The Molecular Weight of rRNA Precursor Molecules and Their Processing in Higher Plant Cells

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Actively dividing callus cells of higher plants *(Petroselinum crispum, Daucus carota, Acer pseudoplatanus)* were used to detect the primary gene product of rDNA *in vivo*. Parsley and carrot cells were labelled with [^{32}P]orthophosphate. Under non-denaturing conditions, in both cases only one high molecular weight rRNA precursor was present on polyacrylamide gels. Its molecular weight did not exceed 2.5×10^6 dalton. Under denaturing conditions, $2.0-2.1\times10^6$ dalton were determined on formamide gels. This rRNA precursor was already present after a labelling period of 5–10 min. In parsley cells labelled mature rRNA (25S and 18S) arrived in the cytoplasm 45 min after onset of incubation.

In *Acer pseudoplatanus* incubated with [^{3}H]uridine two rapidly labelled components did emerge from polyacrylamide gels without formamide; their molecular weights were 2.3 and $3.2-3.4\times10^6$ dalton. After electrophoresis in formamide, the larger component disappeared, thus indicating that it would be an intermolecular aggregate of different RNAs. From these results we have no evidence for the existence of rRNA precursors exceeding the molecular weight of 2.5×10^6 dalton.

Introduction

Synthesis and processing of ribosomal RNA has been extensively studied in plant and animal cells. In plant cells it is evident from a large number of investigations that the 1.4 and 1.0×10^6 components are direct precursors to the mature rRNA molecules. The data about the primary transcription product differ in their molecular weights measured after polyacrylamide gel electrophoresis. The values mostly vary from 2.2×10^6 to 2.6×10^6 dalton. Two outstanding exceptions are the values for root discs of the carrot (2.8×10^6 [1]) and for freely suspended callus cells from *Acer pseudoplatanus* (3.4×10^6 [2]).

In the present paper some experiments concerning the size of the primary product of the ribosomal genes are described. Actively dividing callus cells grown in a liquid medium *(Petroselinum crispum* and *Acer pseudoplatanus)* or on agar *(Daucus carota)* are used to determine the size of the primary transcript of the rDNA of higher plant cells. *Acer pseudoplatanus* was choosen, since Cox and Turnock [2] were able to detect such a large precursor molecule in this species.

The absence of large precursor molecules over 2.6×10^6 dalton in nearly all plant cells might have several reasons. One reason might be that the extraction procedures are not suitable for such long molecules or the molecules are destroyed during extraction and purification. Another reason might be that the processing and the turn-over of these precursor molecules are too rapid to detect these primary gene products. A third possibility could be that the molecular weights determined for *Daucus carota* and *Acer pseudoplatanus* are artifacts as described in ref. [3]. In order to avoid these difficulties, several precautions were taken. 1. The incubation period was varied over a broad range. 2. Isolated nuclei were used to guarantee a careful extraction of the precursor molecules. 3. Cycloheximide which slows down the processing of ribosomal RNA [4] was applied to accumulate the primary gene product. In order to detect artifacts such as unspecific aggregations of different RNA molecules which lead to higher molecular weights, electrophoresis under denaturing conditions was carried out [5].

Materials and Methods

Cell growth conditions

Freely suspended callus cells of *Petroselinum crispum* were propagated according to ref. [6]. *Daucus carota* cells on a solid medium were grown as previously described [7]. Cells of *Acer pseudoplatanus* were propagated as suspensions on a rotary shaker in a medium according to ref. [8]. All cultures were kept in darkness at 26 °C.

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Labelling conditions

Cells of *P. crispum* and *D. carota* from the logarithmic stage of the growth period were transferred into a phosphate-free medium and cultivated for 12 h. After this preincubation period 10 – 20 μCi/ml [^{32}P]orthophosphate (Amersham Buchler, Braunschweig) were added. Cells of *A. pseudoplatanus* (6 days after inoculation) were incubated with [^{3}H]uridine (6 μCi/ml; Amersham Buchler, Braunschweig). For special labelling conditions see figure legends.

Pulse-chase-experiments with P. crispum

At the end of the pulse incubation with [^{32}P]-orthophosphate the radioactive medium was removed by filtration and washing. The cells were resuspended in the normal culture medium containing an excess of [^{31}P]orthophosphate.

