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Maturation Pathway for Novikoff Ascites Hepatoma 5.8 S Ribosomal Ribonucleic Acid

EVIDENCE FOR ITS PRESENCE IN 32 S NUCLEAR RIBONUCLEIC ACID*

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SUMMARY

Evidence that 32 S nRNA contains 5.8 S rRNA was provided by studies on specific oligonucleotide sequences of these RNA species. Purified ^{32}P-labeled 5.8 and 28 S rRNA and 32 S RNA were digested with T_1 ribonuclease, and the products were fractionated according to chain length by chromatography on DEAE-Sephadex A-25 at neutral pH. The oligonucleotides in Peak 8 were treated with alkaline phosphatase and the products were separated by two-dimensional electrophoresis on cellulose acetate at pH 3.5 and DEAE-paper in 7% formic acid. Seven unique oligonucleotide markers for 5.8 S rRNA including the methylated octanucleotide A-A-U-U-Gm-C-A-Gp were present in 32 S RNA but were not found in 28 S rRNA, indicating that 5.8 S rRNA is directly derived from the 32 S nucleolar precursor. These studies confirm a maturation pathway for rRNA species in which 32 S nucleolar RNA is a precursor of 5.8 S rRNA as well as 28 S rRNA.

In eukaryotic cells, labeling kinetics (1–4), hybridization studies (4, 5), and sequence studies (6–9) have shown that the two high molecular weight 28 and 18 S rRNA are transcribed in the nucleolus as portions of a single large precursor molecule, the 45 S nRNA in mammalian cells (Fig. 1). In addition to 18 and 28 S rRNA, cytoplasmic ribosomes of eukaryotic cells also contain two low molecular weight RNA species, 5 S and 5.8 S rRNA. Both are constituents of the 60 S ribosomal subunit in equimolar ratios to 28 S rRNA; the 5.8 S molecule is hydrogen bonded to the high molecular weight component (10–15). A comparison of the labeling kinetics for 5.8 S rRNA with the labeling of high molecular weight RNA and 45 and 32 S nucleolar precursors (11) indicates that the 5.8 S rRNA also may be a cleavage product of these precursors.

Chemical proof for this precursor-product relationship required a demonstration of the 5.8 S rRNA sequence within nucleolar precursor molecules. Studies on the sequences of specific oligonucleotides previously showed that 18 and 28 S rRNA are derived from 45 S rRNA (6–9). In the present study, the origin of 5.8 S rRNA in the nucleolar 32 S precursor is confirmed by the presence of unique oligonucleotide markers in both types of RNA (11).

MATERIALS AND METHODS

Cells and Labeling Conditions—Novikoff hepatoma ascites cells were maintained in male Holtzman rats for 6 days and then labeled *in vitro* with [^{32}P]orthophosphate as described by Mauritzen *et al.* (16). One to two grams of packed cells were incubated at 37° with 30 to 300 mCi of carrier-free [^{32}P]orthophosphate for 5 hours to label the high molecular weight RNA or 18 hours to label the low molecular weight RNA.

Isolation and Purification of ^{32}P-labeled RNAs—After labeling, the cells were collected by centrifugation at 7000 × *g* for 10 min at 0°; the RNA was extracted with sodium dodecyl sulfate-phenol at 65° and precipitated at −20° with 2 volumes of ethanol (17). Low molecular weight RNA was separated on 10 to 40% sucrose density gradients and fractionated by electrophoresis on 10% polyacrylamide gel slabs (18, 19). High molecular weight RNA was dissolved in 50% formamide, incubated at 37° for 10 min to dissociate all remaining low molecular weight components, and fractionated at pH 8.3 by electrophoresis on 1.5% agarose slabs (20). The RNA was detected by autoradiography (Fig. 2) and recovered from the gel by homogenization in water followed by high speed centrifugation (30,000 × *g*, 1 hour) to remove the gel particles.

Digestion of ^{32}P-labeled RNAs and Analyses of Fragments—Purified RNA was precipitated at −20° with 2 volumes of ethanol (2% potassium acetate) and completely digested by T_1 ribonuclease (20:1, substrate to enzyme, w/w) for 30 min at 37° (21). The analytical procedures used to fractionate and analyze the fragments in this study are those described by Tener (22) and by Sanger and Brownlee (21).

