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3'-Terminal processing of ribosomal RNA precursors in mammalian cells

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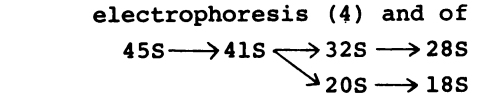
ABSTRACT

The 3'-terminal structures of ribosomal 28S RNA and its precursors from rat and mouse were analyzed by means of periodate oxidation followed by reduction with ^{3}H-borohydride. 3'-terminal labeled nucleoside derivatives produced by RNase T_2 digestion were determined by thin-layer chromatography and oligonucleotides generated by RNase T_1 digestion were analyzed by DEAE-Sephadex chromatography. In the rat, the major 3'-terminal sequences of ribosomal 28S RNA, nucleolar 28S, 32S, 41S, and 45S RNAs were YGU_{oh}, GZ_2U_{oh}, $GZ_{12}U_{oh}$, GZ_2U_{oh}, and GZ_7G_{oh}, respectively, whereas in the mouse corresponding sequences were YGU_{oh}, $GZ_{1,2}$, or $_3U_{oh}$, G_{oh}, U_{oh} and $GZ_{13}U_{oh}$, respectively. (Y: pyrimidine nucleoside, Z: any nucleoside other than guanosine) These results suggest that a "transcribed spacer" sequence is present at the 3'-terminus of the 45S pre-ribosomal RNA, which is gradually removed during the steps of processing.

INTRODUCTION

In animal cells, ribosomal RNA (rRNA) is synthesized as a large common precursor molecule, which is processed in a sequential set of nucleolytic cleavage into mature 28S, 18S, and 5.8S RNA. The 45S RNA, the presumptive primary transcript of rRNA gene in mammals, is thought to have the structure of (5') spacer-18S-spacer-5.8S-spacer-28S(3') (1,2), these "transcribed spacer" sequences being removed stepwise at each maturation step (3). The following general scheme has been established according to the data of kinetic studies with gel electrophoresis (4) and of electron microscopy (1):



Most previous studies of rRNA processing, however, have concerned with the major cleavages of the precursors but not

with detailed steps which might involve the trimming of relatively short sequences.

Previously, we demonstrated that 45S RNA molecules of a mouse hepatoma (MH134) which had been isolated on sucrose gradient had four kinds of pNp at the 5'-terminus, suggesting that this RNA might be subjected to a 5'-terminal processing before it was converted into 41S intermediate (5). Evidence was also presented that even 28S RNA may undergo a 5'-terminal processing in the cytoplasm (6). Although electron microscopic observations placed the 28S RNA sequence at the extreme 3'-end of the 45S RNA (1), trimming of 100-200 nucleotides may not have been detected owing to the limitations inherent to the method. We therefore compared, in this study, the 3'-terminal structure of rRNA precursors with that of mature 28S RNA.

The results indicate that each rRNA precursor has a different 3'-terminus suggesting that a processing is indeed under way at the 3'-terminal sequences of these precursors.

MATERIALS AND METHODS

1) Cells and preparation of RNA

The MH134/C cells were maintained in mice of C3H/He strain. About ten days after intraperitoneal transplantation, the ascites cells were harvested and rRNA was extracted from ribosome fraction by sodium dodecylsulfate-phenol precedure (6). Nucleolar RNA (NoRNA) was extracted from purified nucleoli prepared by a sonication procedure described previously (7), by a modification of the hot sodium dodecylsulfate-phenol method (8, 9). Both RNAs were further fractionated by sucrose density gradient centrifugation, and the peaks of 45S, 41S, 32S, 28S, and 18S RNAs were separately pooled. Ribosomal 28S, nucleolar 28S and 32S RNA were further purified by heat-quenching followed by re-centrifugation (10).