Cell fractionation

Nuclei and ribosomes were prepared from the same culture batch. After the labelling period the cells were disrupted in a potter homogeniser (glas-teflon). Isolation buffer: 50 mM Tris·HCl, 2 mM $MgSO_4$, 250 mM sucrose, pH 7.5. The homogenate was filtered through a series of nylon nets (200 μm, 100 μm, 30 μm, 10 μm). The nuclei were gathered from the last filtrate by centrifugation ($1000 \times g$, 10 min). The supernatant contained the ribosomes. The nuclei were purified by two washings in isolation medium (see above). The centrifugation conditions were $1000 \times g$ for 10 min. Nucleic acids were extracted by the normal phenol method (see below).

The supernatant containing the ribosomes was centrifuged twice at $12\,000 \times g$ for 10 min. The resulting supernatant was treated with sodiumdeoxycholate (final concentration 0.5%; 15 min) to detach membrane bound ribosomes from the endoplasmic reticulum. The ribosomes were sedimented at $105\,000 \times g$ for 90 min. RNA was also extracted by the phenol method.

Preparation of high molecular weight RNA

The RNA from homogenates of *D. carota*, *A. pseudoplatanus* and from the nuclei derived from *D. carota* was prepared after Cox and Turnock [2]. After precipitation in cold ethanol the nucleic acids were collected by centrifugation and dissolved in 0.15 M sodium acetate with 1 mM EDTA (pH 6.0) and precipitated once more by adding 2.5 vol. of ethanol (– 18 °C). RNA from parsley cells was extracted by the phenol method described earlier [9].

Polyacrylamide gel electrophoresis

Electrophoresis was carried out in 2.1% gels according to [9]. A buffer system containing 36 mM Tris·HCl, 30 mM NaH_2PO_4, 1 mM EDTA, pH 7.8 was used. After electrophoresis (2.5 mA/gel, 220 min) the gels were scanned at 260 nm, frozen in dry ice and chopped in 1 mm slices. Radioactivity (^{32}P and ^{3}H) was determined in a scintillation counter.

Formamide gel electrophoresis

Polyacrylamide gels (6×80 mm) were prepared with 98% formamide according to ref. [10]. The running buffer, a 10 mM Na_2HPO_4 solution, was adjusted to pH 9.0 with 10 mM NaH_2PO_4. The nucleic acids were denatured by heating them in buffered formamide at 60 °C for 3 min and layered immediately on the gels. Electrophoretic conditions: 3 mA/gel, 9 h, 22 °C. A buffer cycle (10 – 15 ml/min) was installed between both buffer reservoirs. The gels were analysed for absorbance (260 nm) and radioactivity (see above).

Results

Labelling kinetics with D. carota

To detect the primary gene product of ribosomal DNA, a series of experiments with labelling periods ranging from 5 to 30 min were carried out (Figs 1 and 4 b). The incorporation rate of [^{32}P]orthophosphate was very low after 5 min, only heterogeneous RNA was labelled. After a labelling period of 10 min a distinct peak between DNA and 25S RNA emerged from the gels. Its molecular weight was 2.3×10^6 dalton. Ribosomal RNA from *E. coli* was used as a standard. Mature rRNA (indicated by arrows) was not labelled at that time. After 20 min the label was incorporated into the 2.3×10^6 component and into the 25S and 18S rRNA as well. To avoid labelling of heterogeneous material, in some experiments [^{3}H-methyl]methionine had been used as a tracer since methyl groups are incorporated pre-

