nuclear extracts prepared as described in Fig. 10 were centrifuged for 16h at 200000g, the pellets were resuspended in triethanolamine hydrochloride/NaOH buffer (pH7.7), fixed in 6% (w/v) formaldehyde, and centrifuged in a CsCl gradient for 42h at 35000rev./min. Fractions were collected, precipitated with trichloroacetic acid, collected on to glass-fibre filters, and ^{32}P radioactivity was determined. ●, ^{32}P c.p.m.; ----, CsCl density.

Table 7. Quantitative estimation of the nuclear and messenger RNA species

The plateau values are taken from Figs. 2 and 3, the specific radioactivity of nucleotide precursor as 18200c.p.m./ μg and the ratio of total RNA/DNA as 17.6.

	Plateau value (c.p.m./ μg of DNA)	μg of RNA/ μg of DNA	Percentage of total RNA
hnRNA	2900	0.160	0.9
2.5×10^6 -mol.wt. precursor rRNA	1200	0.066	0.4
1.4×10^6 -mol.wt. precursor rRNA	2500	0.137	0.8
1.0×10^6 -mol.wt. precursor rRNA	175	0.010	0.06
mRNA	10000	0.55	3.1

such as the 1.4×10^6 - and 1.0×10^6 -mol.wt. precursor rRNA species, and therefore the calculated proportions of the pool sizes relative to the 2.5×10^6 -mol.wt. precursor rRNA will be underestimated. The predicted and observed values for the relative pool size of the 1.4×10^6 -mol.wt. precursor rRNA (1.35 and 2.08 respectively) are therefore consistent with this molecule being an obligatory intermediate in the pathway of synthesis of the 1.3×10^6 -mol.wt. rRNA. The relative amount of the 1.0×10^6 -mol.wt. precursor rRNA (0.83 and 0.15) is not, however, consistent with this molecule being an intermediate in the major pathway of synthesis of the 0.7×10^6 -mol.wt. rRNA. If the 1.0×10^6 -mol.wt. precursor rRNA were on the major pathway, it would have to be present in much larger amounts (or be preferentially degraded during extraction), have an extremely short half-life (8–9 min) or be present in nuclear and cytoplasmic pools possessing very different kinetics.

In order to obtain preparations free of cytoplasmic contamination, nuclei were collected by low-speed centrifugation (800g, 6 min). This was sufficient to sediment nuclei and most of the chromatin, but nucleoli or nucleoplasmic components released by nuclear damage were not all sedimented. Some nuclear ribonucleoproteins may also have diffused out of the nuclei during isolation. When 70% of the total DNA was recovered in the nuclear pellet, only 50% of the total 2.5×10^6 - and 1.4×10^6 -mol.wt. precursor rRNA species were recovered. Thus, in relating the synthesis of nuclear precursor rRNA species to that of mature rRNA species in the cytoplasm, it is necessary to correct the nuclear values by this factor. We can use the differentiated form of eqn. (1):

$$\frac{dS}{dt} = S_{\infty} \ln 2 \left(\frac{1}{t_D} + \frac{1}{t_h} \right)$$

and the correction factor above to calculate the rates of synthesis, measured as the incorporation of radioactivity, of the unstable RNA species. These would be 2912, 2509, 196, 4438 and 2913 c.p.m./h per μ g of DNA for the 2.5×10^6 -, 1.4×10^6 -, 1.0×10^6 -mol.wt. precursor rRNA species, hnRNA and cytoplasmic mRNA respectively. To incorporate radioactivity into the 1.3×10^6 -mol.wt. rRNA at 3900 c.p.m./h per μ g of DNA (Fig. 3) the 1.4×10^6 -mol.wt. precursor rRNA must be synthesized at a rate of 4075 c.p.m./h per μ g of DNA (the accurate values of 1.33×10^6 and 1.39×10^6 daltons for artichoke rRNA and precursor rRNA have been used for this calculation). The observed synthesis of the 1.4×10^6 -mol.wt. precursor rRNA was only 2509 c.p.m./h per μ g of DNA; therefore, to make the nuclear and cytoplasmic data comparable, the nuclear data must be multiplied by a factor of 1.6. The adjusted rate of synthesis of the

2.5×10^6 -mol.wt. precursor rRNA (4717 c.p.m./h per μ g of DNA) is still less than the value expected from the rates of incorporation into the mature rRNA species (6780 c.p.m./h per μ g of DNA), but this is probably due to the overestimation of the half-life of the 2.5×10^6 -mol.wt. precursor rRNA, as discussed above. This calculation suggests that the half-life of the 2.5×10^6 -mol.wt. precursor rRNA may be 17 rather than 24 min, and, if the entire correction was due to the overestimation of half-lives, resulting from the lack of equilibration of the nucleotide-precursor pools, then the half-life of the 2.5×10^6 -mol.wt. precursor rRNA could be as little as 10 min. As with the analysis of the pool sizes, the adjusted rate of synthesis of the 1.0×10^6 -mol.wt. precursor rRNA (317 c.p.m./h per μ g of DNA) is inconsistent with this being on the pathway of synthesis of the 0.7×10^6 -mol.wt. rRNA (1750 c.p.m./h per μ g of DNA). The rates of synthesis of cytoplasmic mRNA (2912 c.p.m./h per μ g of DNA) and hnRNA (7190 c.p.m./h per μ g of DNA) suggest that as much as 40% of the hnRNA may be transported to the cytoplasm. This value is, however, based on the assumption that the precursor rRNA is quantitatively processed into the stable rRNA species, under these culture conditions, and a less conserved processing would lower this estimate of the percentage of hnRNA transported to the cytoplasm. Clearly a major portion of the hnRNA must turn over within the nucleus.

