Intranuclear Maturation Pathways of Rat Liver Ribosomal Ribonucleic Acids

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The maturation of pre-rRNA (precursor to rRNA) in liver nuclei is studied by agar/urea-gel electrophoresis, kinetics of labelling *in vivo* with [^{14}C]orotate and electron-microscopic observation of secondary structure of RNA molecules. (1) Processing starts from primary pre-rRNA molecules with average mol.wt. 4.6×10^6 (45 S) containing the segments of both 28 S and 18 S rRNA. These molecules form a heterogeneous peak on electrophoresis. The 28 S rRNA segment is homogeneous in its secondary structure. However, the large transcribed spacer segment (presumably at the 5'-end) is heterogeneous in size and secondary structure. A minor early labelled RNA component with mol.wt. about 5.8×10^6 is reproducibly found, but its role as a pre-rRNA species remains to be determined. (2) The following intermediate pre-rRNA species are identified: 3.25×10^6 mol.wt. (41 S), a precursor common to both mature rRNA species; 2.60×10^6 (36 S) and 2.15×10^6 (32 S) precursors to 28 S rRNA; 1.05×10^6 (21 S) precursor to 18 S rRNA. The pre-rRNA molecules in rat liver are identical in size and secondary structure with those observed in other mammalian cells. These results suggest that the endonuclease-cleavage sites along the pre-rRNA chain are identical in all mammalian cells. (3) Labelling kinetics and the simultaneous existence of both 36 S and 21 S pre-rRNA reveal that processing of primary pre-rRNA in adult rat liver occurs simultaneously by at least two major pathways: (i) $45\text{S} \rightarrow 41\text{S} \rightarrow 32\text{S} + 21\text{S} \rightarrow 28\text{S} + 18\text{S}$ rRNA and (ii) $45\text{S} \rightarrow 41\text{S} \rightarrow 36\text{S} + 18\text{S} \rightarrow 32\text{S} \rightarrow 28\text{S}$ rRNA. The two pathways differ by the temporal sequence of endonuclease attack along the 41 S pre-rRNA chain. A minor fraction (mol.wt. 2.9×10^6 , 39 S) is identified as most likely originating by a direct split of 28 S rRNA from 45 S pre-rRNA. These results show that in liver considerable flexibility exists in the order of cleavage of pre-rRNA molecules during processing.

The maturation pathways of pre-rRNA* in animal cells have been intensively investigated (see Loening, 1970; Maden, 1971; Perry, 1976). Several approaches have been used to establish the subsequent steps in pre-rRNA processing: (a) identification and molecular-weight determination of pre-rRNA species by gel electrophoresis; (b) determination of the pattern of methylated sequences in pre-rRNA; (c) competition-hybridization of rRNA and pre-rRNA; (d) electron-microscopic 'mapping' of secondary structure in pre-rRNA and rRNA; (e) labelling kinetics and actinomycin D chase of nucleolar rRNA species. As a result, several schemes for the maturation of pre-rRNA have been proposed, the best-documented ones being for growing mouse L cells (Wellauer *et al.*, 1974a) and

HeLa cells (Weinberg & Penman, 1970; Maden *et al.*, 1972; Wellauer & Dawid, 1973). Since differences in the maturation pathways in *Xenopus laevis*, mouse L cells and HeLa cells were found, it was suggested that they might be of evolutionary significance (Wellauer *et al.*, 1974a). In rat liver, a model of non-growing animal cells, two schemes for the maturation of pre-rRNA have been proposed, namely by Busch and co-workers (Quagliarotti *et al.*, 1970; Choi *et al.*, 1974):

* Abbreviations: pre-rRNA, precursor to rRNA; PPO, 2,5-diphenyloxazole; dimethyl-POPOP, 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene.

and by Fujisawa *et al.* (1973a,b):

In the above schemes, mol.wt. $\times 10^6$ and assigned *s* values are given. Considerable differences exist between the two schemes, probably owing to uncertainties in the identification and molecular-weight determination of pre-rRNA species and consequently in labelling kinetics studies.

