Oligonucleotide Pattern after Pancreatic Ribonuclease Digestion and the 3′ and 5′ Termini of 5S Ribonucleic Acid from HeLa Cells*

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ABSTRACT: Highly purified 5S ribosomal ribonucleic acid has been prepared from HeLa cells. Sedimentation analysis performed in 0.1 M NaCl or under denaturing conditions indicated that the ribonucleic acid used in the present work was homogeneous in sedimentation properties and was represented by continuous polynucleotide chains. A nucleotide composition analysis showed absence of pseudouridylic acid and methyl groups in 5S ribonucleic acid, in contrast to high molecular weight ribosomal ribonucleic acid. An analysis of the oligonucleotide pattern after pancreatic ribonuclease digestion of 5S ribonucleic acid gave results which were in general agreement with those reported for KB cell 5S ribonucleic acid, with respect to both the identity and the frequency of the partial sequences. However, a trinucleotide was found in considerable amount among the digestion products of HeLa 5S ribonucleic acid which was absent in the KB 5S pattern, and which could not be accounted for by the presence of contaminating ribonucleic acid species. The presence of this trinucleotide, together with the reproducibly much lower than expected molar yield of the larger oligonucleotides, strongly suggests the occurrence of alternate sequences at various sites in the 5S molecule of human cells.

The 3′-terminal nucleoside in HeLa 5S ribonucleic acid has been found to be uridine, as in KB cell 5S ribonucleic acid. Likewise, as in the latter ribonucleic acid, guanine nucleotides were found at the 5′ terminus of HeLa 5S ribonucleic acid; however, in addition to pGp reported for KB cells, the presence of ppGp and pppGp, representing the major portion of the 5′ end, has been clearly demonstrated in HeLa 5S ribonucleic acid. The implications of this finding with regard to the origin of 5S ribonucleic acid are discussed.

Ribosomes from many, and presumably all, organisms contain, in addition to the high molecular weight rRNA species, a low molecular weight RNA component of sedimentation constant about 5S (Rosset and Monier, 1963; Comb and Katz, 1964; Comb *et al.*, 1965; Galibert *et al.*, 1965; Marcot-Queiroz *et al.*, 1965; Bachvaroff and Tongur, 1966; Brown and Littna, 1966; Knight and Darnell, 1967). This RNA is a constituent of the larger ribosomal subunit (one per particle); its function is unknown.

As to the origin of 5S RNA, the possibility has been suggested (see Perry, 1967) that it may derive, as 18 and 28 S, from the 45S rRNA precursor. However, kinetic data (Knight and Darnell, 1967) and RNA–DNA hybridization data (Brown and Dawid, 1968; Brown and Weber, 1968) speak strongly against such a possibility. RNA–DNA hybridization experiments have indicated the presence of several genes for 5S RNA in the bacterial chromosome (about four genes in *Bacillus subtilis* (Smith *et al.*, 1968), about ten genes in *Escherichia coli* (Zehavi-Willner and Comb, 1966)); a redundancy of information for 5S RNA several orders of magnitude higher has been found in the genome of higher organisms (Brown and Weber, 1968; L. E. Hatlen and G. Attardi, in preparation). As in the case of the high molecular weight rRNA species, this multiplicity of genes raises the problem of heterogeneity of 5S RNA. In view of the small size of this rRNA species, an analysis of its primary structure should provide relevant data on this subject. In the present work, the method of oligonucleotide mapping described in a previous paper (Amaldi and Attardi, 1968) has been applied to HeLa cells 5S RNA. The results obtained suggest the existence of multiple forms of 5S RNA in human cells. Furthermore, the presence of pGp, ppGp, and pppGp at the 5′ end of the 5S molecule was demonstrated.

After this work was started, the complete sequence of 5S RNA from *E. coli* (Sanger *et al.*, 1967) and from human carcinoma (KB) cells (Forget and Weissman, 1967b) has been reported. In *E. coli* several forms of 5S molecules differing from one another by one or more bases have been identified (Sanger *et al.*, 1967; Brownlee *et al.*, 1968); in KB cells the published data are consistent with the existence of a relatively small number of very similar 5S molecules.

Materials and Methods

A. Cells and Method of Growth. For the method of growth of HeLa cells reference is made to a previous paper (Amaldi and Attardi, 1968).

B. Labeling Conditions. For long-term labeling with ^{32}P, exponentially growing HeLa cells were washed twice with phosphate-free modified Eagle's medium plus 5% dialyzed calf serum, then resuspended at a concentration of 4–5 $\times 10^4$ cells/ml in medium containing 0.01 mM phosphate and 12–20 μCi/ml of [^{32}P]orthophosphate. After about 50-hr incubation at 37°, the cells were subjected to a 12–13-hr chase with

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0.005 M nonradioactive phosphate, in order to reduce the specific activity of the rapidly labeled nuclear and cytoplasmic RNA fractions (Penman *et al.*, 1963; Scherrer *et al.*, 1963; Houssais and Attardi, 1966; Warner *et al.*, 1966).

For short-term labeling with [^{32}P]orthophosphate, cells were washed and resuspended in phosphate-free modified Eagle's medium with 5% dialyzed calf serum (2×10^5 cells/ml), incubated for 2 hr at 37°, then exposed for 45 min at 37° to [^{32}P]orthophosphate (25 μCi/ml).

