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Early Ribonucleic Acid Synthesis during the Germination of Rye (*Secale cereale*) Embryos and the Relationship to Early Protein Synthesis

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Incorporation studies with radioactive precursors showed that synthesis of protein and RNA is initiated in germinating embryos of rye within the first hour of imbibition of water. By polyacrylamide-gel fractionations of radioactive nucleic acid components, the appearance of products of transcription of the genome was shown to follow the sequence: heterogeneous (ribonuclease-sensitive) RNA, 4S and 5S RNA by 20min, 31S and 25S rRNA by 40min, and 18S RNA by 60min. 'Fingerprint' analysis of T_1-ribonuclease digests show that all the large oligonucleotides present in 25S and 18S RNA are present in the 31S species, indicating that 31S RNA is the precursor rRNA molecule to both 25S and 18S RNA. The importance of these early RNA syntheses and in particular the possible template function of the heterogeneous RNA is discussed in relation to the concept of long-lived mRNA and the coding for protein synthesis in the first hours of germination.

Fertilization in higher plants is followed by active synthesis of RNA and protein, the replication of DNA and the formation of a multi-cellular and differentiated embryo. These initial stages of development precede a period of organized dehydration during which the water content of the seed is decreased to some 10–15% of the seed weight. The synthesis of RNA, DNA and protein is then arrested, and the embryo enters a period of minimal metabolic activity and maximum resistance to extremes of temperature and water stress. Cell growth and the synthesis of nucleic acids and protein can be resumed only when water is again supplied and the cells of the embryo become rehydrated.

The first reports by Dure & Waters (1965) and Waters & Dure (1966) for germinating cotton seeds, by Marcus & Feeley (1964), Marcus *et al.* (1966) and Chen *et al.* (1968*a*) for germinating wheat embryos, and by Barker & Hollinshead (1964) for pea seeds, indicated that renewed protein synthesis could occur in the absence of concomitant synthesis of RNA. This conclusion was based predominantly on evidence for (*a*) the continued incorporation of radioactive amino acids into trichloroacetic acid -insoluble protein in the presence of actinomycin D, (*b*) the failure to detect incorporation of low-specific-radioactivity bases or of radioactive orthophosphate into nucleic acid, and (*c*) the lack of an increase in percentage hybridization of RNA to DNA during early germination.

Since then Barker *et al.* (1971) have questioned the interpretation of each of these results.

In wheat, protein synthesis in excised embryos could be demonstrated within 30min of imbibition of water (Marcus *et al.*, 1966; Chen *et al.*, 1968*b*), whereas newly synthesized rRNA could be detected in the nucleus at 2–3h but in the cytoplasm only at 6h, with no evidence for new mRNA before 12h (Chen *et al.*, 1971). Results of this kind have supported the concept that during the early stages of germination in these seeds, protein synthesis is directed solely by long-lived mRNA, and that this mRNA is synthesized during embryogenesis, stored in the dry seed and reactivated as a template only after imbibition of water at germination. The demonstration by Marcus & Feeley (1966) that a microsomal fraction from dry seeds could be activated to synthesize protein *in vitro* by the addition of ATP and an ATP-generating system, and that the synthesis was associated with the formation of polyribosomes, was additional evidence for the concept of long-lived mRNA in embryos. Weeks & Marcus (1971) then showed that a 'messenger' fraction could be isolated from dry wheat embryos and could sustain protein synthesis in the cell-free system. Schultz *et al.* (1972) subsequently separated ribonucleoprotein particles containing an RNA with mRNA template activity from the ribosomal fraction of dry wheat embryos. These particles appear to resemble the mRNA-containing 'informosomes' described by Spirin & Nemer (1965) and Spirin (1969).

Although such biochemical data add credence to the notion of long-lived mRNA in dry seeds, other studies have indicated the occurrence of an early

synthesis of RNA at germination which could include mRNA.