In the present work we studied the pre-rRNA-maturation pathways in rat liver by determining the molecular weight and labelling kinetics of nucleolar rRNA species, as well as their secondary structure. Advantage was taken of a new agar/urea-gel electrophoresis technique (Dudov *et al.*, 1976), allowing a more accurate fractionation and molecular-weight determination of nuclear rRNA species and their kinetics of labelling. The results obtained show that, in general, the pre-rRNA species and their processing in rat liver are similar to those reported for other mammalian cells. Further, the evidence revealed that at least two pathways of pre-rRNA processing occur simultaneously in adult rat liver, differing by the temporal sequence of endonucleolytic splits along pre-rRNA molecules. Part of these results have been reported (Dabeva *et al.*, 1975).

Experimental

Animals

The experiments were carried out with male albino rats weighing about 150 g. The labelling of RNA *in vivo* was with 25 μ Ci of [14 C]orotate (sp. radioactivity 25 mCi/mmol) per animal, given intraperitoneally at different times before death. The animals were killed by cervical dislocation, and the livers dissected out, rinsed in cold 0.9% NaCl and immediately processed further.

Isolation of nuclei

This was done as described by Chauveau *et al.* (1956), the method being modified as follows. The pooled livers were homogenized (10 g/20 ml of medium) in 2.3 M-sucrose containing 0.01 M-Tris/HCl (pH 7.8), 0.01 M-MgCl₂ and 0.1 mg of dextran sulphate/ml, with five strokes of a glass/Teflon Potter-Elvehjem homogenizer. The homogenate was filtered through gauze cloth, and the volume adjusted to 30 ml with the same sucrose solution. The homo-

genate was layered over 8 ml of 2.3 M-sucrose (as above) and centrifuged for 30 min at 25000 rev./min in the SW 27 rotor of a Beckman L2-65B ultracentrifuge at 0°C. The supernatant was aspirated, the tube walls were cleaned and the nuclear pellet was rinsed with cold 0.25 M-sucrose in the same medium. All procedures were carried out in the cold with pre-cooled solutions.

Fractional extraction of nuclear RNA

This was done by consecutive extraction with phenol at different temperatures by a modification of the techniques previously used in this laboratory (Hadjiolov *et al.*, 1974; Dabeva *et al.*, 1976). The nuclear pellet from 10 g of liver was suspended in 40 ml of a 1:1 (v/v) mixture of 0.1 M-Tris/acetate buffer (pH 5.5)/1 mM-EDTA plus phenol, saturated with the same buffer and containing 0.1% 8-hydroxyquinoline. The suspension was homogenized with five strokes of a loose Potter-Elvehjem homogenizer. The mixture was shaken for 20 min at 4°C and centrifuged for 20 min at 3000 g. The water layer, containing nuclear-sap RNA, was removed, and the interphase layer was suspended in 40 ml of the same mixture and shaken for 15 min at 50°C. The mixture was chilled on ice and centrifuged for 20 min at 3000 g in the cold. The water layer was collected and sodium dodecyl sulphate added to 0.5%, followed by an equal volume of phenol. The mixture was shaken at 4°C for 10 min and centrifuged as above. The water layer was aspirated, mixed with a 1:1 (v/v) mixture of phenol/chloroform, shaken for 10 min and centrifuged as above. A third deproteinization of the water layer was done with an equal volume of chloroform, and RNA from the final water layer was precipitated at -10°C overnight with 2 vol. of 95% (v/v) ethanol containing 1% sodium acetate. The sediment was collected by centrifugation, dissolved in a small volume (1-2 ml) of 0.01 M-NaCl/0.01 M-EDTA and reprecipitated as above. The RNA fraction obtained represents mainly nucleolar RNA (Dabeva *et al.*, 1976) and is designated further as 50°C nu-RNA.

Fractionation of RNA by agar/urea-gel electrophoresis

This was done as described by Dudov *et al.* (1976). The E_{260} of the dried electrophoretograms was

recorded as described by Hadjiolov *et al.* (1974). The labelled RNA components were detected by radioautography or by direct counting of radioactivity of slices from the dried agar film. In the latter procedure, the agar film was cut in 1 mm slices, put into counting vials and incubated for 2 h at 60°C in toluene/water/Protosol (10:1:9, by vol.). The samples were cooled, 4.5 ml of toluene/PPO/dimethyl-POPOP phosphor (5 g of PPO and 0.25 g of dimethyl-POPOP in 1 litre of toluene) was added, and the mixtures were counted for radioactivity in a Packard Tri-Carb model 3320 liquid-scintillation spectrometer. The specific radioactivity of each electrophoretic fraction was calculated by dividing the total radioactivity by the area under the E_{260} peak.