For sequence analysis 5.8 S rRNA digests were fractionated directly using two-dimensional electrophoresis on cellulose acetate at pH 3.5 and DEAE-paper in 7% formic acid. For oligonucleotide comparisons the RNA fragments first were separated according to chain length on DEAE-Sephadex A-25 columns (1.0 × 75 cm) (Pharmacia Fine Chemicals) eluted with a linear NaCl gradient from 0.05 to 0.4 M in 7 M urea-0.005 M Tris-HCl, pH 7.5 (total volume, 2000 ml per gradient); 5-ml fractions were collected every 10 min and counted to determine the radioactivity (22). The fragments in Peak 8 were treated with 10 µl of alkaline phosphatase (0.1 mg per ml in 10 mM Tris, pH 9.0) for 2 hours at 37° and fractionated further by the two-dimensional electrophoresis procedure.

The individual fragments were characterized by their nucleotide compositions and for further sequence analysis of oligonucleotides in 5.8 S RNA, by digestion with pancreatic or U_2 ribonuclease or spleen phosphodiesterase as described by Brownlee

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(23). For nucleotide compositions alkaline digests (20 μl of 0.3 N NaOH for 18 hours at 37°) were separated by electrophoresis on Whatman No. 3MM paper at pH 3.5. For sequence analysis the oligonucleotides were digested further with 20 μl of pancreatic ribonuclease (Sigma, St. Louis, Mo.) (0.1 mg per ml of 0.01 M Tris-HCl-0.001 M EDTA, pH 7.4, for 60 min at 37°), 20 μl of U_2 ribonuclease (Sankyo, Tokyo, Japan) (0.5 unit per ml of 0.05 M sodium acetate-0.002 M EDTA, pH 4.5, for 30 to 120 min at 37°), or 20 μl of spleen phosphodiesterase (Worthington, Freehold, N.J.) (0.2 mg per ml of 0.1 M ammonium acetate-0.002 M EDTA-0.05% Tween 80, pH 5.7, for 1 to 2 hours at 37°). Products from pancreatic ribonuclease digestion were separated by electrophoresis on DEAE-paper at pH 1.9, while those from U_2 ribonuclease or phosphodiesterase digestion were separated by electrophoresis on DEAE-paper at pH 1.9, in 7% formic acid or by two-dimensional electrophoresis on cellulose acetate at pH 3.5 and DEAE-paper in 7% formic acid. The nucleotide compositions of the products were subsequently determined by alkali hydrolysis.

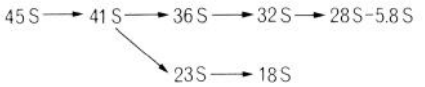

FIG. 1. Proposed maturation pathway for rRNA in Novikoff ascites hepatoma cells. This maturation pathway is a summary of reports based on labeling kinetics (1–4), competitive hybridization (4, 5), and partial sequence analyses (6–9).

Commercial preparations of U_2 ribonuclease and spleen phosphodiesterase contain contaminating endonuclease activities (28) which produce additional cleavage products. In some instances these fragments were valuable in confirming the over-all sequence (Table I).

RESULTS

Oligonucleotide Markers for 5.8 S rRNA—Fig. 3 shows an autoradiograph for a two-dimensional fractionation of a T_1 ribonuclease digest of ^{32}P-labeled Novikoff hepatoma 5.8 S rRNA obtained in studies of its primary sequence (24, 25). Seven of the 22 major spots in this digest were found to be unique marker fragments containing eight or more nucleotides. Complete se-