Tanifuji *et al.* (1969), using methylated albumin–kieselguhr columns and sucrose density gradients, found the synthesis of heterogeneous RNA within 2–5h of germination of pea seeds. Deltour (1970), using radioautography, showed that nuclear RNA synthesis occurred by 4h in germinating maize. Rejman & Buchowicz (1971) and Dobrzanska *et al.* (1973) reported mRNA synthesis in wheat embryos after only 15min germination. In their experiments, however, protein synthesis was not observed until 3h, with rRNA synthesis at 12h and tRNA synthesis at 18h.

At present, no clear conclusion can be drawn for the relative importance of long-lived or newly synthesized mRNA in coding for the first protein synthesis that occurs when embryos imbibe water at germination. Radioautographic studies (Osborne *et al.*, 1974) have shown that in rye, nRNA synthesis occurs in root cells within 10min of imbibition of water.

The experiments described here are designed to determine the time and sequence of synthesis of the different classes of RNA during the early hours of germination of rye and to shed further light on whether long-lived or newly synthesized mRNA could be involved in early protein synthesis.

Experimental

Materials

Rye (*Secale cereale* var. Lovasz patonai) grain of 99% viability (1973 harvest) was obtained from Nunns Corn and Coal Ltd., Quayside, Woodbridge, Suffolk, U.K.

Embryos were separated from the rest of the grain by the mass isolation technique of Johnston & Stern (1957).

Radiochemicals

G-^{14}C-labelled amino acid mixture (25mCi/mg-atom of C), [U-^{14}C]uridine (482 or 489mCi/mmol), [U-^{14}C]adenine (287mCi/mmol) and [^{32}P]P_i (75Ci/mg of P) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K.

Ribonuclease T_1 (Sankyo Co.) was obtained from Calbiochem Ltd., London W.1, U.K. Cellulose acetate strips (Schleicher and Schuell) measuring 75cm×3cm were from Anderman and Co. Ltd., London S.E.1, U.K., and plastic-backed thin layers of PEI (polyethyleneimine)–cellulose (Macherey, Nagel and Co.) measuring 40cm×20cm were from Camlab, Cambridge, U.K. Kodirex X-ray film was from Kodak Ltd., London HP2 7EU, U.K.

Methods

Measurement of incorporation of radioactive precursors into trichloroacetic acid-insoluble material. Rye embryos were germinated at 25°C on germinating medium which consisted of sucrose (2%, w/v) with chloramphenicol at 10μg/ml. After various times from the start of germination, embryos were blotted dry and placed in fresh germinating medium containing the appropriate radioactive precursor for 1h (2h for the RNA time-course).

After incubation in radioactive precursors, embryos were rinsed and homogenized in germinating medium containing a 150-fold excess of the appropriate non-radioactive precursor. Portions of this suspension were pipetted on to GF/A glass-fibre filters and air dried. These samples enabled the total uptake of the precursor by the embryos to be checked and the results are not presented.

The rest of the homogenate was mixed with an equal volume of trichloroacetic acid (10%, w/v) at 0°C. For the amino acid incorporation, the precipitate was heated for 15min at 95°C to deacylate the aminoacyl-tRNA and then cooled to 0°C. Precipitates were collected on GF/A glass-fibre filters and sequentially washed with trichloroacetic acid (5%, w/v) containing a 150-fold excess of non-radioactive precursor, ethanol, ethanol–ether (3:1, v/v) and finally ether. The air-dried dry filters were placed in 10ml of scintillant {0.7% butyl-PBD[5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole] in toluene} and counted for radioactivity to a standard error of 1% or less in a liquid-scintillation spectrometer with a ^{14}C-counting efficiency of 80–90%.

Polyacrylamide-gel electrophoresis of nucleic acids. Embryos were germinated at 25°C for various lengths of time in germinating medium containing [^{14}C]adenine or [^{14}C]uridine. Total RNA was extracted by the method of Parish & Kirby (1966) as described by Loening (1969), by using the detergents sodium tri-isopropylnaphthalenesulphonate and sodium 4-aminosalicylate, and a phenol–cresol mixture for deproteinization.

The precipitate was dissolved in sodium dodecyl sulphate (0.5%) in 0.15M-sodium acetate buffer, pH6.0, and precipitated with 2vol. of ethanol to remove contaminating traces of phenol and extraction detergents. The sample was then stored overnight at −20°C.