Poly(A)-containing hnRNA

Some of the hnRNA is polyadenylated, the proportion showing some variation between the two different physiological states of the tubers used in these experiments (Table 4). The proportion of poly(A)-containing hnRNA is rather similar to that reported in animal cells (Greenberg & Perry, 1972; Brandhorst & McConkey, 1975; Lewin, 1975). The rates of turnover of poly(A)-containing hnRNA and hnRNA lacking poly(A) appear to be identical. This similarity, and the value for the half-life of the hnRNA, is comparable with the results reported for mouse L cells by Brandhorst & McConkey (1974, 1975).

The size of the poly(A) tract was found by nuclease digestion and subsequent gel electrophoresis to be 40–80 nucleotides long (Fig. 6). However, the average size of the poly(A)-containing hnRNA (0.72×10^6 daltons; Fig. 5b) and the proportion of the c.p.m. resistant to ribonucleases A and T₁ (8.4%; Table 1), indicate an average length of 180 nucleotides per poly(A) tract. This suggests that there may be more than one poly(A) segment per molecule, or that a single tract of poly(A) may be cleaved, owing to either a lack of enzyme specificity or the presence of other bases within the poly(A) tract. The large variability in the sizes reported for plant poly(A)

(Van de Walle, 1973; Sagher *et al.*, 1974; Schmid *et al.*, 1975; Key & Siflow, 1975) suggests that before the size of the plant poly(A) tract can be rigorously defined a much more careful analysis must be undertaken.

Fractionation of nuclei

The kinetic experiments indicate that at least 60% of the hnRNA is not transported from the nucleus to the cytoplasm. It is interesting to note that a similar proportion of the hnRNA remains associated with the chromatin throughout the nuclear fractionation procedures used in these studies.

Approx. 30% of the ^{32}P -labelled RNA was released from chromatin after sonication of nuclei, and 50% after lysis in EDTA. The polyacrylamide-gel analysis of fractions from sucrose gradients indicated that the hnRNA was uniformly distributed down the gradients rather than sedimenting as a broad peak around 70–80S, as reported in HeLa cells (Pederson, 1974) and sea-urchin embryos (Levner *et al.*, 1975), or a narrow 50–60S band, as in slime mould (Firtel & Pederson, 1975). Only slowly sedimenting particles were removed from nuclei during incubation in buffer.

Metrizamide-density-gradient centrifugation separated ribonucleoprotein from deoxyribonucleoprotein only when the chromatin structure had been destroyed by prolonged sonication. This resulted in deproteinization of deoxyribonucleoprotein and possibly ribonucleoprotein. With less sonication, or after nuclear lysis in EDTA, complete separation was not possible. Under the gradient conditions used, free RNA and chromatin banded at very similar densities, 1.17–1.20 g/cm³, so that it was not possible to determine whether the failure to separate most of the ribonucleoprotein from chromatin was because the ribonucleoprotein was bound within the chromatin structure or that the ribonucleoprotein had been released from the chromatin, but also deproteinized, with the result that the RNA banded at a similar density to the chromatin. This fact detracts from the possible use of metrizamide in studies of this type. Material released from nuclei by incubation in buffer banded in metrizamide at higher densities (Fig. 10b), which corresponded to the dense side of the main ^{32}P -labelled band from EDTA-lysed nuclei. The banding pattern obtained from EDTA-lysed nuclei was similar to that obtained from mouse L cells by Monahan & Hall (1975) in that the major peak of ^{32}P -labelled material was displaced towards the denser side of the [^3H]thymidine (chromatin) band and a second region of ^{32}P -labelled material banded as a small broad peak between 1.24 and 1.30 g/cm³. The density in CsCl of the ribonucleoprotein particles containing hnRNA was much higher than that usually found in mammalian cells (Lukanidin *et al.*,

1972; Pederson, 1974), indicating that the ratio of protein to RNA is much lower. However, Levner *et al.* (1975) have reported the presence of dense ribonucleoprotein particles in lysed sea-urchin-embryo nuclei.

It therefore appears that more than 50% of the hnRNA in isolated artichoke nuclei is tightly bound to the chromatin, and is not released until the chromatin structure has been destroyed. The proportion of hnRNA that is released during lysis presumably represents the soluble phase of the nuclear hnRNA, possibly the transitory pool *en route* to the cytoplasm. The hnRNA in this soluble phase contains a similar proportion of polyadenylated molecules (15–20%) to that associated with the chromatin. This lack of enrichment for poly(A)-containing hnRNA in the soluble phase could indicate the tight coupling between polyadenylation and transcription, and also suggests that the soluble-phase component is not selected on the basis of polyadenylation. The significance of the higher proportion of polyadenylated cytoplasmic mRNA species (50%; D. Baulcombe, personal communication) is not clear from these results, since it could be interpreted in terms of selection for polyadenylated molecules, or cytoplasmic adenylation.

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