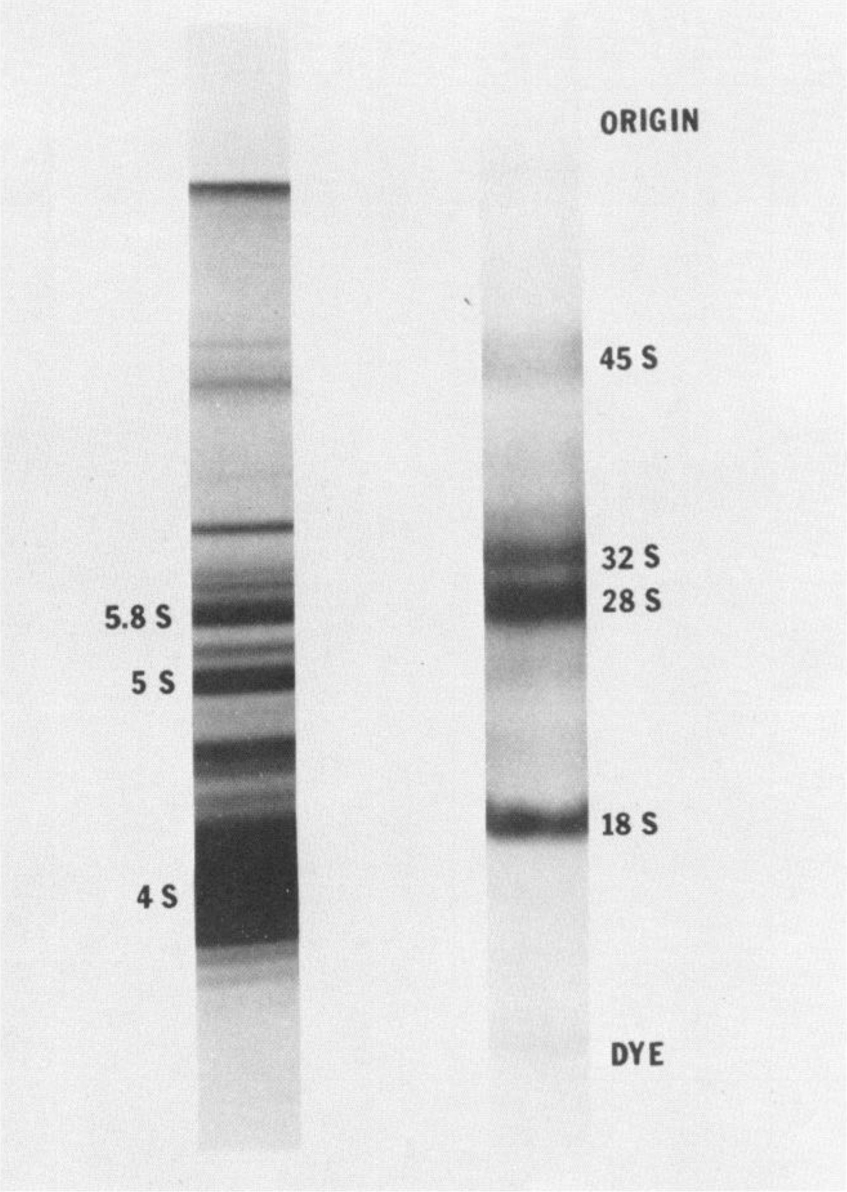

FIG. 2. Autoradiographs for portions of preparative gels for ^{32}P-labeled high and low molecular weight RNAs in Novikoff ascites hepatoma cells; 5.8 S rRNA was prepared on 10% polyacrylamide gels, pH 7.2 (*left*), and high molecular weight 28, 32, and 45 S RNAs were fractionated on 1.5% agarose gels, pH 8.3 (*right*). The RNAs and approximate positions of the origins and bromphenol blue dye markers are identified in the margins.

Fig. 3

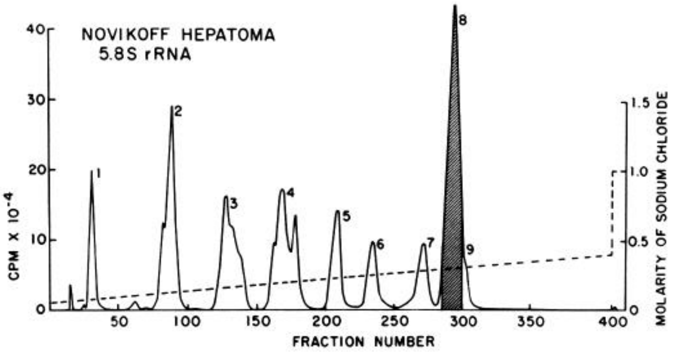

FIG. 3. Patterns of separation for T_1 ribonuclease digests of ^{32}P-labeled Novikoff hepatoma 5.8 S RNA. *Left*, fractionation by two-dimensional electrophoresis from right to left on cellulose acetate, at pH 3.5 and from top to bottom on DEAE-paper in 7% formic acid; *right*, fractionation by DEAE-Sephadex A-25 column chromatography at neutral pH. The *shaded area* represents those fractions which were pooled as Peak 8 for subsequent analysis.

quences for these were deduced by further digestion with pancreatic or U_2 ribonuclease and spleen phosphodiesterase (Table I). The octanucleotide A-A-U-U-Gm-C-A-Gp is the most characteristic for 5.8 S rRNA because it contains the alkali-stable dinucleotide Gm-Cp (24, 25), which also has been found in HeLa cell (26) and wheat embryo (27) 5.8 S rRNA.