Nucleic acids weref ractionated in 2.2% and 7.5% polyacrylamide gels as described by Loening (1967, 1969). Electrophoresis was at room temperature (approx. 20°C) in 'E buffer' [36mM-Tris, 30mM-NaH_2PO_4, 1mM-EDTA (disodium salt) and 0.2% sodium dodecyl sulphate, pH7.8 (Loening, 1967)] at a constant voltage of 50V and with a current of approx. 5mA/gel. The gels were scanned at 260nm in a modified Hilger–Gilford spectrophotometer,

frozen with solid CO_2 and cut into 1 mm slices. Each slice was dried, placed in 10 ml of scintillant, and counted for radioactivity in a liquid-scintillation spectrometer as described above.

'Fingerprint' analyses of 31S, 25S and 18S RNA. Approximately 100 embryos were germinated at 25°C for 24h in 1 ml of germinating medium and 1 mCi/ of $^{32}P_i$ to obtain 25S and 18S RNA at high specific radioactivity. The medium was then replaced with fresh germinating medium containing 9 mCi of $^{32}P_i$ for 2h to label the 31S RNA, after which RNA was extracted as described above.

The RNA obtained was fractionated overnight at 50V in a 13cm×15cm×1.5mm-deep slab of 2.4% polyacrylamide gel (Loening, 1969) and the 31S, 25S and 18S RNA bands were located by radioautography. The bands were cut out, frozen and sliced into 1 mm sections. The sections were thawed into 10 ml of 0.4M-NaCl, shaken overnight at room temperature and centrifuged. The gel sections were re-extracted and the pooled supernatants were concentrated and freed of soluble acrylamide by acid precipitation (Brownlee & Cartwright, 1971).

Each species of RNA was digested to completion with T_1 ribonuclease at an enzyme/substrate ratio of 1:40 by incubating the sample at 37°C for 30min as previously described (Payne *et al.*, 1973). The resulting oligonucleotides were fractionated in two dimensions: cellulose acetate electrophoresis at 5kV for 1.5h in 5% (v/v) acetic acid–0.5% (v/v) pyridine–7M-urea, pH3.5, in the first dimension (Brownlee, 1972) and t.l.c. on PEI–cellulose for 16h at 35°C in 4.0M-formic acid–7.5% (v/v) pyridine–7M-urea in the second dimension (Griffin, 1971). The positions of the oligonucleotides were detected by radioautography.

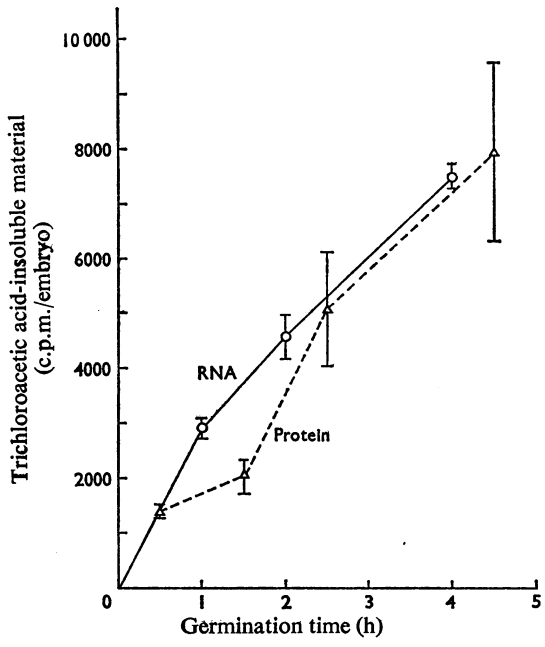


Fig. 1. *Incorporation of [^{14}C]uridine and ^{14}C-labelled amino acid mixture into trichloroacetic acid-insoluble material during early hours of germination in the presence of germinating medium*

For [^{14}C]uridine (10μCi/ml; ○), embryos were pulsed for 2h and the value for c.p.m. incorporated per embryo is located at the mid-point of the pulsing period along the germination time axis. For ^{14}C-labelled amino acids (50μCi/ml; △), embryos were pulsed for 1h and the point was similarly located at the mid-point of the pulsing period. The bar represents range of duplicate samples of five embryos.