2) *In Vitro* labeling of 3'-terminal groups

The labeling procedure was essentially the same as that described by RajBhandary (11).
500 µg of mixed rRNA (18S + 28S RNA) or 250 µg of an equimolar

mixture of NoRNAs (45S, 41S, 32S, and nucleolar 28S RNA), were dissolved in 0.6 ml of 0.1 M sodium acetate (pH 5.1). 50 µl of 0.1 M sodium meta-periodate was added to the RNA solution, and the mixture was kept at 0°C in the dark. After 60 min, 25 µl of 10% (vol./vol.) ethylene glycol was added and the mixture stood for 10 min to destroy excess periodate. The RNA was then precipitated and redissolved in 0.3 ml of 0.4 M potassium phosphate (pH 7.0) containing 5 µmoles of NaB^3H_4 (330 mCi/m mole, NEN) or 5 µmoles of NaB^3H_4 (10 Ci/m mole, NEN) to label rRNA or NoRNA, respectively. After a 4 h period in the dark at 0°C, 50 µl of 1 M acetic acid was added. The mixture was filtered through a column of Sephadex G-50 equilibrated with 0.01 M Tris-HCl, 0.3 M NaCl, 1 mM ethylene diamine tetraacetic acid, 7 M urea (pH 7.6), and the ultraviolet-absorbing material in void volume was recovered by precipitation with cold ethanol. 3'-terminally labeled RNA was fractionated into each molecular species by sucrose density gradient centrifugation and further purified by either 1% agarose or, 2% polyacrylamide-0.6% agarose gel electrophoresis (12). Labeling indices (^3H-labeled 3'-terminal nucleoside moles/total RNA moles) were calculated by assuming that 1.0 A_{260} corresponded to 50 µg RNA and the lengths of 45S, 41S, 32S, 28S and 18S RNAs were 1.4, 1.0, 0.7, 0.5 and 0.2 x 10^4 nucleotides, respectively.

3) Identification of ^3H-labeled 3'-terminal nucleoside derivatives

Purified 3'-terminally labeled RNA was dissolved in 0.05 M sodium acetate (pH 4.6) and digested with 5 units of RNase T_2 (Sankyo Pharmaceutical Co., Tokyo) at 37°C for 24 h. Then, the digest was mixed with four kinds of unlabeled nucleoside trialcohols (here termed C', A', U' and G') prepared according to RajBhandary (11), spotted on an Avicel-SF cellulose glass plate (Funakoshi Co. 10 x 10 cm), and chromatographed with a solvent system consisting of tert butanol-methylethylketone-concentrated NH_4OH-water (40:30:10:15) (13). After development, four nucleoside trialcohols were localized by an UV lamp. Strips of adsorbent, 5 mm wide, were

removed from the glass plate by means of a scraper, and counted for radioactivity (14). For further sequential analysis, ^{3}H-labeled RNA and 25 μg of unlabeled rRNA were dissolved in 50 mM Tris-HCl, pH 7.5 and digested with 10 units of T_1-RNase (Sankyo Pharmaceutical Co., Tokyo) at 37°C for 2 hr. The digest was fractionated on a DEAE-Sephadex column as described previously (15).

RESULTS

1) 3'-terminal sequences of 18S and 28S rRNA

We first analyzed 3'-terminal sequences of 18S and 28S rRNA from MH134/C cells. When 18S and 28S rRNA from MH134 cells were labeled with $NaB^{3}H_4$, and centrifuged through sucrose gradient, ^{3}H radioactivity was specifically incorporated into RNA (Fig. 1). The labeling indices as defined by ^{3}H-labeled 3'-terminal nucleoside moles/total RNA moles of 18S and 28S rRNA calculated from Fig. 1 were 48% and 40%, respectively. The radioactivity on the top was not in RNA but probably in polysaccharide or glycoprotein that had contaminated this RNA. Labeled 18S and 28S rRNA were further purified by agarose gel electrophoresis, and subjected to the following sequence analysis. As shown in Fig. 2, 18S and 28S rRNA had the 3'-terminal sequences of A_{oh} and YGU_{oh}, respectively. 3'-terminal sequences of both rRNAs prepared from rat liver were the same as those from MH134 (data not shown). These results were consistent with Shine & Dalgarno's data (17) in the 3'-terminal structure of both 18S and 28S rRNA. This confirmed the usefulness of this method for the purpose of determining the 3'-terminus of high molecular weight RNA.