To determine their chain lengths by an alternate method, the T_1 ribonuclease digest also was analyzed by chromatography on DEAE-Sephadex A-20 columns at pH 7.5 (Fig. 3). The seven most characteristic oligonucleotide markers were eluted in Peak 8 (−9 charge) and the adjacent shoulder, Region 9. The shoulder contained the nonanucleotide C_5U_3G, which eluted earlier than other nonanucleotides and therefore was collected partially with Peak 8 (Fig. 3). A similar chromatographic behavior was found for other polypyrimidines of the same nucleotide composition in Novikoff hepatoma 28 S rRNA (28). For more discrete separations of marker oligonucleotides, the seven components in Peak 8 were treated with alkaline phosphatase and separated by two-dimensional electrophoresis on cellulose acetate at pH 3.5 and DEAE-paper in 7% formic acid (Fig. 4). Of the seven spots observed, five were clearly separated but the two fastest moving fragments, Spots 5 and 6 ($C_3A_2U_2G$), were not resolved. As indicated above, the nonanucleotide C_5U_3G (Spot 12) was collected only partially in Peak 8 and therefore appears as a relatively minor spot.

5.8 S rRNA Markers in Nucleolar Ribosomal Precursor RNAs—As indicated in Fig. 1, labeling studies (11) have suggested that nucleolar 32 S RNA is the immediate precursor of 5.8 S rRNA. Accordingly, it was analyzed for the presence of 5.8 S rRNA oligonucleotide markers; comparisons were made with 28 S RNA. For these studies, ^{32}P-labeled high molecular weight RNAs were isolated by electrophoresis on 1.5% agarose gels at pH 8.3 (Fig. 2). To ensure that 5.8 S RNA was not present as a noncovalently hydrogen-bonded contaminant of the high molecular RNAs, they were extracted at 65° (15), thoroughly desalted, and layered on the gel in 50% formamide after incubation at 37° for 10 min.

Fig. 4 shows the autoradiographs for two-dimensional separations of Peak 8 for 5.8, 28, and 32 S RNA after digestion with T_1 ribonuclease, separation on DEAE-Sephadex columns (29) as shown in Fig. 3 for 5.8 S rRNA and subsequent treatment with alkaline phosphatase. The 32 S RNA contained 26 major spots while 28 and 5.8 S rRNA contained only 16 and 7 spots, respectively. Five of the 5.8 S marker oligonucleotides were clearly present as distinct spots in 32 S RNA and absent in 28 S RNA (Fig. 4). The remaining spots co-migrated with two fragments from 28 S rRNA (Table II). Analyses of nucleotide composition and digestion with pancreatic ribonuclease or spleen phosphodiesterase provided further evidence for the presence of the seven marker oligonucleotides in 32 S RNA (Table II). Five additional spots labeled 8–11, 8–17, 8–22, 8–25, and 8–26 were found in 32 S RNA that were absent from both 5.8 and 28 S rRNA. This result indicates the presence of nonconserved sequences in 32 S RNA (9, 29–31) in addition to 28 and 5.8 S rRNA.

DISCUSSION

In this study seven oligonucleotide markers that represent one-third of the total nucleotide sequence of 5.8 S rRNA were present in 32 S RNA but were not found in 28 S RNA. These results confirm the earlier suggestion (11) that 5.8 S rRNA is derived directly from the 32 S nucleolar RNA precursor (Fig. 1). Similar results have been obtained with the 45 S nRNA but the identity of

TABLE I

Unique oligonucleotide markers in 5.8 S RNA produced by complete digestion with T_1 ribonuclease

After the digests were fractionated by two-dimensional electrophoresis (Fig. 3), the radioactivity was determined and each spot was eluted and identified by its nucleotide composition and by further digestion with pancreatic or U_2 ribonuclease or partial stepwise digestion with spleen phosphodiesterase as described under "Materials and Methods." The molar yields which are averages for five determinations were calculated assuming a 180-nucleotide chain length for 5.8 S rRNA (25). The pancreatic ribonuclease digestion products are expressed as number of moles of each product relative to 1 mol of the products ending in Gp.