Results

Incorporation into trichloroacetic acid-insoluble products

The general pattern of incorporation of [^{14}C]-uridine and ^{14}C-labelled amino acids into trichloroacetic acid-precipitable material shows a steady rise for both components during the first 5h of germination (Fig. 1).

Incorporation into nucleic acids fractionated by gel electrophoresis

Separations of total nucleic acid from embryos incubated in [^{14}C]adenine or [^{14}C]uridine for various lengths of time by fractionation in 2.2% polyacrylamide gels (Figs. 2*a*, 2*c*, 2*e* and Fig. 3) show that a ribonuclease-sensitive, heterogeneous product is the first material to become labelled (20min germination). By 40min (Fig. 2*c*) the radioactivity in this fraction of the RNA has increased, a 31S RNA fraction is labelled and low radioactivity is associated with the 25S RNA; other minor radioactive fractions between the 31S species and the ribosomal RNA are also apparent. In embryos germinated in [^{14}C]uridine for 60min (Fig. 2*e*) all the major RNA fractions are radioactive. At later times (3 and 6h incubations) the histogram patterns change with respect to the proportion of radioactivity in the various fractions (Figs. 3*a*, 3*b*), the intermediate products between the 31S RNA and the ribosomal RNA are no longer apparent and most of the label is present in the 25S and 18S RNA: correspondingly lower proportions of the total radioactivity are then found in heterogeneous RNA and the 31S RNA.

On the 7.5% gels, newly synthesized 4S and 5S RNA can be detected at the earliest time (20min) and the amount of radioactivity incorporated into these two