Electron microscopy of RNA molecules

The RNA components of the 50°C nu-RNA were fractionated by preparative-scale agar/urea-gel electrophoresis. The gel bands (containing the separate RNA species) were cut out, and each fraction was homogenized in 0.1 M-Tris/acetate buffer (pH 5.5) containing 0.5% sodium dodecyl sulphate and 0.01 M-EDTA. The suspension was mixed with an equal volume of phenol, saturated with 0.1 M-Tris/acetate buffer (pH 5.5), containing 1 mM-EDTA and 0.1% 8-hydroxyquinoline. The mixture was shaken vigorously and centrifuged for 30 min at 3000g in the cold. The water phase was deproteinized with an equal volume of phenol/chloroform and then twice with chloroform. The RNA from the final water layer was precipitated with 2.5 vol. of 95% ethanol at -10°C overnight. The RNA precipitate was collected, dissolved in 5 ml of extraction buffer (see above) and deproteinized with chloroform. The purity of the fraction and the integrity of the separate RNA components were checked by agar/urea-gel electrophoresis. The separate RNA samples were prepared for electron microscopy after partial denaturation in formamide/urea as described by Wellauer & Dawid (1974). The samples were spread and adsorbed on carbon-coated Formvar films, stained with uranyl acetate and shadowed with WO_3 instead of Pt/Pd (I. Emanuilov, unpublished work). The observations were carried out with a Hitachi HU 11A electron microscope at 75 kV.

Materials

Analytical-grade reagents were used throughout. PPO, dimethyl-POPOP and agar no. 1 (neutralized) were from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Protosol was from New England Nuclear Corp., Boston, MA, U.S.A.; dextran sulphate (mol.wt. 1×10^5) was from Pharmacia, Uppsala, Sweden, and $[6-^{14}C]$ orotate (sp. radioactivity 25 mCi/mmol) was from NAEI Institute for Isotopes, Budapest, Hungary.

Results

(a) Agar/urea-gel electrophoresis: identification and molecular weights of pre-rRNA and rRNA species

Phase-contrast and electron-microscopic survey of the nuclear fraction showed that well-preserved clean nuclei are obtained. Also, undegraded rRNA and

Fig. 1. Agar/urea-gel-electrophoresis fractionation of rat liver 50°C nu-RNA

The RNA was labelled *in vivo* for 120 min with 25 μ Ci of $[^{14}C]$ orotate per animal. The nuclear 50°C nuRNA fraction was isolated and analysed as described in the Experimental section. ----, E_{260} ; —, radioactivity recorded at 550 nm from the blackening of the radioautogram. The arrows indicate the separate RNA components as listed in Table 1.

pre-rRNA species can be extracted reproducibly from the nuclear fraction isolated with 2.3M-sucrose in low-ionic-strength buffer containing 0.01 M-Mg²⁺ and dextran sulphate. This is shown by the observation that, on agar/urea-gel electrophoresis (i.e. under conditions of partial denaturation), the 50°C nu-RNA fraction is resolved into several distinct RNA components with a very low background radioactivity (Plate 1a).

The E_{260} and radioactivity profiles of rat liver 50°C nu-RNA show that all nucleolar RNA fractions form neatly delineated peaks (Fig. 1). The main 28S and 18S rRNA peaks are prominent and symmetrically shaped, thus indicating the absence of even incipient degradation (Venkov & Hadjiolov, 1969). Six RNA components (A-F) with mobilities lower than that of 28S rRNA are reproducibly observed. The most prominent fraction is F (32S), but fractions C (41S) and E (36S), described in HeLa cells as 'minor' nucleolar RNA fractions (Weinberg & Penman, 1970), are also clearly seen. Fraction B (45S) is characterized by its early labelling and appears to comprise at least three components, a finding which extends to rat liver the reported heterogeneity of 45S pre-rRNA in L 5178Y mouse cells (Tiollais *et al.*, 1971) and 40S pre-rRNA in *Xenopus laevis* (Loening

et al., 1972). In addition, we consistently observed small but reproducible peaks constituting the components designated as A ('55S') and D ('39S'). Several components (a-f) could be identified also in the zone between 28S and 18S rRNA. Among these, fraction c (21S) is characterized by its prominent radioactivity peak.