For labeling with [^{14}C]RNA precursors, HeLa cells were incubated for 48 hr in the presence of 0.12 μCi/ml of [8-^{14}C]adenosine (55 mCi/mmole), then subjected to a 12-hr chase with 0.001 M unlabeled adenosine; in other experiments, [2-^{14}C]uridine (0.12 μCi/ml; 50 mCi/mmole) was utilized, followed by a 12-hr chase with 0.001 M unlabeled uridine.

To label the methyl groups of HeLa cell RNA and DNA, exponentially growing cells were washed twice with methionine-free Eagle's medium plus 5% dialyzed calf serum, resuspended in the same medium at a concentration of $4–5 \times 10^4$ cells/ml; after 2.5-hr incubation at 37°, L-[*methyl*-^{14}C]-methionine (9.3 mCi/mmole) was added at 0.20 μCi/ml. This concentration of methionine was sufficient to allow two generations of growth, after which the cells were harvested.

One experiment of double labeling was performed by incubating HeLa cells for 48 hr in modified Eagle's medium (containing 0.01 M phosphate), with 5% calf serum, in the presence of 0.06 μCi/ml of [8-^{14}C]adenosine (50 mCi/mmole) and 6 μCi/ml of [^{32}P]orthophosphate. The cells were washed and resuspended in fresh medium, supplemented with 0.001 M unlabeled adenosine, then allowed to grow another 16 hr at 37°; at the end of this chase period, the cells were harvested.

C. Isolation of the Ribosome–Polysome Fraction and Extraction of RNA. All operations described below were carried out at 2 to 4°. The procedure used for isolating the ribosome–polysome fraction was a modification of that described previously (Attardi and Smith, 1962). HeLa cells washed three times in NKM[1] (0.13 M NaCl–0.005 M KCl–0.001 M $MgCl_2$) were resuspended in three volumes of 0.002 M $MgCl_2$, and immediately homogenized in a Potter-Elvehjem homogenizer for 30 sec (five to six strokes); one-third volume of four-times-concentrated medium A was then added (medium A is 0.25 M sucrose–0.025 M KCl–0.004 M $MgCl_2$–0.05 M Tris buffer, pH 7.4). The homogenate was centrifuged 10 min at 600*g* to sediment nuclei, large debris, and unbroken cells; the resulting supernatant was treated for 30 min with 0.5% sodium deoxycholate, and, after centrifugation at 6600*g* for 15 min, centrifuged for 2 hr at 105,000*g* to yield a ribosome–polysome pellet.

The ribosome–polysome pellet was suspended in TKM (0.05 M Tris buffer (pH 7.4)–0.025 M KCl–0.0025 M $MgCl_2$), and the RNA extracted from it as previously described (Attardi *et al.*, 1966). After the final ethanol precipitation, the pellet of RNA was dissolved in 0.1 M NaCl, at a concentration of 0.5–10 mg/ml, as determined on the basis of ultraviolet absorption measurements. The value of 214 for $\epsilon^{1\,cm}_{1\%}$ at 260 mμ previously used for 18S and 28S RNA (Amaldi and Attardi, 1968) was also employed for 5S RNA.

For the isolation of free polysomes from HeLa cells pulse labeled with [^{32}P]orthophosphate, the cells were homogenized as described above in 0.002 M $MgCl_2$; one-eighth volume of 2.0 M sucrose in TKM was then added, and the homogenate centrifuged 15 min at 6600*g*. The resulting supernatant was layered onto a 15–30% linear gradient of sucrose in TKM and centrifuged 90 min at 25,000 rpm in a Spinco SW25.1 rotor. Sucrose gradient fractions corresponding to the polysome band were pooled, and the RNA was phenol extracted.

D. Fractionation of RNA on Sephadex G-100. ^{32}P- or ^{14}C-labeled RNA extracted from the ribosome–polysome pellet was fractionated on a 1.8×180 cm column of Sephadex G-100 (Galibert *et al.*, 1965), equilibrated with 0.1 M NaCl, at 22°. The flow rate was 30 ml/hr, and 2-ml fractions were collected. The fractions were analyzed by counting a small aliquot (1–100 μl) in 15 ml of Bray's solution, and/or by determining the optical density at 260 mμ. After the first run through Sephadex, the central fractions of the 5S peak were pooled, precipitated with ethanol, dissolved in a small volume of 0.1 M NaCl, and run on a second column, usually with the addition of unlabeled RNA extracted from the ribosome–polysome pellet; the use of carrier tended to increase the recovery of 5S RNA in the rerun, up to almost 100%. The central fractions of the 5S peak were again pooled, precipitated with ethanol, and dissolved in a suitable medium for the analysis to be performed.

E. Extraction of DNA. DNA was extracted from the 600*g* pellet obtained from the total homogenate of L-[*methyl*-^{14}C]-methionine-labeled cells. The extraction was carried out according to the Marmur procedure (Marmur, 1961) with three additional deproteinization steps using phenol containing 0.1% 8-hydroxyquinoline (Kirby, 1962).