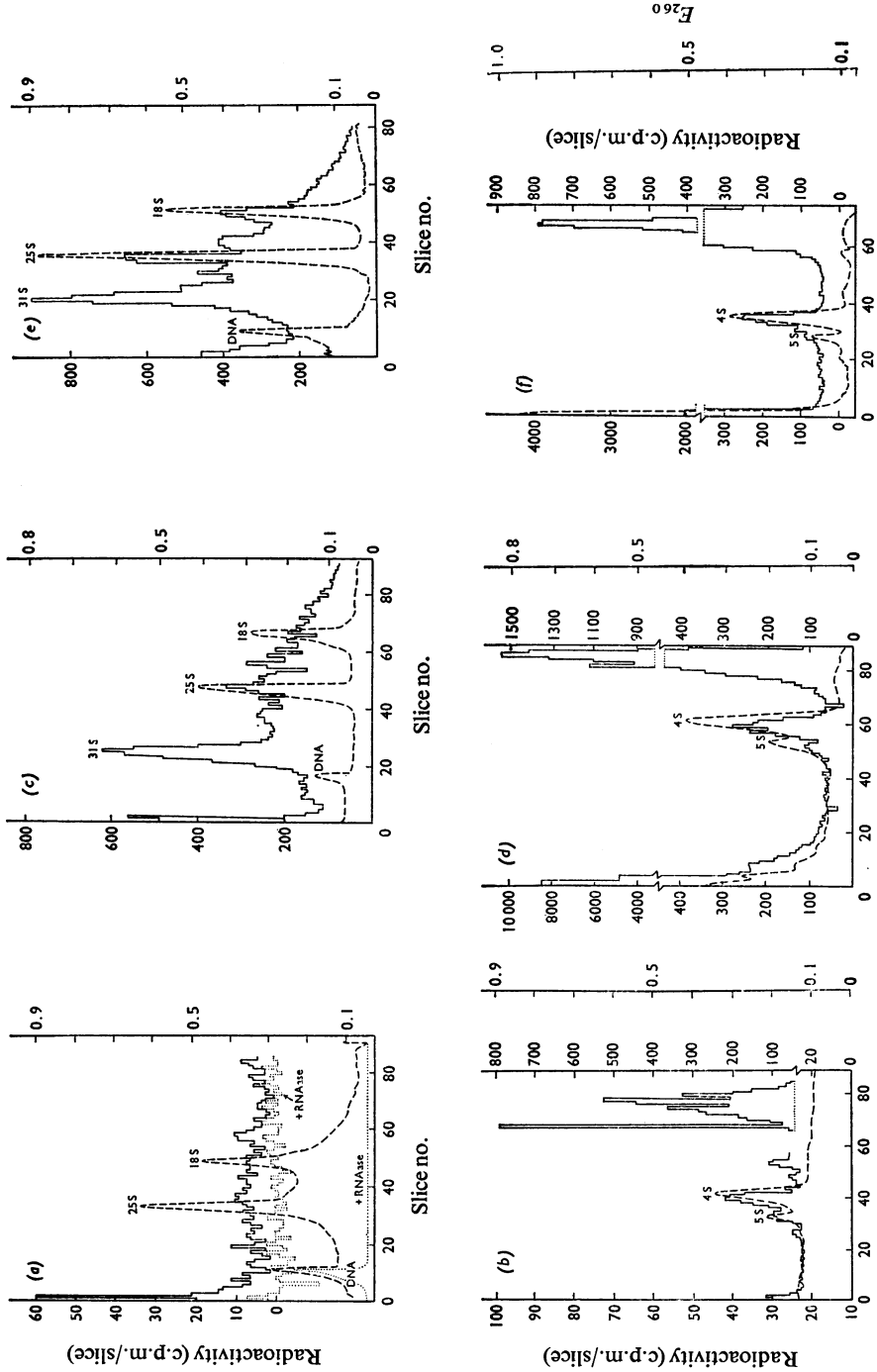
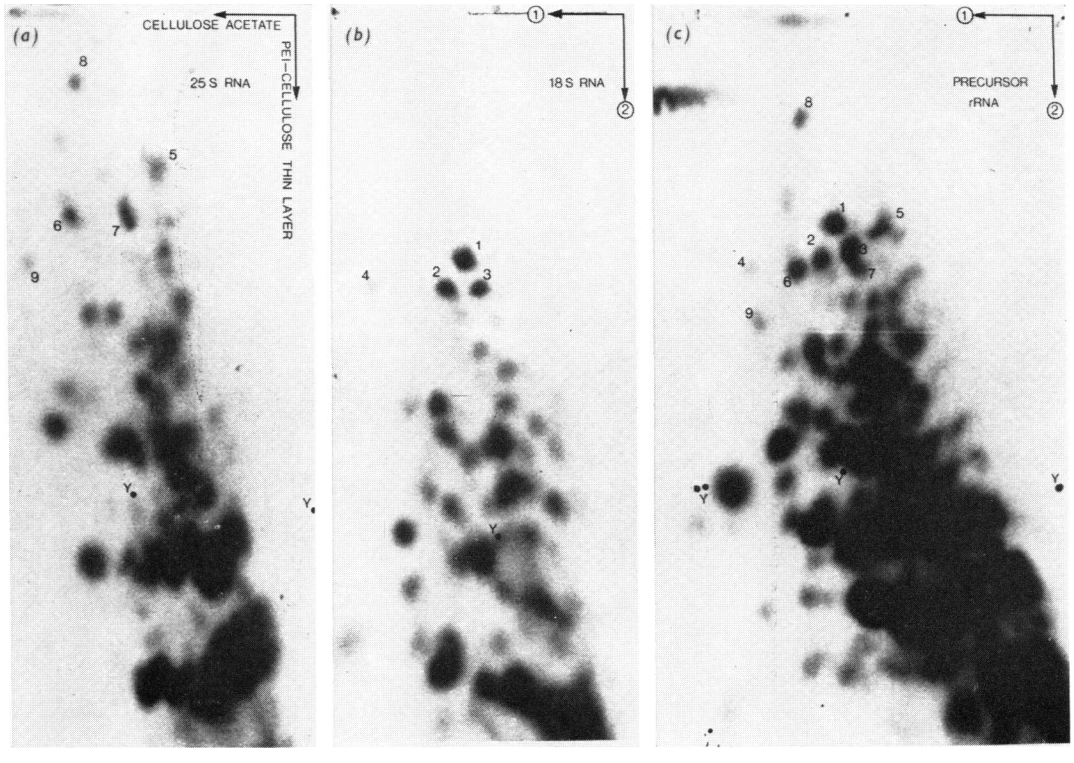