The molecular weights of the separate rat liver 50°C nu-RNA components determined from their electrophoretic mobilities in agar/urea gels are listed in Table 1. Owing to differences in the fractionation and analysis techniques used, a direct comparison with the results on rat liver pre-rRNA species reported previously (Fujisawa *et al.*, 1973a; Choi *et al.*, 1974) is not possible. However, taking the molecular-weight estimates as reference, a tentative correlation between the results obtained in different laboratories could be established. It is noteworthy that in several cases, i.e. fractions C (41S), F (32S) and c (21S), the identity of the separate pre-rRNA components can be established unambiguously on a molecular-weight basis, whereas assigned *s* values may be misleading. The *s* values assigned in the present work to liver pre-rRNA are also arbitrary and correspond to the most commonly used *s* values to designate pre-rRNA components of identical molecular weight (see Table

Table 1. *Molecular weights of rat liver pre-rRNA and rRNA species*

Our results were determined by agar/urea-gel electrophoresis. The values are compared with those reported by others for rat liver or other mammalian cells. The designation of the separate rRNA species is as given in Fig. 1. The *s* values assigned by us are given in parentheses. They correspond to the *s* values most commonly used to designate pre-rRNA or rRNA species with a molecular weight similar to or identical with that determined in this work. The *s* values assigned to our fractions A and D are arbitrary. Those assigned by other authors are given when different from ours. For HeLa and L cells the molecular weight was determined by electron-microscopic length measurements. References: (a) Fujisawa *et al.* (1973a); (b) Choi *et al.* (1974); (c) Wellauer & Dawid (1973); (d) Wellauer *et al.* (1974a).

RNA species	No. of independent expts.	10 ⁶ × mol.wt.			HeLa cells (ref. c)	Mouse L cells (ref. d)
		Rat liver				
		Present work (mol.wt. ± s.d.)	Ref. (a)	Ref. (b)		
A ('55S')	14	5.85 ± 0.31	—	—	—	—
B (45S)*	16	4.61 ± 0.17	4.34	4.24	4.71	4.66
C (41S)	16	3.25 ± 0.11	3.16 (37S)	—	3.30	3.30
D ('39S')	15	2.96 ± 0.13	—	—	—	—
E (36S)	16	2.60 ± 0.09	2.88	2.87 (35S)	—	2.66
F (32S)	16	2.15 ± 0.07	2.11	2.07 (28S)	2.27	2.19
28S	16	1.74 ± 0.02	1.65	1.7	1.76	1.74
a (24S)	16	1.39 ± 0.04	—	—	1.33	—
c (21S)	16	1.05 ± 0.05	0.94	1.37 (23S)	0.99	—
f	15	0.83 ± 0.04	—	—	—	—
18S	16	0.68 ± 0.03	0.65	0.68	0.68	0.68

* The 45S pre-rRNA peak is heterogeneous. The value given corresponds to the components situated in the middle of the peak.

EXPLANATION OF PLATE I

(a) *Radioautogram of liver 50°C nuRNA fractionated by agar/urea-gel electrophoresis*

Labelling *in vivo* was with [^{14}C]orotate for 90 min. The designation of pre-rRNA fractions is as given in Fig. 1 and Table 1. For details, see the text.

(b)–(e) *Electron micrographs of rat liver 45 S pre-rRNA (fraction B) spread after partial denaturation with formamide/urea*
The bar represents 0.25 μm . Each molecule is oriented with its 28 S rRNA segment to the left.