2) Labeling of 3'-termini of nucleolar pre-ribosomal RNAs

Nucleolar 28S, 32S, 41S, and 45S RNAs prepared and mixed as described in Materials and Methods were labeled at the 3'-termini and separated again by 2.0% polyacrylamide-0.6% agarose gel electrophoresis. Usual RNases are known to cleave phosphodiester bonds to generate 2', 3' phosphate (...Np/N...). These nicks would not interfere with our experiments, because

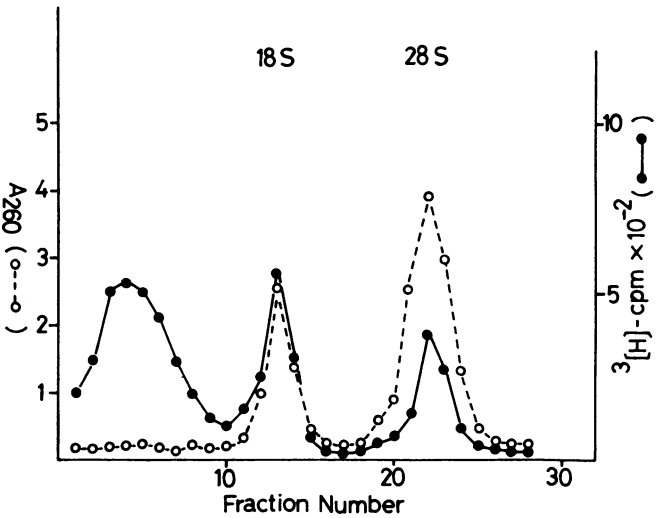


Fig. 1 Sucrose gradient sedimentation analysis of ^{3}H-labeled rRNA.

18S and 28S rRNA from MH134 were labeled with ^{3}H-borohydride as described in Materials and Methods. ^{3}H-labeled RNA which was filtered on Sephadex G-50 column was layered on 10 to 30% sucrose gradient and centrifuged for 15 hr at 30,000 rpm in a Hitachi 40T rotor, TCA (trichloroacetic acid)-insoluble radioactivity in 40 µl of each fraction (0.4 ml) and ultraviolet absorption were determined.

O·····O A_{260}, ●——● ^{3}H-radioactivity.

they could not be labeled by this procedure. However, if these large RNA molecules contained internal nicks that would generate free 2', 3'-OH groups (...N/pN/...), these artificial 3'-ends would be labeled in addition to native 3'-ends. To assess this possibility, the extent of 3'-terminal labeling was estimated by checking the labeling index of each RNA. As shown in Fig. 3, all the pre-ribosomal RNAs were labeled with almost the same, and sufficiently low indices, although the real labeling indices must have been even lower in view of the presence of non-RNA material shown in Figs.4 and 5. We also heat-quenched the labeled RNAs before applying to the gel to remove further high molecular weight RNA with internal nicks. Each RNA was recovered from the gel, and used for the