Fig. 2. *Electrophoretic fractionation of total nucleic acids on polyacrylamide gels (2.2% and 7.5%) after 20, 40 and 60 min of germination in the presence of germinating medium containing ^{14}C-labelled precursors*

The dashed line represents the E_{260} and the histogram represents the radioactivity as c.p.m./1 mm gel slice. For (*a*) and (*b*), the precursor is [^{14}C]adenine (700 μCi/ml); for (*c*), (*d*), (*e*) and (*f*) the precursor is [^{14}C]uridine (700 μCi/ml). (*a*), (*c*), (*e*), Electrophoretic fractionation on 2.2% gels of high-molecular-weight nucleic acid extracted after 20, 40 and 60 min of germination respectively. The dotted line in (*a*) indicates the E_{260} and the dotted histogram the radioactivity present in the RNA in a sample of the extract subjected to digestion with pancreatic ribonuclease A (1 mg/ml) before electrophoresis. Molecular weights of the RNA fractions relative to *Escherichia coli* rRNA are 31S = 2.3×10^6, 25S = 1.3×10^6 and 18S = 0.7×10^6. (*b*), (*d*), (*f*), Electrophoretic fractionation on 7.5% gels of low-molecular-weight nucleic acid extracted after 20, 40 and 60 min of germination respectively. ——, Radioactivity; – – – –, E_{260}.



EXPLANATION OF PLATE 1

'Fingerprint' patterns of (*a*) 25*S RNA*, (*b*) 18*S RNA and* (*c*) 31*S RNA after digestion with ribonuclease* T_1

Fractionation was by electrophoresis in cellulose acetate (direction 1) and chromatography on thin layers of PEI–cellulose (direction 2). Some of the larger oligonucleotides are numbered to ease comparison of the three 'fingerprints'. The locations of the marker Orange G in the second dimension are denoted by Y.