EXPLANATION OF PLATE 2

Electron micrographs of rat liver pre-rRNA and rRNA spread after partial denaturation with formamide/urea

(a) 41S pre-rRNA; (b) two 36S pre-rRNA molecules; (c) 32S pre-rRNA; (d) '39S' RNA; (e) 28S rRNA. The designations are as given in Table 1. The bar represents 0.25 μ m.

1). Further, the molecular-weight estimates in the present work for the main pre-rRNA components (B, C, E, F and *c*) coincide very well with the values reported for HeLa and L cells (Wellauer & Dawid, 1973; Wellauer *et al.*, 1974a). Thus it may be concluded safely that the main pre-rRNA and rRNA species found in mammalian cells are identical in size.

(b) Electron-microscopic characterization of secondary structure of pre-rRNA and rRNA molecules

When spread from formamide/urea under partly denaturing conditions, animal cell pre-rRNA and rRNA molecules display characteristic and reproducible secondary-structure patterns (see Wellauer & Dawid, 1975). The main feature is the presence of rather long hairpin loops showing a typical form and size and located at the same sites along the polynucleotide chain. Most likely, these loops correspond to the high-molecular-weight G+C-rich segments (up to 78% G+C content) isolated from partial nuclease digests of animal cell rRNA (see Hadjiolov *et al.*, 1975). The presence of these typical loops at identical sites in both pre-rRNA and rRNA molecules gives an independent approach in defining pre-rRNA-processing pathways in animal cells. The electron micrographs of rat liver pre-rRNA and rRNA species are presented in Plate 1(b)–1(e) and Plate 2(a)–2(e). The characteristic secondary-structure pattern of mature 28S rRNA (Plate 2e) is found in all larger pre-rRNA molecules and allows their unambiguous identification. The marker double-stranded loops at the 28S rRNA side of pre-rRNA molecules include: one terminal single loop, one double loop located in the middle of the 28S rRNA segment and one triple loop near the internal side of the 28S rRNA segment. On the basis of these features we can identify our fractions B (45S), C (41S), E (36S) and F (32S) as containing a single 28S rRNA segment and therefore possessing the structural requirements to be precursors to 28S rRNA. These four pre-rRNA molecules are further identified by the presence of the two typical neighbour single loops (close to the 28S rRNA segment) as well as by their size.

It should be stressed that the 28S rRNA segment of the molecules in our fraction B (45S) is remarkably homogeneous in its secondary-structure pattern, starting always with a typical double-stranded loop. On the other hand, considerable heterogeneity in size and secondary structure is observed at the opposite end of 45S pre-rRNA molecules (see Plate 1b–1e). This observation shows that the terminal large transcribed spacer segments in 45S pre-rRNA are heterogeneous, a finding which correlates with the heterogeneity of this group of molecules revealed by agar/urea-gel electrophoresis.

The bulk of the molecules observed in fraction E (36S) contain the complete 28S rRNA sequence and therefore they could not be spuriously derived by

endonuclease split from the other end of 45S pre-rRNA, as reported for HeLa cells (Wellauer & Dawid, 1973). However, we have observed also the occasional presence of molecules lacking the 28S rRNA segment (Plate 2d). By their size they correspond to the minor electrophoretic fraction D (39S), but, owing to the minute amount of this fraction, an unambiguous distinction between the molecules in our fractions D and E could not be made.

Identification of the 18S rRNA segment in pre-rRNA molecules is less clear-cut, since it is devoid of characteristic secondary structure. Also, the small single loops in fraction *c* (21S) are not observed in all molecules present in this fraction. However, the 21S RNA molecules are identical in size and give an average 21S/18S RNA ratio of 1.66, a value reasonably close to the ratio of 1.54 expected on the basis of molecular-weight estimates. The presence of a long stretch devoid of secondary structure at one of the ends of the 41S pre-rRNA molecule is in accordance with the possibility that the 18S rRNA sequence is included, though obviously missing at the stage of 36S and 32S pre-rRNA.

Summarizing, it may be stated that the secondary-structure pattern of rat liver pre-rRNA and rRNA molecules is remarkably close to the pattern reported for mouse L cells (Wellauer *et al.*, 1974a). Further and more detailed studies are needed to ascertain their complete identity. Although the size and secondary-structure pattern of 28S rRNA and the respective segment in pre-rRNA are remarkably reproducible, the large transcribed spacer segment in 45S primary pre-rRNA molecules displays considerable heterogeneity.