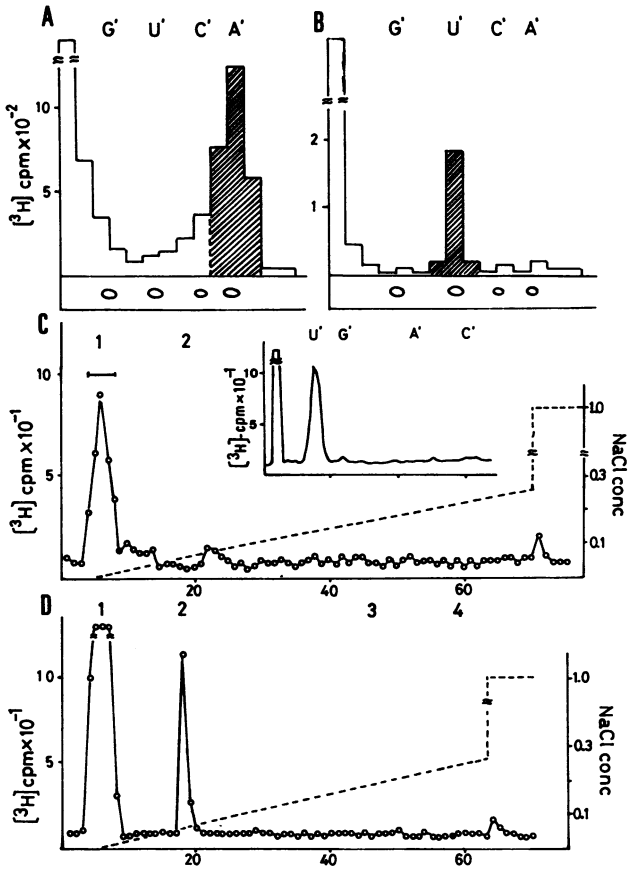


Fig. 2 3'-terminal analysis of 18S and 28S rRNA
^{3}H-labeled 18S and 28S rRNA separated by sucrose density gradient centrifugation as in Fig. 1 and further purified by gel electrophoresis were used for 3'-terminal analysis.
(A) and (B): Thin-layer chromatography of T_2-RNase digests of ^{3}H-labeled 18S (A) and 28S (B) rRNA
(C) and (D): DEAE-Sephadex chromatography of T_1RNase (C) and Pancreatic (D) RNase digests of ^{3}H-labeled 28S rRNA.
Inset in C : Phosphocellulose chromatography (16) of the radio-active fraction eluted with the void volume in C.
G', U', C', A' in figures show the positions of each nucleoside trialcohols in the chromatogram. 1-4 on the top of Fig. C and D show the elution position of oligonucleotide trialcohols containing 1-4 nucleoside residues. The radioactivity remained at the origin in (A) or (B), and eluted with void volume in (D), contained no 3'-terminal material as determined by re-digestion and rechromato-graphy on thin-layer or by rechromatography on phosphocellulose column.

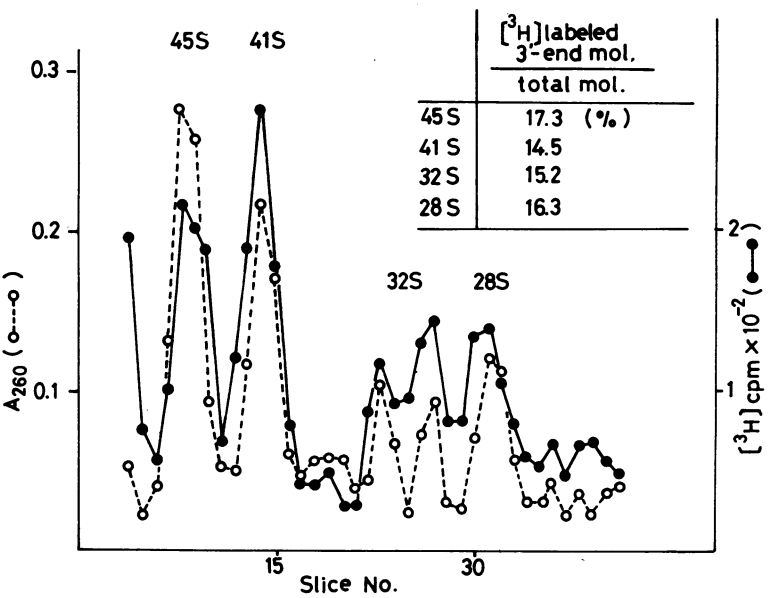


Fig. 3 Gel electrophoresis of ^{3}H-labeled NoRNA.
Rat liver NoRNAs mixed and 3'-terminally labeled as described in Materials and Methods were heat-quenched in 10 mM Tris-0.5 % SDS pH 7.4 at 60°C, for 5 min and electrophoresed on 2% polyacrylamide-0.6% agarose gel. After electrophoresis, gel was sliced at 2 mm width, and RNA was eluted from each slice by incubation with 2 ml of 40 mM Tris-20 mM sodium acetate-2 mM EDTA-0.5% SDS,pH 7.8. Then, A_{260} of aqueous portion and TCA-insoluble radioactivity of 0.3 ml of aqueous phase were counted. RNA molarity and ^{3}H-labeled 3'-end molarity indicated in the inset were calculated from A_{260} and TCA insoluble radioactivity, respectively. Counting efficiency of [^{3}H] was postulated to be 0.3. NoRNA from MH134/C gave almost the same pattern (data not shown).

following sequence analysis.