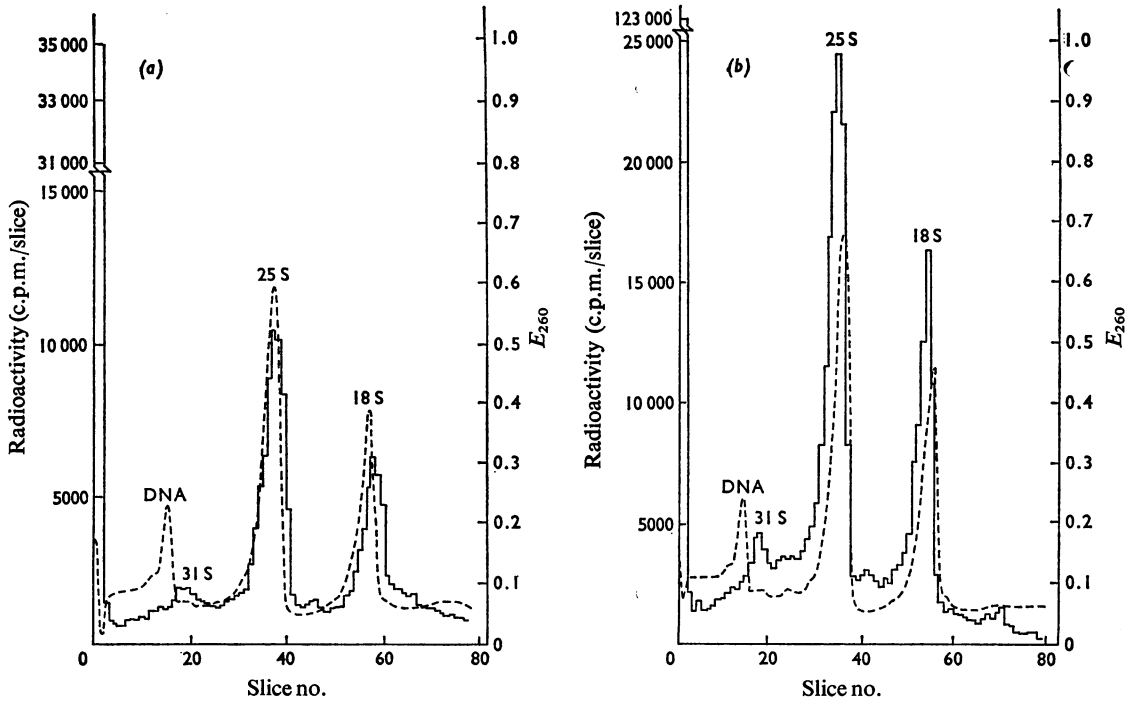


Fig. 3. *Electrophoretic fractionation on 2.2% polyacrylamide gels of high-molecular-weight nucleic acid extracted after (a) 3 h and (b) 6 h of germination in [^{14}C]uridine*

Molecular weights of the RNA species are as indicated in Fig. 2.

species increases during germination (Figs. 2*b*, 2*d* and 2*f*). Considerable radioactivity is present in an unidentified low-molecular-weight material at the base of these gels.

'Fingerprint' analyses of 31S, 25S and 18S RNA

'Fingerprint' patterns of oligonucleotides obtained after T_1-ribonuclease digestion of 31S, 25S and 18S RNA are shown in Plate 1. Electrophoresis in the first dimension and chromatography in the second were adjusted to give a clear separation of the larger oligonucleotides. In this way a characteristic, reproducible, 'fingerprint' pattern was obtained for each RNA species. The distribution of the oligonucleotides in the 25S and 18S RNA 'fingerprints' are clearly very different from each other (Plates 1*a* and 1*b*): the former, for instance (Plate 1*a*), has a greater number of large oligonucleotides rich in uridine residues (Griffin, 1971). The 31S RNA 'fingerprint' has approximately twice the number of large oligonucleotides as the other two (Plate 1*c*). Further, the distribution of spots is similar to the combined 'fingerprint' patterns of both 25S and 18S RNA (when the radioautographs are overlaid). A similar distribution of oligonucleotides to the 31S RNA has also been obtained by 'fingerprinting' digests of 25S and 18S RNA that had previously been mixed in equimolar amounts.

These results would be predicted if 31S RNA was a precursor of both 25S and 18S RNA.

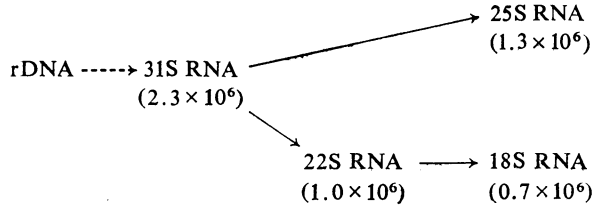
Discussion

The data presented in Fig. 1 demonstrate that protein synthesis occurs in rye embryos within the first hour of germination (RNA synthesis within 2 h) and the rate increases as germination proceeds. Electrophoretic separation of extracted RNA at 60 min (Figs. 2*e* and 2*f*) shows that all the major classes of RNA are then synthesized, including 31S, 25S, 18S, 5S and 4S. This pattern is not peculiar to rye and has been demonstrated also for wheat embryos at 1 h of germination (S. Sen, unpublished work). Separations of the RNA from rye at earlier times (20 and 40 min) indicate the probable sequence of activation of RNA transcription. At 20 min (Fig. 2*a*) much of the [^{14}C]adenine incorporated is present as heterogeneous ribonuclease-sensitive material

which probably corresponds to heterogeneous nuclear RNA (hnRNA) (Naora, 1973). Part of this is of high molecular weight and could be of the precursor-mRNA type. In addition, 4S and 5S RNA are clearly labelled at 20min (Fig. 2*b*), so this part of the genome is also activated very early in hydration. As far as 4S RNA is concerned, however, it seems unlikely that the synthesis of this component is a limiting factor for germination: our earlier work (Roberts *et al.*, 1973) has shown that even in non-viable embryos, where no RNA synthesis occurs, extracts containing tRNA can be charged and will sustain protein synthesis in a cell-free system as readily as will those from viable and germinating embryos. Although radioactive 5S RNA (Fig. 2*b*) can be detected at 20min, no other radioactive ribosomal components are then discernible in the scans, but this does not exclude their presence in small amounts. Certainly by 40min (Fig. 2*c*) the largest proportion of radioactivity is found in 31S RNA, with a small proportion clearly detectable in the 25S species. By 3–6h (Fig. 3), radioactivity is predominantly in 25S and 18S ribosomal RNA, with a relatively small (though still increasing) amount of other RNA components compared with earlier times.