(c) Labelling kinetics of rat liver pre-rRNA and rRNA

To obtain information about pre-rRNA-processing pathways in rat liver, we carried out experiments where labelling with [¹⁴C]orotate *in vivo* was used for time-periods ranging from 20 to 200 min. The changes with time of the labelling patterns of the 50°C rRNA fraction are presented in Fig. 2. These results show that the label appears initially in 45S (and '55S') pre-rRNA. Reproducible differences in the labelling kinetics of the 45S and '55S' RNA components could not be established. Further, the radioactivity enters the presumably intermediate pre-rRNA fractions C (41S), D ('39S'), E (36S) and F (32S) to appear at the end in nucleolar 28S and 18S rRNA. The labelling of the fractions located in the zone between 28S and 18S rRNA remains relatively low at all labelling periods studied, their specific radioactivity being comparable with that of 28S rRNA. In comparison, the labelling of fractions *a* ('24S') and *c* (21S) predominates. Fraction *a* ('24S') is neatly delineated as a separate highly labelled peak only in the initial periods of [¹⁴C]orotate labelling. At later stages it is obscured by the increasing

Fig. 2. Agar/urea-gel electrophoresis of rat liver 50°C nuRNA labelled *in vivo* with [^{14}C]orotate for 20 min (a), 35 min (b), 50 min (c), 70 min (d), 90 min (e), 120 min (f), 160 min (g) and 200 min (h)

The experimental conditions and the designation of the separate RNA components (arrows) are as in Fig. 1 and Table 1. —, Radioactivity recorded at 550 nm from the blackening of the radioautogram. The amount of E_{260} units of fractionated RNA and the exposure time for all electrophoretograms are the same. For details, see the text.

labelling of neighbouring fractions, thus making uncertain the determination of its labelling kinetics. On the other hand, the labelling of fraction c (21 S) constitutes a well-shaped peak at all times of labelling investigated. It should be added that interference by

highly labelled non-ribosomal RNA species is highly unlikely, since we have shown (Dabeva *et al.*, 1976) that only 1–2% of the labelling of the 50°C nu-RNA fraction may be due to poly(A)-containing RNA.

The good resolving power of agar/urea-gel electro-

phoresis and the virtual absence of cross-contamination for most pre-rRNA fractions, as well as the possibility of recording both their E_{260} and ^{14}C -labelling, made possible the accurate determination of the kinetics of labelling of these RNA fractions. The time-courses of specific-radioactivity changes for the components of the 50°C nu-RNA fraction are given in Fig. 3. Analysis of the labelling kinetics specifies further the conclusions derived from observations of the gel-electrophoresis patterns. A precursor-product relationship is established for the conversion $45\text{S} \rightarrow 41\text{S}$ pre-rRNA. The labelling kinetics data are also compatible with the subsequent conversions: $41\text{S} \rightarrow 32\text{S} \rightarrow 28\text{S}$ rRNA and $41\text{S} \rightarrow 21\text{S} \rightarrow 18\text{S}$ rRNA. It should be noted that over short labelling periods the specific radioactivities of 32S and 21S pre-rRNA are of the same order, as expected from the postulated conversion $41\text{S} \rightarrow 32\text{S} + 21\text{S}$ pre-rRNA. However, over longer periods of labelling, the specific radioactivity of 21S pre-rRNA is appreciably lower than that of 32S pre-rRNA. This difference is most likely explained by the contamination of the RNA component *c* (21S) by the markedly less labelled neighbour component *d* (see Fig. 1). In

such a case, the specific radioactivity of 21S pre-rRNA (possibly higher than that of 32S pre-rRNA) would be lowered by the contaminating component *d*. The positions of the RNA components D ('39S') and E (36S) in the pattern of sequential labelling deserve further comment. It is noteworthy that the conversion $41\text{S} \rightarrow 36\text{S}$ pre-rRNA displays a very rapid attainment of isotopic steady state, which may reflect the direct supply of the 36S pre-rRNA pool from 45S pre-rRNA. The kinetic data are also compatible with the possibility of a direct conversion $45\text{S} \rightarrow \text{'39S'}$ RNA. Further, the specific radioactivity of 28S rRNA is consistently higher than that of 18S rRNA. This difference is due to the fact that the 18S rRNA in the 50°C nu-RNA fraction is contaminated by 18S rRNA from the nuclear sap characterized by a markedly lower labelling (Dabeva *et al.*, 1976).