Spot	Nucleotide Composition	Molar Yield	Pancreatic RNase Digestion Products	U_2 RNase[a] Digestion Products	Spleen Phosphodiesterase[b] Partial Digestion Products	Sequence
T17a	$C_3A_2U_2G$	1.0 (1)	AU(0.9),G(1.0),AC(1.1), C(1.6),U(1.0)	AC_2UG(0.54),C_2UG,CUA,A	C_2AUG(0.56),C_2UG,CUG,CG	A-U-C-A-C-U-C-Gp[c]
T17b	$C_3A_2U_2G$	1.0 (1)	G(1.0),AC(2.2),C(1.0), U(1.7)	C_3AU_2G(0.18),C_2U_2AG(0.20), C_2U_2G(0.23),CA,A	C_2U_2AG(0.20),C_2U_2G(0.23), CU_2G,CUG,CG,C_2AU_2(0.48), CAU_2,C_2AU,CAU,C_2A_2	A-C-A-C-U-U-C-Gp[c]
T18	$C_2A_3U_2G$	0.9 (1)	G(1.0),AU(1.0),AC(2.1), U(0.8)	CAU_2G(0.22),AU_2G,U_2G,U_2, C_2A_2,C_2A,CA,A	$C_2A_2U_2G$(0.16),CA_2U_2G(0.17), AU_2G,U_2G	A-C-A-C-A-U-U-Gp
T19	C_5U_3G	1.0 (1)	G(1.0),C(4.5),U(2.9)		C_5U_2G(0.20),C_5UG(0.52), C_4UG(0.56),C_3UG(0.61), C_3G,C_2G	U-U-C-C-U-C-C-C-Gp
T20	A_3U_2GmCG	1.0 (1)	A_2U(1.1),AG(1.0), GmC(1.0),U(1.2)	U_2GmCAG(0.08), U_2GmCA(0.33),G,A	U_2GmCAG(0.08), UGmCAG(0.10),GmCAG	A-A-U-U-Gm-C-A-Gp
T21	$C_2A_2U_3G$	1.2 (1)	AG(1.0),AC(1.2), C(1.0),U(3.0)	C_2U_3A(0.40),CU_2A,U_2A, AG,A	U_2AG,C_2U_3A(0.16), C_2U_3(0.20),CU_3,C_2AU, U_2	A-C-U-C-U-U-A-Gp
T22	A_4U_3G	0.9 (1)	A_2U(1.7),G(1.0), U(1.2)	A_2UG,AUG,UGA_2U_2,AU_2, A_2U,U_2,A	A_2U_3G(0.07),A_2U_2G(0.17), A_2UG,AUG,UG	A-A-U-U-A-A-U-Gp

[a] Under the digestion conditions used (see "Materials and Methods") some partial and nonspecific cleavage products were observed (28). These are listed when they were used to confirm the proposed sequence. Products which were present as mixtures are excluded. The electrophoretic mobilities relative to the blue marker (DEAE-paper, 7% formic acid) of the longer fragments are noted in parentheses.

[b] Under the digestion conditions used (see "Materials and Methods") some nonspecific cleavage products were produced by contaminating endonuclease activity (28). These are listed when they were used to confirm the proposed sequence. Products which were present as mixtures are excluded. The electrophoretic mobilities relative to the blue marker (DEAE-paper, 7% formic acid) of the longer fragments are noted in parentheses.

[c] The two sequential isomers in spot T17 were completely separated by further extended electrophoresis on DEAE-paper in 7% formic acid. Molarities and sequences were determined for the separated fragments.