3) Analysis of 3'-terminal nucleotide sequence of pre-ribosomal RNAs

First, the ^{3}H-labeled pre-ribosomal RNAs that were purified by the gel, were recovered from the gel and digested with T_2-RNase. Then the released nucleoside derivatives were determined by thin-layer chromatography. As shown in Fig. 4 and Table 1, 45S, 41S, 32S, and 28S NoRNA from MH134 had

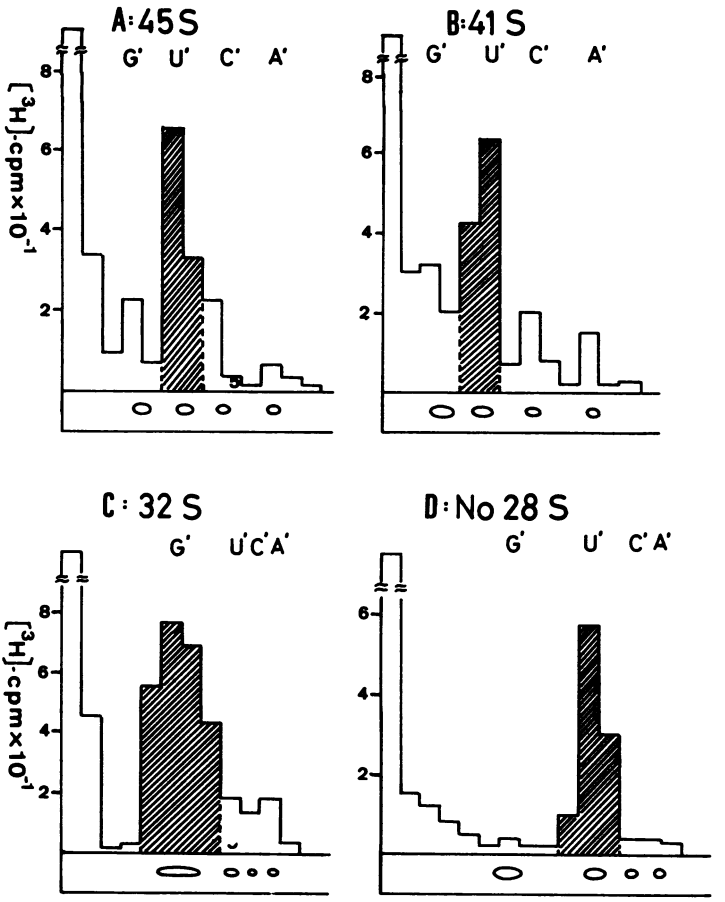


Fig. 4 Thin-layer chromatography of T_2-RNase digests of 3'-terminally-labeled NoRNAs from MH134. Each of ^{3}H-labeled NoRNAs was recovered from the gel in Fig. 3, and digested with T_2-RNase. The digests were chromatographed on thin-layers, as described in Materials and Methods, and strips of adsorbent, 5 mm wide, were counted for radioactivity.

A: 45S, B: 41S, C: 32S, D: 28S NoRNA.

single major 3'-terminal nucleosides; U_{oh}, U_{oh}, G_{oh}, and U_{oh}, respectively. These 3'-termini seemed to be not so heterogeneous. 45S and 41S RNA had 3'-termini different from 32S RNA, and 32S RNA had that different from 28S NoRNA. In the case of rat liver, 45S, 41S, 32S, and 28S NoRNA had also

RNA speciss		3'-terminal nucleoside(%)			
		G_{oh}	U_{oh}	C_{oh}	A_{oh}
mouse hepatoma (MH134)	No 45S RNA	21	58	14	7
	41S	27	55	10	8
	32S	86	6	2	6
	28S	-	>90	-	-
	r 28S RNA	-	90	-	-
rat liver	No 45S RNA	70	21	9	-
	41S	17	60	7	16
	32S	24	63	7	6
	28S	11	65	19	5
	r 28S RNA	-	>90	-	-