Generalizing, it should be stressed that most specific-radioactivity curves reach saturation at about 120–160 min, and for several couples of RNA molecules a typical precursor-product relationship cannot be derived. The rapid attainment of isotopic steady state suggests that the processing of pre-rRNA in adult rat liver may not constitute a pure catenary system, i.e. some of the product RNA may have two or more precursors with different specific radioactivities. Therefore, at the present stage, only groups of sequentially labelled pre-rRNA molecules can be safely delimited, thus specifying the conversions: $45\text{S} \rightarrow 41\text{S}$, '39S', $36\text{S} \rightarrow 32\text{S}$, 21S pre-rRNA $\rightarrow$ 28S, 18S rRNA.

Discussion

The results obtained in the present work add information to the existing knowledge about pre-rRNA-processing pathways in animal cells and their possible regulation. The following aspects deserve further comment.

(a) Similarity of pre-rRNA molecules in mammalian cells

Our results reveal that the main pre-rRNA species in rat liver are remarkably similar in molecular weight and secondary structure to the respective molecules in HeLa (Wellauer & Dawid, 1973) and mouse L (Wellauer *et al.*, 1974a) cells. This observation suggests that the structural pattern of pre-rRNA molecules is preserved in all animal species and that previously reported differences reflect technical imperfections rather than the existence of basic dissimilarities among cell types and species. It may be inferred further that the sites of endonuclease attack along the primary pre-rRNA polynucleotide chain are similar or identical for all animal species. This conclusion is supported by the established identity of terminal nucleotides in mature animal 28S and 18S rRNA (Winicov & Perry, 1975). Consequently, processing

Fig. 3. Kinetics of labelling of pre-rRNA and rRNA components in rat liver nuclear 50°C nu-RNA

The experimental animals (weighing $150 \pm 5\text{g}$) were each injected intraperitoneally with $25\text{ }\mu\text{Ci}$ of [^{14}C]orotate (sp. radioactivity 18.2mCi/mmol). The rats were killed at different time-intervals (in groups of five animals), and 50°C nu-RNA was isolated and fractionated by agar/urea-gel electrophoresis as described in the Experimental section. The amount of each RNA component was determined as the surface area of the respective peak recorded at 260nm (see Fig. 1). The radioactivity of each peak was determined by liquid-scintillation spectrometry of the dried agar gel cut in 1mm slices (see the Experimental section). Δ , Fraction B (45S); $\bullet$, fraction C (41S); $\circ$, fraction D ('39S'); $\square$, fraction E (36S); $\blacksquare$, fraction *c* (21S); ∇ , fraction *a* (24S); $\blacktriangledown$, 28S rRNA; $\diamond$, 18S rRNA. The designation of the separate RNA components is as given in Table 1.

of primary pre-rRNA may be conceived as a relatively simple process specified by four major endonucleolytic attacks along the primary pre-rRNA chain (see Perry, 1976). The role of primary and/or secondary structure of pre-rRNA and endoribonuclease specificity in determining these four cleavage sites remains to be clarified.