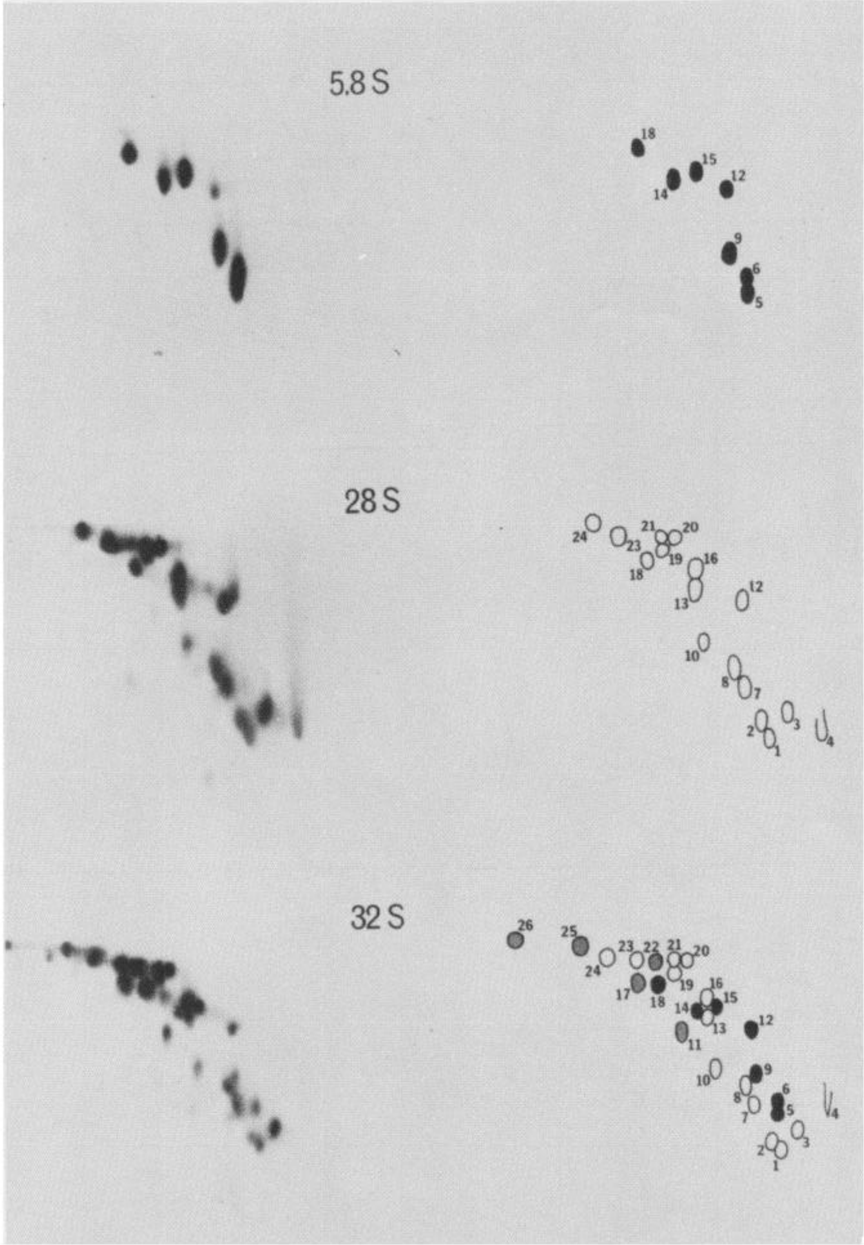

FIG. 4. Two-dimensional fingerprints for the octanucleotide fraction of T_1 ribonuclease digested Novikoff hepatoma RNAs. The RNA digests were fractionated on DEAE-Sephadex A-25 columns (Fig. 3) and Peaks 8 were treated with alkaline phosphatase (text) before electrophoresis from right to left on cellulose acetate, pH 3.5, and from top to bottom on DEAE-paper in 7% formic acid. The fingerprints include all fragments which migrated between the blue and yellow dye markers in the first dimension and between the origin and blue dye markers in the second dimension. A key to identifying the spots is provided on the right for each autoradiograph.

markers was less clear because of their lower radioactivity and the greater complexity of the fragments. Since it has been shown unambiguously (1–9) that 32 S RNA is derived from the 45 S precursor, 5.8 S rRNA must originate in 45 S nRNA (Fig. 1).