Table 1: 3'-terminal nucleosides of rRNA precursors from rat liver and MH134.
Percentages were caluculated from the chromatograms in Fig. 4 and 5. - -; not detectable.

sufficiently homogeneous 3'-termini; G_{oh}, U_{oh}, U_{oh} and U_{oh}, respectively (Fig. 5). The radioactivity remained on the origin of thin-layer plate in Fig. 4 and 5 was not derived from 3'-terminal nucleosides as determined by re-digestion with T_2-RNase and re-chromatography (data not shown), but probably derived from some sugar material that contaminated RNA. Further sequence analyses were carried out by DEAE-Sephadex column chromatography of T_1-RNase digests of labeled NoRNAs. In this chromatography, oligonucleotides were fractionated according to the chain length. T_1-RNase digestion of 45S, 41S, 32S and 28S NoRNA from rat liver gave rise to Z_7N', Z_2N', $Z_{12}N'$ and Z_2N', respectively (Fig. 6). Thus, as summarized in Table 2, the major 3'-terminal sequence of 45S, 41S, 32S, and 28S NoRNA from rat liver were GZ_7G_{oh}, GZ_2U_{oh}, $GZ_{12}U_{oh}$, and GZ_2U_{oh}, respectively. In the case of MH134, 45S RNA released $Z_{13}N'$ by T_1-RNase digestion and No 28S RNA three species of terminal fragment; Z_1N', Z_2N', and Z_3N' (Fig. 7).

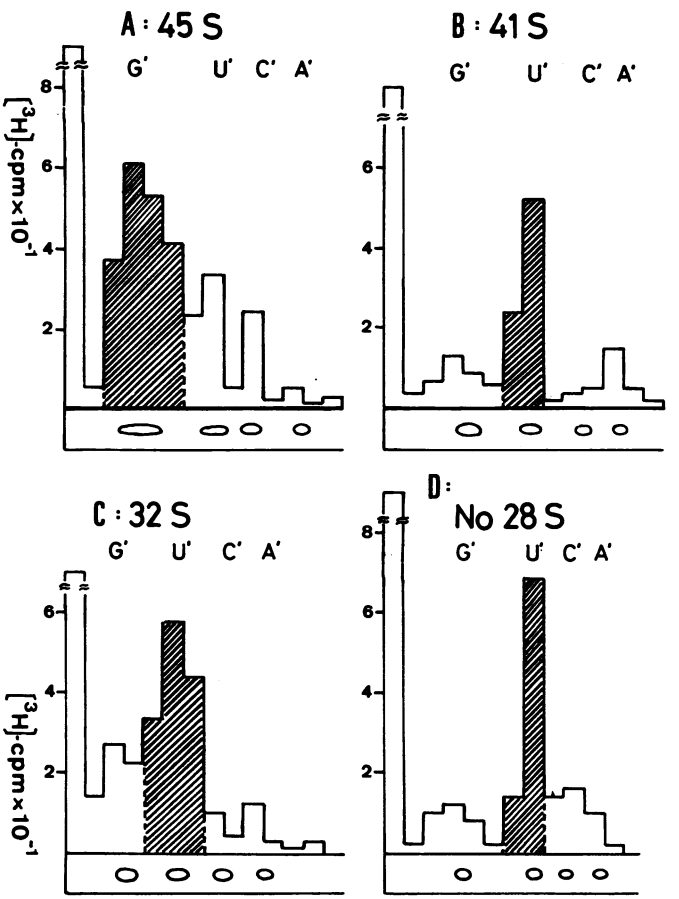


Fig. 5 Thin-layer chromatography of T_2-RNase digests of 3'-terminally-labeled NoRNAs from rat liver.
Details were the same as Fig. 4.
A: 45S, B: 41S, C: 32S, D: 28S NoRNA.