(b) Heterogeneity of primary pre-rRNA molecules

The heterogeneity of primary pre-rRNA molecules on gel electrophoresis was observed initially in plants (Grierson *et al.*, 1970; Loening, 1970), and these observations were extended to primary pre-rRNA of *Xenopus laevis* (Loening *et al.*, 1972) and a mouse cell line (Tiollais *et al.*, 1971). In our present studies rat liver 45S pre-rRNA constituted invariably a rather heterogeneous peak, unlike the peaks of all other pre-rRNA species. Since partly denaturing conditions are used in the fractionation of nucleolar RNA by gel electrophoresis, the reproducible split of the 45S pre-rRNA into two or three components could hardly be explained solely by conformation differences among the molecules constituting this peak. This interpretation is supported further by the observed variations in size and secondary structure of transcribed spacer segments in the molecules from the 45S pre-rRNA peak (see Plate 1b–1e). The difference among 45S pre-rRNA components probably extends to primary structure, as demonstrated by the distinct 'fingerprints' obtained from the '45S' and '47S' constituents of the primary pre-rRNA from a mouse cell line (Galibert *et al.*, 1975). Our electron-microscopic observations indicate that the heterogeneity among 45S pre-rRNA molecules reflects differences in the large transcribed spacer segment, the remaining parts of the molecule being remarkably constant in their secondary-structure pattern. Thus it is likely that the established heterogeneity in the structure of non-transcribed spacers (Wellauer *et al.*, 1974b) is extended to the large transcribed spacer segment of the rRNA transcriptional unit in some eukaryotes. This conclusion is also supported by electron microscopy of active genes from amphibian oocytes and other eukaryotic cells, where noticeable variations in the size of rRNA transcriptional units were found (Scheer *et al.*, 1973; Spring *et al.*, 1974). The possible location of transcribed spacer segments at the 5'-end of 45S pre-rRNA (see Perry, 1976) leaves open the alternative possibility that heterogeneity of primary pre-rRNA molecules may be due to limited and unequal processing occurring simultaneously with transcription (see also Slack & Loening, 1974).

(c) Multiplicity of pre-rRNA-processing pathways

Maturation of primary pre-rRNA along a single processing pathway depends on the strict temporal sequence of endonuclease attack at the four cleavage sites along the polynucleotide chain. In general,

nuclease attack follows a rather rigid sequence in order that in growing cells a single processing pathway prevails (see Perry, 1976). However, in liver cells, as a model of resting cells operating under steady-state conditions, this does not seem to be true. The simultaneous occurrence of 36S, 32S and 21S pre-rRNA, established in the present work, cannot be accommodated within a single maturation pathway. The presence of 36S pre-rRNA (constituting about 50% of the u.v.-absorbing material of 32S pre-rRNA) is unambiguously proved by electron microscopy of its secondary structure and by labelling kinetics data. On the other hand, evidence for identification of 21S pre-rRNA as an intermediate pre-rRNA species is: (i) kinetics of labelling (the present work and Fujisawa *et al.*, 1973a); (ii) labelling with [*Me*-³H]-methionine (Muramatsu & Fujisawa, 1968); (iii) competition-hybridization (Egawa *et al.*, 1971). Consequently, maturation of pre-rRNA in adult rat liver proceeds simultaneously by at least two pathways resulting from the reversal of the sequence of endoribonuclease attack at cleavage sites 2 and 3 (Fig. 4).

It is not yet clear whether the generation of intermediate pre-rRNA species could not proceed by an even greater flexibility in the temporal sequence of endoribonuclease attack. In particular, identification of the minor '39S' pre-rRNA fraction suggests a direct cleavage of 45S pre-rRNA at site 4. Further, generation of 36S pre-rRNA by a direct endonucleolytic attack of 45S pre-rRNA at cleavage site 3 should be also considered an open possibility compatible with our labelling kinetics studies.

The generation of 'minor' rRNA fractions by alteration of the order of cleavage steps was first proposed by Weinberg & Penman (1970), but suggestive information on the possible physiological role of this flexibility in the sequence of endoribonuclease attack was published only recently. Thus it was found that in resting lymphocytes both 36S and 32S pre-rRNA are formed, and phytohaemagglutinin stimulation caused a fourfold increase in the rate of processing through 32S pre-rRNA only (Purtell & Anthony, 1975). Also, the ts 422E mutant of BHK cells, altered in the conversion of 32S pre-rRNA into 28S rRNA (Toniolo *et al.*, 1973; Toniolo & Basilico, 1976), produces rRNA via 32S pre-rRNA at the permissive temperature, whereas processing through 36S pre-rRNA is observed at the non-permissive temperature (Winicov, 1976; Toniolo & Basilico, 1976).

Fig. 4. Scheme of liver 45S pre-rRNA showing the order of endoribonuclease cleavage during processing

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