F. Sedimentation Analysis of 5S RNA. Sedimentation analysis of the 5S component isolated by Sephadex G-100 chromatography was carried out on a 5–20% sucrose gradient in 0.1 M NaCl–0.01 M sodium acetate buffer (pH 5.0), in the presence of [^{3}H]4S RNA marker. The gradient was run 11.5 hr at 62,000 rpm in a Spinco SW65 rotor, at 4°.

For the analysis of sedimentation properties under denaturing conditions, 5S RNA samples mixed with a 4S RNA marker were heated at 80° for 3 min in 0.001 M Tris buffer (pH 7.0) containing 0.00025 M EDTA, quickly cooled to 0°, and run through a 5–20% sucrose gradient in the same buffer; alternatively, samples were heated at 70° for 5 min in 18% neutralized formaldehyde containing 0.001 M EDTA, quickly cooled, diluted two times with H_2O, and run on a 5–20% sucrose gradient in 0.02 M sodium phosphate buffer (pH 7.4), 0.1 M NaCl, containing 1% formaldehyde. The conditions of centrifugation for 5S RNA after denaturation were the same as described above. Fractions from the sucrose gradient were collected directly into scintillation vials, and after addition of Bray's solution, were counted in the scintillation counter.

G. Base Composition and Sequence Analysis. The technique of nucleotide analysis after alkaline hydrolysis has been previously described (Attardi *et al.*, 1966). In some experiments aimed at elucidating the 3′ end of the 5S RNA molecule, an elution step with about 40 ml of 0.005 M formic acid was introduced prior to the 0.02 M formic acid, in order to separate the nucleosides from the 2′,3′-cytidylic acid. In other experiments, directed toward determining the nature of

[1] Abbreviations used are: NKM, 0.13 M NaCl–0.005 M KCl–0.001 M $MgCl_2$; TKM, 0.05 M Tris buffer (pH 7.4)–0.025 M KCl–0.0025 M $MgCl_2$. Medium A is 0.25 M sucrose–0.025 M KCl–0.004 M $MgCl_2$–0.05 M Tris buffer, pH 7.4.

the 5′ end of the 5S RNA molecule, after elution of the 2′,3′-guanylic acid peak, a linear gradient of 0.5–4.0 M formic acid (75 ml of each concentration) was applied, followed by stepwise elution with 4.0 M formic acid (90 ml), 0.2 M ammonium formate in 4.0 M formic acid (55 ml), 0.4 M ammonium formate in 4.0 M formic acid (70 ml), and 0.8 M ammonium formate in 4.0 M formic acid (125 ml) (modified from Hurlbert *et al.*, 1954). Aliquots were dried on planchets under an infrared lamp (under these conditions the ammonium formate was removed) and counted in a low-background gas-flow counter. Fractions of the eluate to be further analyzed were lyophilized (this step likewise resulted in removal of virtually all the ammonium formate).

Oligonucleotide mapping after pancreatic ribonuclease digestion was carried out as previously reported (Amaldi and Attardi, 1968). Elution of the RNase digestion products up to trinucleotides and first tetranucleotides was carried out with a nonlinear concentration gradient of NH_4HCO_3 buffer formed by means of a Varigard six-chamber system as previously described (Amaldi and Attardi, 1968); after about 800 ml of eluate had been collected (the molarity of NH_4HCO_3 buffer in the effluent being 0.40–0.50 M, as determined by a direct conductivity measurement), a new nonlinear concentration gradient of NH_4HCO_3 buffer (pH 8.6) was formed by using four chambers containing each 150 ml of buffer at the following concentrations: 0.40–0.50, 0.60, 1.0, and 1.5 M.

For the identification of the various peaks of the DEAE-cellulose chromatographic patterns, individual components, after removal of NH_4HCO_3 by brief heating at 80° (Amaldi and Attardi, 1968), were hydrolyzed with 0.5 M NaOH for 16 hr at 30° or digested with T1 ribonuclease (Sankyo Co., Ltd., Tokyo) at a concentration of 6 units/ml in 0.025 M Tris buffer (pH 7.4) for 16 hr at 30°; the digestion products were then run through Dowex 1-X8 (formate form). In some cases, the component under investigation was treated with *E. coli* alkaline phosphatase (Worthington Biochemical Corp., Freehold, N. J.) for 1 hr at 37° at a concentration of 3 units/ml in 0.04 M Tris buffer (pH 8.3) in the presence of 2 mg/ml of unlabeled 2′,3′-ribonucleotides, then extracted with phenol, and digested with snake venom phosphodiesterase (Worthington Biochemical Corp.) for 20 min at 22° at a concentration of 0.20 mg/ml in 0.05 M Tris buffer (pH 7.4) with 0.15 mg per ml added unlabeled 5′-guanylic acid (to minimize the effects of any possible 5′-nucleotidase activity) (modified from Holley *et al.*, 1964); subsequent to this treatment, the material was analyzed on a Dowex 1-X8 column.