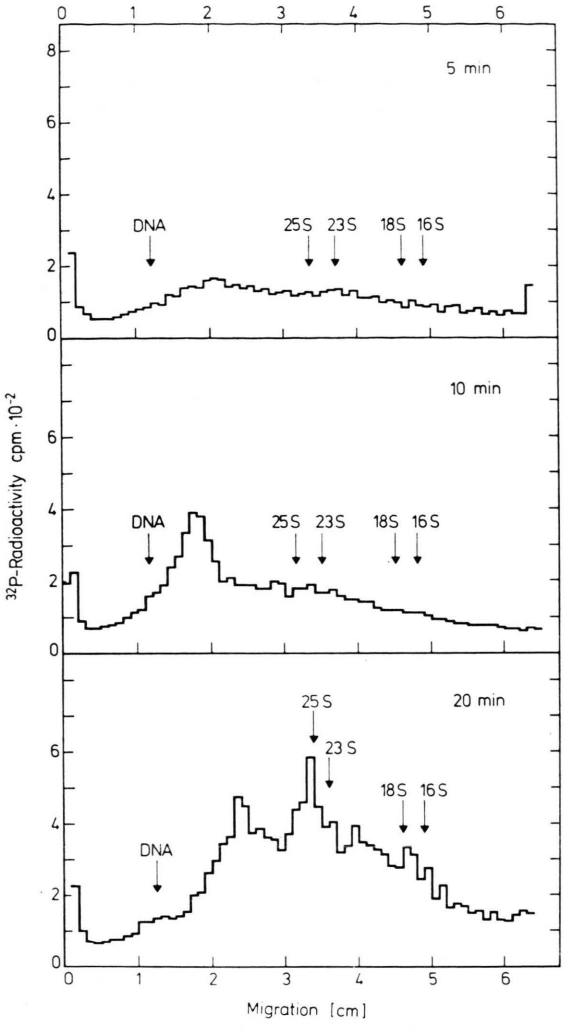

Fig. 1. Polyacrylamide gel electrophoresis (2.1%) of pulse labelled RNA from callus cells of Daucus carota after various incubation periods (5, 10 and 20 min) with [^{32}P]-orthophosphate (20 μCi/ml). The RNA was extracted according to [2]. The arrows indicate the maxima in absorbance at 260 nm. *E. coli* RNA (23 and 16S) was used as a standard.

ferentially into ribosomal RNA. In the case of carrot cells this method was not successful, because the label was mainly incorporated into pectinic acids instead of rRNA (data not shown).

The data presented here for carrot cells do not point to a rRNA precursor with a molecular weight over 2.3×10^6 dalton. The cells needed 5 – 10 min to synthesise precursor RNA. Approximately 15 min were necessary for both processes, transcription and processing of RNA.

Rapidly labelled RNA in isolated nuclei

The specific activity of RNA extracted from isolated nuclei was much higher than such extracted from whole cells (Fig. 2). This is true for carrot and parsley cells as well. Only one precursor RNA did emerge from the polyacrylamide gels. Its molecular weight varied from 2.3 to 2.6×10^6 dalton. In this context it is noteworthy that the specific activity of the 18S RNA was very low compared with that of 25S RNA. This striking difference might be the result of different velocities in transport of both components from nucleus to cytoplasm.

The time requirements for synthesis and transport of rRNA has been revealed by pulse-chase experiments with parsley cells (Figs 3 a and b). In this case RNA was extracted from nuclei and ribosomes prepared from the same homogenate. A 30 min labelling period had been followed by a chase treatment of 15 min. 45 min after onset of incubation labelled rRNA was already present in the cytoplasm, strictly speaking in mature ribosomes (Fig. 3 b). It is obvious that after a 15 min chase treatment we caught just the right moment in which the newly synthesised material reached the cytoplasm.

Cycloheximide experiments

Protein synthesis in parsley cells treated with 5 μg/ml cycloheximide for 1 h was completely suppressed, whereas RNA was synthesised up to 70% of the control [4]. RNA synthesis in carrot cells was much more affected by cycloheximide. On polyacrylamide gels (Fig. 4 a) only one labelled component was visible under these conditions. The molecular weight of this component was 2.3×10^6 dalton. A processing of precursor RNA apparently did not occur. An additional rRNA precursor with a molecular weight higher than 2.3×10^6 dalton was not observed. Electrophoresis under denaturing conditions (formamide gels) brought the same result: only one RNA component which emerged from the gels in a very sharp peak was accumulated in the presence of cycloheximide. These results are documented in Table I.