DISCUSSION

In all organisms, large and small rRNAs are transcribed as a common transcriptional unit. 45S RNA, the probable primary transcript of rRNA genes in mammals, was thought to be organized as (5') spacer-18S-spacer-5.8S-spacer-28S(3')by electron microscopic (1) and UV-irradiation studies (2). However, there has been no demonstration that 45S RNA has a

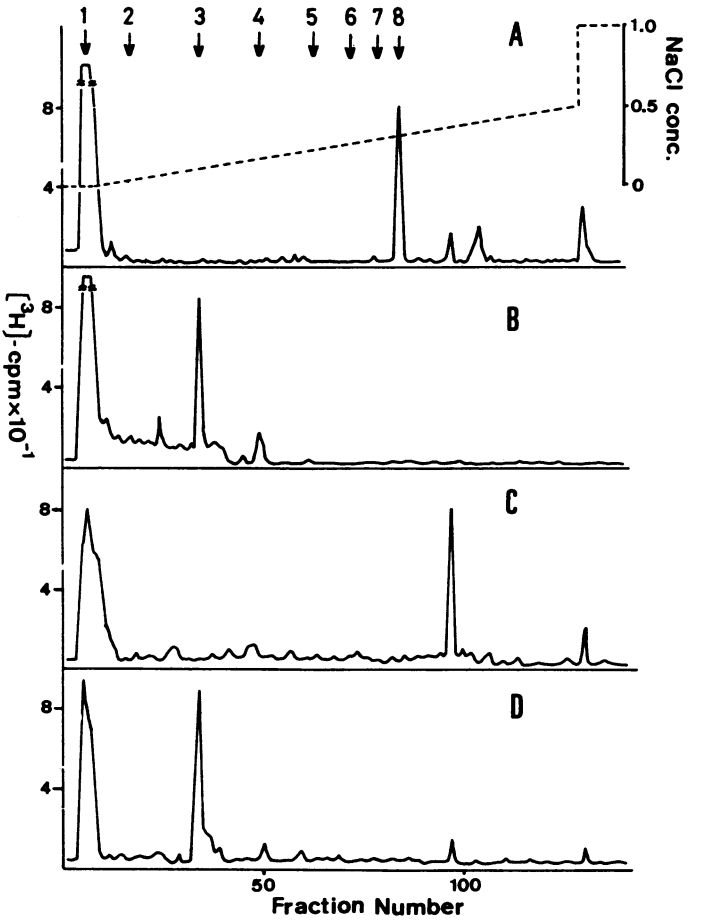


Fig. 6 DEAE-Sephadex chromatography of T_1-RNase digests of 3'-terminally-labeled NoRNAs from rat liver.

T_1-RNase digests of unlabeled rRNA and ^3H-labeled NoRNAs obtained from gel in Fig. 3, were applied onto a DEAE-Sephadex column (0.5 x 30 cm) and eluted with an 80 ml gradient of 0 to 0.5 M NaCl in 7 M urea and 0.05 M Tris-HCl, pH 7.6. Arrows labeled 1-8 refer to the elution positions of oligonucleotide trialcohols containing 1-8 nucleoside residues. The radioactivity eluted in the void volume contained no 3'-terminal material (as determined by phosphocellulose column rechromatography).

A: 45S, B: 41S, C: 32S, D: 28S NoRNA

The major 3-terminal nucleotides sequences of r RNA precursors

mouse hepatoma (MH134)	No 45 S RNA	$GZ_{-13}\text{-}U_{oh}$
	41 S	U_{oh}
	32 S	G_{oh}
	28 S	$GZ_{1,2,3}U_{oh}$
	r 28 S RNA	YGU_{oh}
rat liver	No 45 S RNA	GZ_7G_{oh}
	41 S	GZ_2U_{oh}
	32 S	$GZ_{-12}\text{-}U_{oh}$
	28 S	GZ_2U_{oh}
	r 28 S RNA	YGU_{oh}

Table 2: Major 3'-terminal sequences of rRNA precusors from rat liver and MH134.

transcribed spacer sequence at its 3'-terminus other than 28S RNA sequence. The present study demonstrates for the first time that various precursors to 28S rRNA have 3'-terminal sequences different from that of mature 28S rRNA. This implies that a spacer sequence, the size of which was not determined in our study, is located at the 3'-terminus of 45S RNA. Recently, 37S RNA, the primary transcript of rRNA genes in yeast, was shown to contain an additonal sequence 3'-distal to the mature 26S rRNA (18). The present data also show that the pre-ribosomal RNAs of various stages have different 3'-terminal sequences. Therefore, it is most likely that this 3'-terminal transcribed spacer sequence is gradually removed during the course of rRNA maturation. Although such processing or triming mechanism seems to be the cleavage that generates free 3'-OH group, further details, such as whether exonucleolytic or endonucleolytic remain to be determined.