As an aid in the resolution of mixed-oligonucleotide peaks, chromatography on columns of DEAE-Sephadex A-25 was employed. The sample was charged onto the column (0.6 × 20 cm), then eluted with a 200-ml linear gradient of 0–0.40 M NaCl in 7 M urea–0.02 M Tris (pH 7.6) (Takanami, 1967). Fractions of 2 ml were collected at a flow rate of 11 ml/hr at 22°, then analyzed by scintillation counting of an 0.50-ml aliquot in 15 ml of Bray's solution. The nucleotide composition of peaks from the column was determined, after removal of salt and urea on columns of Bio-Gel P-2 (Bio-Rad, Richmond, Calif.), by alkali digestion and Dowex 1-X8 chromatography.

Chromatography on DEAE-Sephadex A-25, as described above, was also utilized to analyze the products larger than mononucleotides produced by alkali digestion of ^{32}P-labeled 5S RNA.

Chromatography on Dowex 1-X2 (Takanami, 1967) was used for the identification of the component(s) eluting in the region of 5′-GDP on Dowex 1-X8. The appropriate fractions from the Dowex 1-8X column were pooled, lyophilized, incubated 18 hr at 37° in 0.5 N KOH, and neutralized with $HClO_4$; the resulting precipitate of $KClO_4$ was removed by centrifugation and washed twice with water (2°), and the washes were combined with the first supernatant. The pooled supernatant, together with 0.5 mg each of unlabeled 5′-ADP, 5′-UDP, and 5′-CDP, was loaded on a 0.5 × 17 cm column of Dowex 1-X2 (minus 400 mesh), and 2.5-ml fractions were eluted with a 100-ml linear gradient of NaCl (0–0.25 M) in 0.01 N HCl. After optical density measurement, fractions were dried at 100° in glass scintillation vials, 2,5-diphenyloxazole–1,4-bis[2-(5-phenyloxazolyl)]benzene–toluene scintillation mixture was added, and radioactivity in ^{14}C and ^{32}P was measured in a scintillation counter.

Liberation of free bases from RNA and DNA by $HClO_4$ treatment and subsequent analysis on Dowex 50 (Cl) columns were performed as outlined previously (Brown and Attardi, 1965). The RNA sample was hydrolyzed with 0.1 ml of 70% $HClO_4$ at 100° for 1 hr, diluted with 0.3 ml of water, and neutralized with KOH; the insoluble $KClO_4$ was removed by centrifugation and the pellet was washed twice with water (2°); these washings were combined with the original supernatant (final volume about 1 ml). This was brought to 0.5 N HCl and loaded on a 0.55 × 5 cm Dowex 50 column (prepared in a disposable Pasteur pipet). Elution was carried out with 2.0 N HCl at a flow rate of 5 ml/hr; 1-ml fractions were collected and analyzed as described for the Dowex 1-X2 column above. This technique was also utilized in the identification of the bases at the 3′ and 5′ ends of the 5S molecule.

Results

A. Purification of 5S RNA. Purification of HeLa 5S RNA by gel filtration through long columns of Sephadex G-100 was carried out as described in Materials and Methods (section D). Figure 1a shows a typical elution pattern of ^{32}P-labeled RNA from the ribosome–polysome fraction, with three peaks appearing in this order: high molecular weight rRNA and mRNA, 5S RNA, and 4S RNA. A rerun of the central fractions of the 5S peak through Sephadex G-100 resulted in an effective purification of this component (Figure 1b). Reconstruction experiments performed with labeled high molecular weight RNA added to unlabeled RNA from the ribosome–polysome fraction showed the absence of any appreciable contamination of the 5S peak by degradation products of high molecular weight rRNA and mRNA arising during the chromatographic run.

In order to investigate the possible contribution of mRNA to the 5S component, RNA extracted from free polysomes of cells labeled with [^{32}P]orthophosphate for 45 min (*i.e.*, at a time when about 70% of the labeled RNA heavier than 5S is in mRNA, and the rest in 18S rRNA) was analyzed by Sephadex chromatography. As shown in Figure 2, a small amount of radioactivity (about 4% of that present in the void volume) was eluted in close correspondence with the 5S optical density profile. The nucleotide composition showed that this labeled RNA was markedly different from mRNA extracted from cells labeled for 30 min with [^{32}P]orthophosphate (at a time when there is no appreciable 18S labeling)

TABLE I: Nucleotide Composition of Fractions of Free Polysomal RNA from ^{32}P Pulse-Labeled HeLa Cells.[a]

RNA Fraction	Labeling Time (min)	Mole %					
		Cp	Ap	Up	ψp	Gp	G-C
High molecular weight RNA	45	26.3	23.3	20.9	2.2	27.4	53.7
5S RNA	45	26.4	21.0	24.0		28.5	54.9
4S RNA	45	33.5	17.0	16.1	4.0	29.6	63.1
mRNA[b]	30	21.4	24.8	27.9		25.8	47.2

[a] RNA was extracted from free polysomes of HeLa cells labeled for 45 min with [^{32}P]orthophosphate, as described under Materials and Methods, and chromatographed on Sephadex G-100. The ^{32}P-labeled RNA which was eluted in correspondence with the OD_{260} peaks of high molecular weight RNA, 5S RNA, and 4S RNA, respectively, was analyzed for nucleotide composition as described in Materials and Methods (section G). Sedimentation analysis on a sucrose gradient of 45-min ^{32}P pulse-labeled polysomal RNA showed that, after this labeling time, about 30% of the radioactivity sedimenting faster than 5 S is in 18S RNA, the balance being associated with mRNA. [b] Attardi and Attardi (1967).

and rather similar to fully labeled 5S RNA (Table I). Base composition of the high molecular weight RNA was fairly close to that expected for a mixture of mRNA and 18S RNA; the pulse-labeled "4S" material had a base composition somewhat different from that of uniformly labeled 4S RNA.