Formamide gel electrophoresis

Rapidly labelled RNA from *D. carota* (Fig. 5) and *A. pseudoplatanus* was separated under dena-

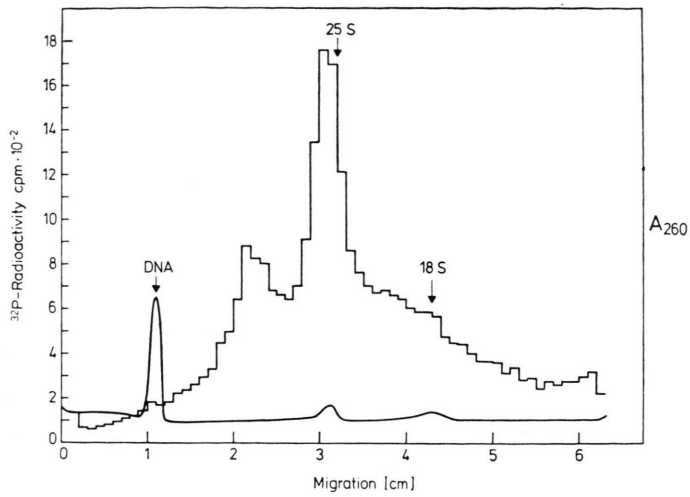

Fig. 2. Polyacrylamide gel electrophoresis of pulse labelled RNA (30 min [^{32}P]orthophosphate, 20 μCi/ml) extracted from isolated nuclei of *Daucus carota*. For purification procedures see Materials and Methods. ——— Absorbance at 260 nm, ⌐¬ ^{32}P-radioactivity.

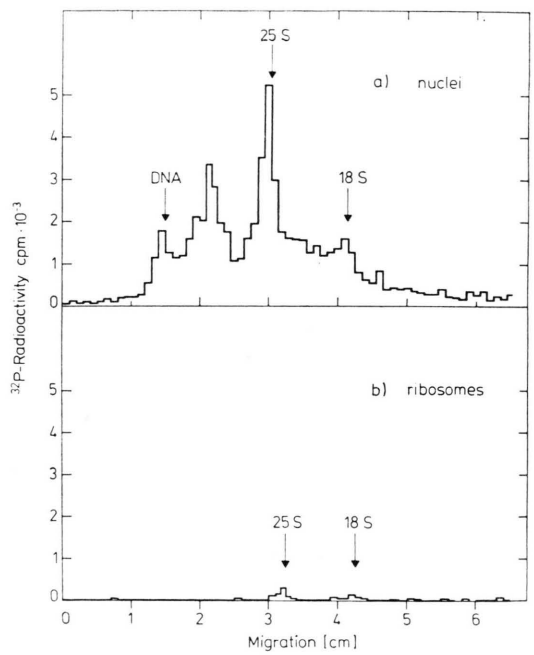

Fig. 3. Pulse-chase experiment with callus cells of *Petroselinum crispum*. The RNA was extracted from nuclei and ribosomes derived from the same homogenate. The cells were incubated for 30 min with [^{32}P]orthophosphate (10 μCi/ml) and transferred into unlabelled phosphate for 15 min. Nuclei and ribosomes were purified as described in Materials and Methods. RNA was extracted from both fractions and separated by gel electrophoresis. a) ^{32}P-radioactivity of RNA extracted from nuclei, b) ^{32}P-radioactivity of RNA from isolated ribosomes. The UV-peaks (260 nm) are indicated by arrows.

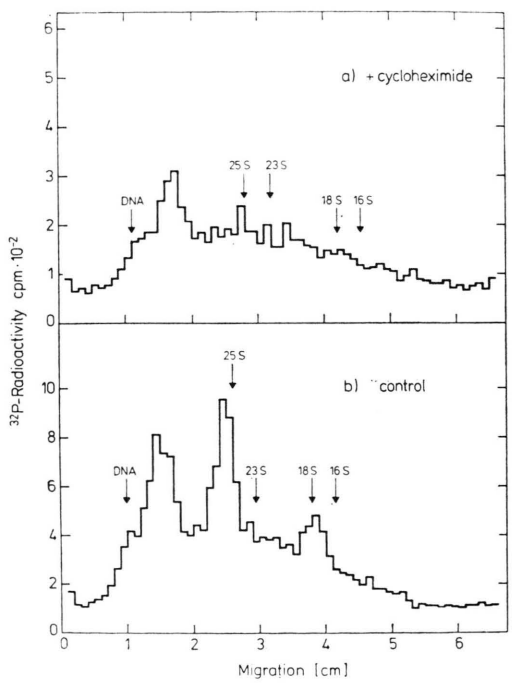

Fig. 4. Effect of cycloheximide on the processing of rRNA in callus cells of *Daucus carota*. The cells were incubated with [^{32}P]orthophosphate (20 μCi/ml) in the presence of cycloheximide (5 μg/ml). Extraction and separation of RNA was carried out according to [2]. Standard RNA originated from *E. coli* (23S and 16S). The UV-peaks (260 nm) are indicated by arrows.

turing conditions. In both cases only one rRNA precursor was visible. The molecular weight of this RNA molecule was determined for carrot and sycamore. It was $2.0-2.1\times10^6$ dalton. The peak was very sharp in comparison to the peak in normal polyacrylamide gels; this gives us a hint for the

Table I. Comparison of molecular weights of rRNA precursors from *Petroselinum crispum*, *Daucus carota*, and *Acer pseudoplatanus*.