As far as our partial investigation was concerned the 3'-terminal sequences of a set of precursors described here did not show any homology. This may mean that different

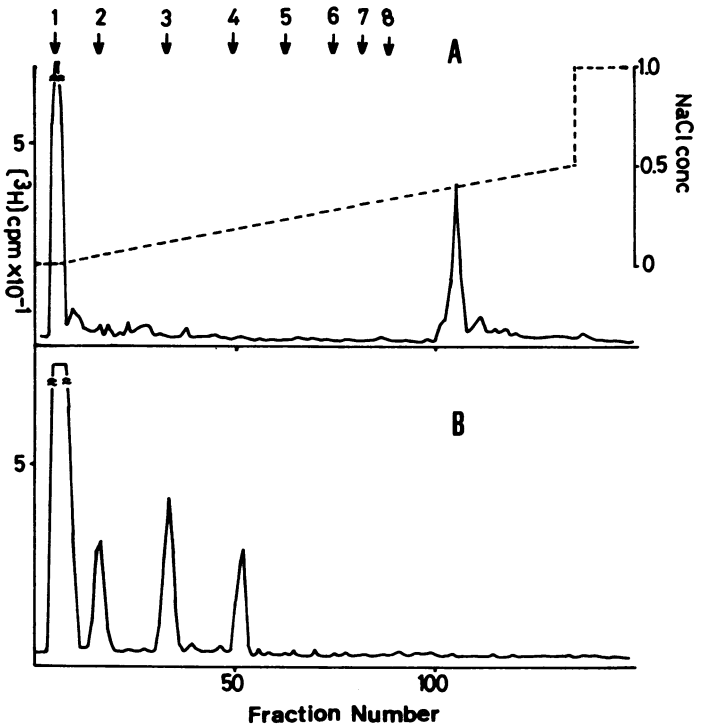


Fig. 7 DEAE-Sephadex chromatography of T_1-RNase digests of 3'-terminally labeled NoRNAs from MH134.
Details are the same as Fig. 6. The radioactivity eluted with the void volume contained no 3'-terminal material.
A: 45S, B: 28S NoRNA
(41S and 32S NoRNA were not determined)

enzymes may be involved for different steps, although the possibility cannot be ruled out that the processing enzymes recognize internal sequences. 3'-terminal sequences of corresponding precursors were different between the mouse and the rat, although mature 28S rRNA had an identical sequence between these species. This phylogenetical heterogeneity of spacer sequences among species, may reflect a somewhat lower selection pressure imposed on this sequence than on the 3'-terminal sequence of 28S rRNA. It is also noteworthy that 3'-terminal sequence of No 28S RNA is heterogenous and

different from that of mature 28S rRNA. We have previously demonstrated that heterogeneous 5'-termini of newly synthesized 28S rRNA is gradually processed into one mature form, pCp..., during the course of transport from nucleus to the cytoplasm, and even while in polysomes (6). The possibility of a similar 5'-terminal processing has recently been reported for SV40 late mRNAs (19). In the same manner, 3'-terminus of 28S RNA appears to be processed to a homogeneous mature ... YGU_{oh}during transport from the nucleus to the ribosome. The 45S RNA is usually thought to be the primary transcript of rRNA genes in mammals, although its 5'-terminus seems to be processed quite rapidly (5). The 3'-terminal sequence of 45S RNA is GZ_7G_{oh}in rat liver, and $GZ_{13}U_{oh}$ in mouse hepatoma. In both species, 45S RNA contain a fairly long T_1-RNase resistant sequences at the 3'-end. It would be interesting to see if these sequences represent U-rich ones, in view of the suspected role of U-clusters as a termination signal (18, 20).

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