When centrifuged through a sucrose gradient in sodium acetate–NaCl buffer, the 5S component isolated by two cycles of Sephadex chromatography moved as a fairly sharp peak sedimenting about 20% faster than the 4S marker, sometimes with a small amount of trailing material (Figure 3a). Also after exposure to denaturing conditions (heat (Figure 3b) or formaldehyde treatment (Figure 3c)) the 5S RNA sedimented as a homogeneous component, running ahead of the 4S RNA, in general with a small tail. These experiments indicated that the 5S component isolated by gel filtration is sub-

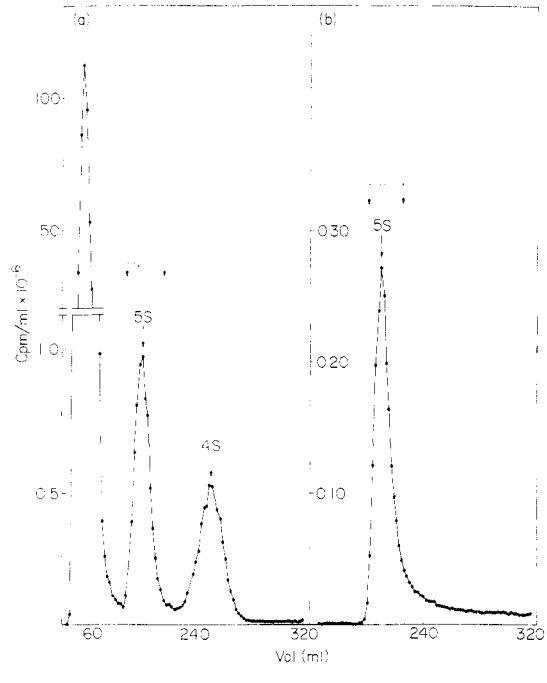

FIGURE 1: Purification by gel filtration through Sephadex G-100 of HeLa 5S RNA. (a) RNA was phenol extracted from the ribosome–polysome fraction of HeLa cells labeled with [^{32}P]orthophosphate, then run through a Sephadex G-100 column equilibrated with 0.1 M NaCl. (b) The fractions indicated by arrows in part a were pooled, precipitated with ethanol, dissolved in 0.1 M NaCl, and rerun through Sephadex G-100.

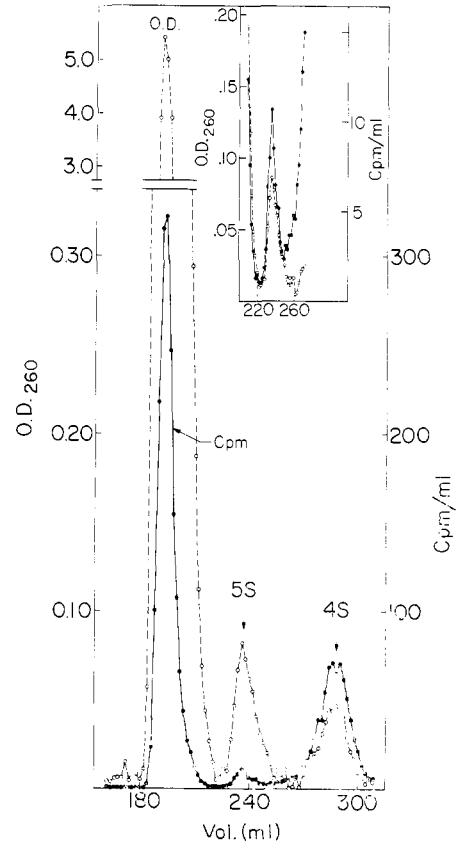

FIGURE 2: Gel filtration through Sephadex G-100 of RNA phenol-extracted from polysomes of HeLa cells labeled for 45 min with [^{32}P]orthophosphate. The insert shows on a larger scale the elution pattern in the 5S region. (O---O) OD_{260} and (●—●) counts per minute of ^{32}P.