Plant material	Preparation Electrophoretic conditions	Molecular weight approximate values ($\times 10^6$ daltons)
P. crispum	total homogenate without formamide	2.3
D. carota	total homogenate without formamide	2.3
	purified nuclei without formamide	2.3 – 2.5
	total homogenate formamide gels	2.0 – 2.1
	cycloheximide total homogenate without formamide	2.3
	cycloheximide total homogenate formamide gels	2.0 – 2.1
A. pseudoplatanus	total homogenate without formamide	2.3 and 3.2 – 3.4
	total homogenate formamide gels	2.0 – 2.1

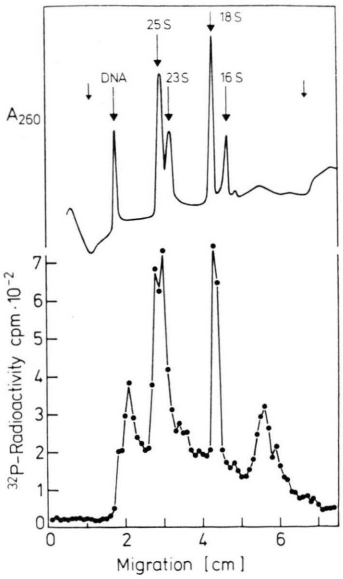

Fig. 5. Formamide electrophoresis of nucleic acids from *D. carota*. The cells were pulse labelled for 30 min with [^{32}P]orthophosphate. Nucleic acids were extracted according to [2]. Electrophoresis of the denatured RNA (dissolved in 99% formamide pH 9.0, heated at 60 °C for 3 min) was carried out as described in Materials and Methods. ——— Absorbance at 260 nm. *E. coli* RNA was used as a standard. ●—● ^{32}P-radioactivity. Wire pieces applied as length markers near the gel ends are indicated by arrows.

homogeneity of the fraction. In all experiments carried out no additional component was observed.

Discussion

Cycloheximide strongly suppressed the processing of rRNA precursors. Assuming that the primary product with a molecular weight higher than 2.3×10^6 dalton is transcribed, one would expect that cycloheximide treatment leads to an accumulation of this precursor RNA, since its processing is impaired. In all inhibitor experiments carried out with *P. crispum* [4] and *D. carota* no precursor with a molecular weight exceeding 2.3×10^6 was accumulated. Therefore, we have good evidence that a precursor larger than approximately 2.3×10^6 is not synthesised by this type of plant cells.

In order to prevent intermolecular aggregation of different RNAs, molecular weights of rRNA were determined after formamide electrophoresis. *E. coli* rRNAs were used as markers. The molecular weights obtained on formamide gels were lower than many values reported for higher plants using polyacrylamide gels without formamide (for review se [11]. Our values for *D. carota* and *A. pseudoplatanus* ($2.0 - 2.1 \times 10^6$ dalton) are in good agreement with results for other species [12, 10]. Spohr *et al.* [3] measured identical molecular weights after repeated electrophoresis in formamide. Therefore, it is rather unlikely that RNA is degraded during fractionation.

Parsley and carrot cells contained only one large precursor with a molecular weight of approximately 2.3×10^6 dalton in normal polyacrylamide gels and of 2.1×10^6 *(D. carota)* in formamide gels. With sycamore cells different results were obtained. On polyacrylamide gels without formamide two distinct peaks were present; their molecular weights were about 2.3×10^6 and $3.2 - 3.4 \times 10^6$ dalton. These findings confirmed the earlier results of Cox and Turnock [2], but on gels containing formamide only one rRNA precursor was present ($2.0 - 2.1 \times 10^6$ dalton). These results are a very strong hint that the large component of about 3.4×10^6 dalton could be an aggregate not stable under denaturing conditions.

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