contains all the large oligonucleotides present in both 25S and 18S RNA. This result clearly implies that the 31S RNA is a precursor molecule to the two mature rRNA species.

In common with others (Grierson & Loening, 1972; Cox & Turnock, 1973) we also detected a rapidly labelled component of mol.wt. 1.1×10^6, which in 'chase' experiments quickly loses its radioactivity. Presumably, as suggested by these other authors, it is an immediate precursor to 18S RNA. In contrast with Leaver & Key (1970), Grierson & Loening (1972), Cox & Turnock (1973) and Rosner *et al.* (1973), we could not detect a rapidly labelled component of mol.wt. 1.4×10^6, which is thought to be the immediate precursor to 25S RNA, although occasionally a fraction occurred with mol.-wt. 2.1×10^6. The 31S RNA would, however, be too small to accommodate both this molecule (as precursor for 25S RNA) as well as the 1.0×10^6-mol.wt. component precursor for 18S RNA. In rye, therefore, 25S RNA must have either a precursor with a molecular weight that is indistinguishable from 25S RNA by gel electrophoresis, or it may be absent altogether. A simple scheme for the synthesis of rRNA in rye embryos is hence:



The nature of the 31S RNA, which has an approximate molecular weight of 2.3×10^6, is of considerable interest. A similar RNA component has been detected in nucleoli-enriched preparations from germinating pea embryos (Tanifuji *et al.*, 1970) and in several other species of plants. It is thought to be a precursor molecule to both 25S and 18S RNA on the basis of (1) kinetics of radioactive labelling (Tanifuji *et al.*, 1970; Leaver & Key, 1970; Rogers *et al.*, 1970), (2) base composition (Rogers *et al.*, 1970), (3) incorporation of radioactive methyl label (Cox & Turnock, 1973) and (4) molecular hybridization by competition (Miassod *et al.*, 1973). Most of this evidence is circumstantial. In the present study, evidence that the 31S RNA of rye is the precursor to the 25S and 18S RNA has followed from the digestion of all three molecules with T_1 ribonuclease and the fractionation of the products by two-dimensional electrophoresis. The distribution of spots on the 'fingerprints' (Plate 1) indicates that the 31S RNA

Our results are of special interest in relation to the concept of long-lived messenger RNA in plants, for they show that in the rye embryo, the genome is activated very early in germination; in the coleorhiza this probably occurs as soon as the cells imbibe water (Osborne *et al.*, 1974). If hydration alone is the requirement for renewed transcription from the DNA template, then it may be that the nucleolus becomes hydrated somewhat later than the non-nuclear chromatin, for heterogeneous RNA, tRNA and 5S RNA can be detected earlier than high-molecular-weight ribosomal components. It is possible, however, that the relatively high proportion of radioactivity associated with the heterogeneous RNA could obscure the contribution of a 31S component in the gels of the 20min germination.

Characteristics of the heterogeneous RNA described for rye resemble those of hnRNA of animal cells, and the rye product may, as in animals, be the precursor for mRNA. Because the heterogeneous

RNA is the first RNA product to be detectable in rye, there is the possibility that before the start of protein synthesis some mRNA could be processed from the hnRNA, then transferred to the cytoplasm and there function as a template for some proteins synthesized in the early hours of germination. It is equally possible, of course, that this early heterogeneous RNA is degraded within the nucleus and has no function as a template (Naora, 1973). Whether or not the early synthesized RNA of rye functions as a messenger must await evidence from further biochemical studies. Meanwhile, the results presented here reveal a very early activation of the genome on hydration and indicate the possibility that the early protein synthesis initiated at germination could be coded for in part by newly synthesized mRNA.

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