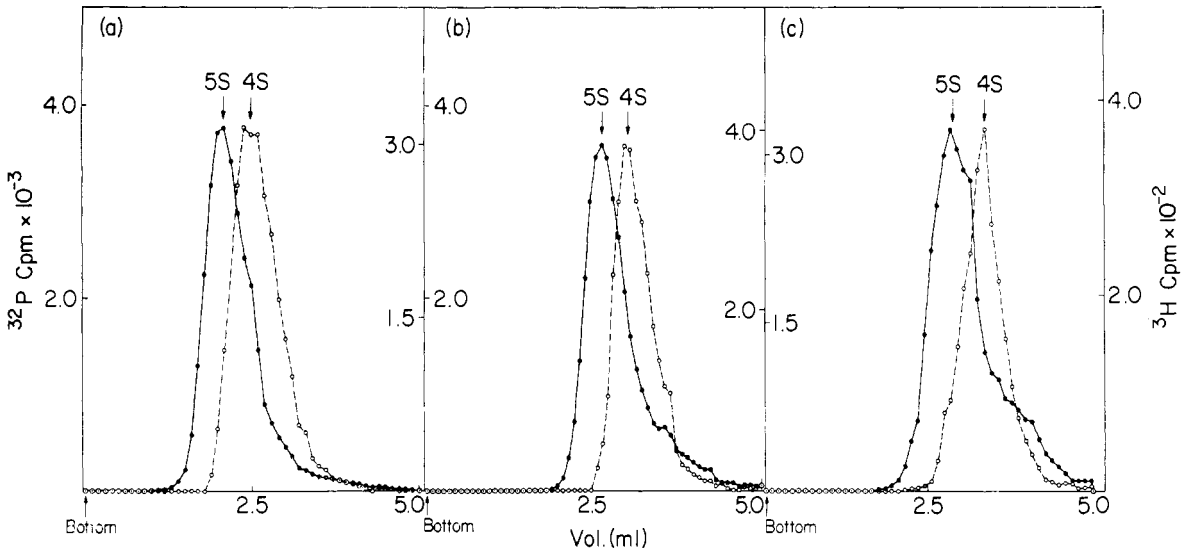

FIGURE 3: Sedimentation analysis of 5S RNA under denaturing conditions. (a) Purified ^{32}P-labeled 5S RNA and ^{3}H-labeled 4S RNA were run through a 5–20% sucrose gradient in NaCl-acetate buffer in a Spinco SW-65 rotor at 62,000 rpm for 11.5 hr at 4°. (b) ^{32}P-labeled 5S RNA and ^{3}H-labeled 4S RNA were heat denatured in low ionic strength buffer and run through a 5–20% sucrose gradient in the same buffer under conditions of centrifugation described in part a. (c) ^{32}P-labeled 5S RNA and ^{3}H-labeled 4S RNA were denatured with formaldehyde and run in a 5–20% sucrose gradient in the presence of formaldehyde under the conditions of centrifugation specified in part a. (●——●) counts per minute of ^{32}P and (O----O) counts per minute of ^{3}H.

TABLE II: Nucleotide Composition of RNA Species from HeLa Cells.[a]

RNA Component	Mole %					1 N HCl Eluate (% of Total)	No. of Determ
	Cp	Ap	Up	ψp	Gp		
^{32}P 4 S	28.4	19.0	17.7	3.9	31.0	7.0	(3)
^{32}P 5 S	28.7	18.8	22.9	<0.03	29.7	3.3	(5)
Unlabeled 5 S	29.0	18.3	22.3		30.3		(1)
^{32}P 18 S[b]	27.3	20.2	20.5	1.52	30.5	5.1	(4)
^{32}P 28 S[b]	32.3	16.0	15.6	1.10	35.0	2.3	(5)

[a] The nucleotide composition of the unlabeled 5S RNA was determined from the optical density measurements on the basis of the extinction coefficients of the four 2′,3′-nucleotides. (The extinction coefficients reported by Beaven *et al.* (1955) were used, after correction for the different pH of the individual elution media.) The nucleotide composition of the ^{32}P-labeled RNA components was determined from the distribution of label among the 2′,3′-nucleotides; the amount of radioactivity eluted with 1 N HCl was not included in this calculation. The proportion of pseudouridylic acid was determined by two successive reruns through Dowex 1-X8 (Amaldi and Attardi, 1968). [b] Amaldi and Attardi (1968).

stantially homogeneous in sedimentation properties, with the great majority of the molecules being represented by continuous polynucleotide chains.

B. Nucleotide Composition of 5S RNA. Table II shows the nucleotide composition of ^{32}P-labeled and unlabeled 5S RNA and, for comparison, that of 4S, 18S, and 28S RNA from HeLa cells. No pseudouridylic acid (<0.03%) was detected in 5S RNA. The majority of the radioactive material eluted with 1 N HCl could be accounted for by the 2′,5′–3′,5′-nucleoside di-, tri-, and tetraphosphates released by alkali digestion from the 5′ end of the 5S molecules (see section D).

The 5S RNA isolated from cells labeled for two generations in the presence of L-[*methyl*-^{14}C]methionine showed a small amount of radioactivity incorporated (Figure 4). This low level of labeling was previously observed by others, but not further analyzed (Knight and Darnell, 1967); if associated with methyl groups, this would have corresponded to 1 methyl group/200 nucleotides, or less than 1 methyl group/5S molecule. Analysis of the distribution of ^{14}C among the bases liberated by perchloric acid treatment showed that the radioactivity was eluted in perfect correspondence with the guanine and adenine marker peaks, and was therefore presumably associated with the purine rings (as a result of the contribution of the *methyl*-^{14}C group of methionine to the pool of 1-carbon compounds used in purine biosynthesis (Figure 5)). Likewise, in a Dowex 1-X8 chromatography run of alkali-digested L-[*methyl*-^{14}C]methionine-labeled 5S RNA, the radio-