One goal of studies in this laboratory is to define the topography of ribosomal precursor RNA. Although present in the 32 S precursor, the exact position of 5.8 S rRNA relative to the 28 S rRNA sequence remains to be defined. Moreover, the presence of at least five octanucleotides in 32 S RNA (Fig. 4) that are absent from 28 or 5.8 S rRNA confirms earlier reports that 32 S RNA contains nonconserved sequences (9, 29–31). In the Novikoff hepatoma these sequences are approximately 1200 nucleotides long (4) but their distribution relative to the 5.8 and 28 S rRNA sequences also remains to be defined. Fourteen different models for the topography of 32 S RNA may be proposed. The simplest assumes a single nonconserved region, *e.g.*

5′ <u>   28 S   </u> - - - - - - - - - <u>5.8 S</u> 3′

and the most complex requires three such regions, *e.g.*

5′ - - - <u>5.8 S</u> - - - <u>   28 S   </u> - - - 3′

A minimum of two cleavage steps, therefore, would be required for the maturation of 32 S precursor to 5.8 and 28 S rRNA. This could be accomplished by a single endonuclease or a group of enzymes. The various markers which have been identified in this study should prove useful in further topographic studies on 32 S RNA required to answer these questions.

TABLE II

Analyses of oligonucleotide marker fragments in 32 S RNA

Spot	Nucleotide Composition[a]	Pancreatic RNase Digestion Products in 5.8S RNA	Pancreatic RNase Digestion Products in 32S RNA
Peak 8-1	C_2AU_2NmN[b]G		
-2	$C_3A_2U_2G$		
-3	$C_3A_2U_2G$		
-4	C_5U_2G		
-5 (T17a)	$C_3A_2U_2G$	AUp, ACp, Cp, Up	AUp, ACp, Cp, Up
-6 (T17b)			
-7	$C_3A_2U_2G$		
-8	$C_2A_3U_2G$		
-9 (T18)	$C_2A_3U_2G$	AUp, ACp, Up	AUp, ACp, Up
-10	$C_4A_2U_2G$		
-11	$C_2A_3U_2G$		
-12 (T19)	C_5U_3G	C_5UG, C_4UG, C_3UG, C_3G	C_5UG[c], C_4UG, C_3UG, C_2UG, CUG, C_2G
-13	$C_2A_2U_3G$		
-14 (T20)	A_3U_2GmCG	A_2Up, GmCp, AG, Up	A_2Up, GmCp, AG, Up
-15 (T21)	$C_2A_2U_3G$	AG, ACp, Up, Cp	AG, ACp, Up, Cp
-16	$C_2A_2U_3G$		
-17	A_4U_3G		
-18 (T22)	A_4U_3G	A_2Up, Up	A_2Up, AUp[d], AG, Up
-19	C_2AU_4G		
-20	C_3U_4G		
-21	C_2AU_4G		
-22	CA_2U_4G		
-23	CA_2U_4G		
-24	CA_2U_4G		
-25	A_3U_4G		
-26	CAU_5G		

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[a] Since the oligonucleotides were treated with alkaline phosphatase prior to fractionations the G termini were not detected after alkali digestion but are assumed to be present as these are T_1 ribonuclease digestion products.

[b] This alkali stable dinucleotide migrates slightly faster than adenylic acid on Whatman No. 3MM paper at pH 3.5 consistent with AmCp or CmAp.

[c] Spot 8-12 was partially digested with spleen phosphodiesterase rather than pancreatic ribonuclease. In earlier studies on sequences in 28 S rRNA (28) two polypyrimidines with the nucleotide composition C_5U_3G were detected, C-U-U-C-U-C-C-C-Gp and C-U-C-C-U-C-U-C-Gp. Since neither of these could give rise to C_5UG the presence of this product in 32 S RNA is consistent with the 5.8 S RNA sequence.

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Addendum—Our findings that long oligonucleotide markers for 5.8 S rRNA are present in 32 S ribosomal precursor RNA of Novikoff hepatoma cells are in good agreement with those obtained by Maden and Robertson (26) whose study on HeLa cells appeared after this paper was submitted.

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[d] Spot 8-18 was also present in 28 S RNA but in 32 S RNA the relative radioactivity in this spot was doubled, consistent with the presence of both the 5.8 S marker oligonucleotide and a fragment derived from 28 S rRNA. A-Up and A-Gp were found in the fragment from 28 S RNA.

Maturation pathway for Novikoff ascites hepatoma 5.8 S ribosomal ribonucleic acid. Evidence for its presence in 32 S nuclear ribonucleic acid.

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