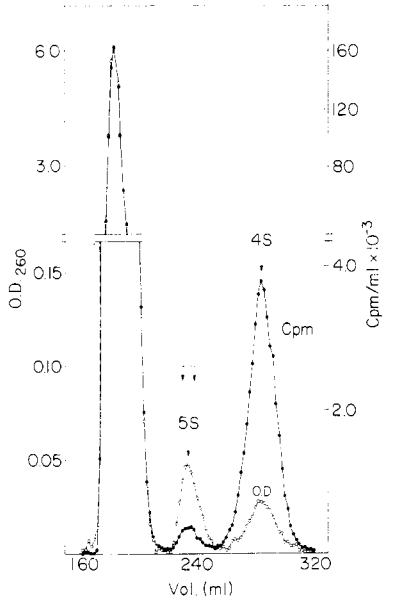

FIGURE 4: Gel filtration through Sephadex G-100 of RNA phenol extracted from the ribosome–polysome fraction of HeLa cells labeled for two generations with L-[*methyl*-^{14}C]methionine. The fractions indicated by arrows were utilized for nucleotide and base analysis (see Figure 5, Table III, and text). (O——O) OD_{260} and (●—●) cpm ^{14}C.

activity profile coincided with the optical density peaks of the marker adenylic and guanylic acids; the 1 N HCl eluate, which would contain any ribose-methylated oligonucleotides, had only 2.5% of the label, *i.e.*, the amount expected to be associated with the 5′-terminal guanosine di-, tri-, and tetraphosphates (see section D). As a control, the specific activity

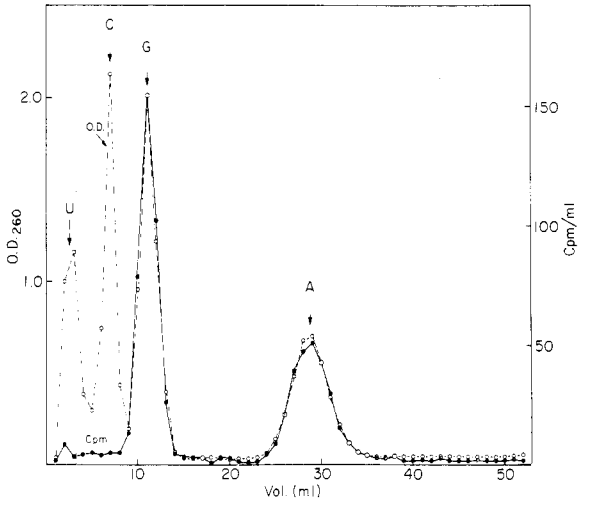

FIGURE 5: Separation on Dowex 50 (Cl^-) of the products of perchloric acid hydrolysis of 5S RNA isolated from cells labeled with L-[*methyl*-^{14}C]methionine. Fractions indicated by arrows in the Sephadex G-100 pattern of Figure 4 were pooled and ethanol precipitated, and the final precipitate was dissolved in water, then lyophilized. After perchloric acid hydrolysis, the bases were separated on Dowex 50 (Cl^-) as detailed in Materials and Methods (section G). U, uracil; C, cytosine; G, guanine; A, adenine. (O----O) OD_{260} and (●—●) cpm ^{14}C.

TABLE III: Specific Activity of Bases in 5S RNA from Methionine-Labeled HeLa Cells.[a]

Base	Specific Activity (dpm/μmole) Found	Expected for 100% of Label in Purine Rings[b]
Adenine	225,000	230,000
Guanine	201,000	210,000

[a] 5S RNA was isolated from HeLa cells grown for two generations in the presence of L-[*methyl*-^{14}C]methionine (Figure 4) and the distribution of radioactivity among its bases analyzed by perchloric acid treatment–Dowex 50 (Cl^-) chromatography (Figure 5). Since insufficient amounts of 5S RNA were available to permit accurate optical density measurements, the specific activities of adenine and guanine were calculated indirectly. For this purpose, the proportion of ^{14}C radioactivity in adenine (40%) and guanine (58%) in the Dowex 50 (Cl^-) pattern was determined. From this information, from the known molar content of adenine (18.7%) and guanine (30.3%) in 5S RNA (Table II; the guanylic acid content was corrected to include the 5′-terminal guanosine di-, tri-, and tetraphosphates eluted with HCl (see section D of Results)), and from the specific activity of [*methyl*-^{14}C]methionine-labeled 5S RNA (105,000 dpm/μmole), the specific activities of adenine and guanine could be calculated as follows: specific activity of adenine = (105,000)·(0.40)/(0.187) dpm/μmole and specific activity of guanine = (105,000)(0.58)/(0.303) dpm/μmole. [b] Determined from the labeling of adenine and guanine in DNA extracted from the same batch of L-[*methyl*-^{14}C]methionine-labeled cells as used for 5S RNA analysis (see text). The bases were liberated by perchloric acid treatment of DNA and chromatographed on Dowex 50 (Cl^-). The specific activities were determined directly from the radioactivity and OD_{260} measurements by using the extinction coefficients of Beaven *et al.* (1955), corrected for the pH of the sample.

of adenine and guanine in DNA from the same preparation of labeled cells was determined (HeLa DNA is known not to contain any unusual methylated bases besides 5-methylcytosine (Brown and Attardi, 1965)); by using these specific activity values and the base composition of 5S RNA, essentially all the radioactivity in the 5S component could be accounted for by the labeling of the purine rings (Table III).

C. Oligonucleotide Mapping of 5S RNA after Pancreatic Ribonuclease Digestion. Figure 6 shows a typical DEAE-cellulose chromatographic pattern of the pancreatic RNase digestion products of 5S RNA. The components in the numbered peaks were identified as described in Materials and Methods (section G). The data for the alkali and T1 ribonuclease digestion products of fractions corresponding to the various peaks are given in Table IV; Table V summarizes the results of additional tests carried out for the identification of some pancreatic RNase digestion products. Peaks numbered 1–8, 11, 12, 14, and 16 were identified in the previously published partial sequence analysis of 18S and

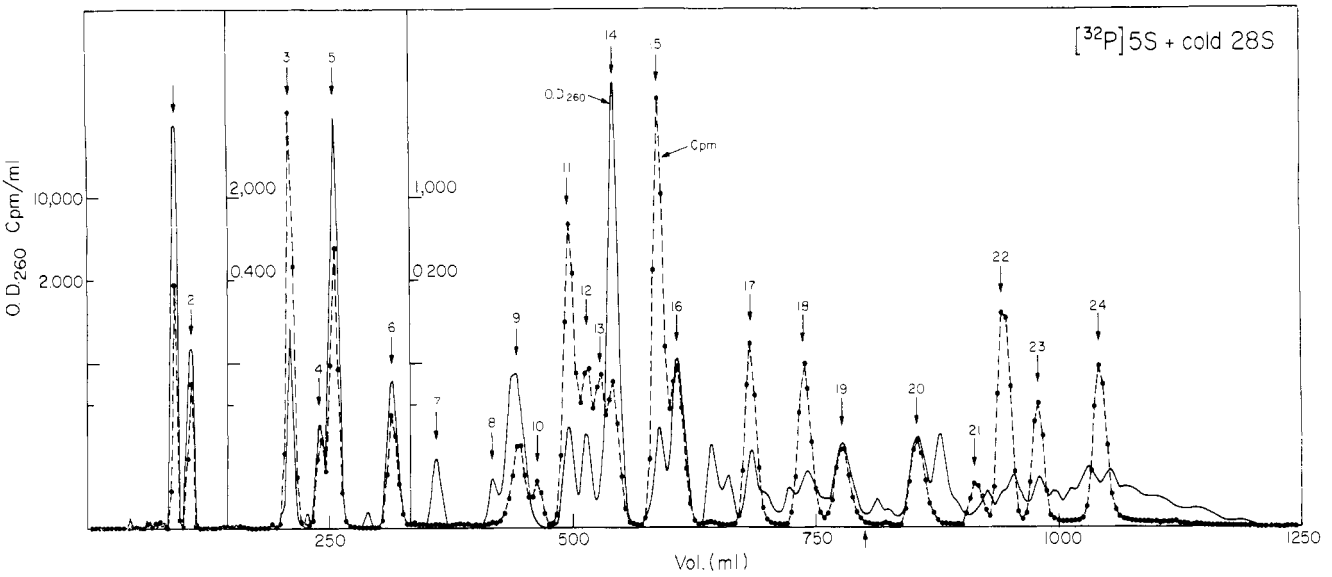

FIGURE 6: Typical pattern of DEAE-cellulose chromatography of the pancreatic RNase digest of a mixture of ^{32}P-labeled 5S RNA and unlabeled 28S RNA from HeLa cells. (See Amaldi and Attardi (1968) and Materials and Methods (section G) for details concerning the conditions of RNase digestion and chromatography.) The arrow on the axis of the abscissa indicates the position where the second concentration gradient of NH_4HCO_3 buffer was started. For the identification of the numbered peaks see Table IV. (——) OD_{260} and (●----●) cpm ^{32}P.

TABLE IV: Identification of the Components in the Various Peaks of the DEAE-Cellulose Chromatographic Pattern.[a]

	Alkali Digestion cpm (%)					T1 RNase Digestion cpm (%)					
Peak	Cp	Ap	Up	Gp	1 N HCl Eluate	Cp	Ap	Up	Gp	1 N HCl Eluate	Major Component(s) in Peak
1	98.1	0.3	1.4	0.2	0						Cp
2	<0.1	<0.1	99.8	0.1	0						Up
3	46.0	46.8	0.7	1.9	4.6						ApCp
4	1.0	49.0	47.2	0.9	1.9						ApUp
5	49.4	1.0	2.1	46.5	1.0						GpCp
6	1.0	1.1	47.4	46.2	4.3						GpUp
7	29.3	62.6	1.1	3.2	3.8						ApApCp
8	2.3	55.7	29.5	2.9	9.6						ApApUp
9	30.1	34.2	1.9	30.5	3.4	30.6	1.9	0.1	4.7	62.8	ApGpCp
10	5.1	3.7	32.2	6.4	52.7	2.3	2.3	26.1	4.6	64.6	pGpUp[b]
11	0.6	32.4	31.8	29.5	5.7	0.1	2.3	2.9	25.0	69.8	GpApUp
12	1.0	31.6	31.4	30.1	5.9	0.2	1.5	26.2	5.9	66.2	ApGpUp
13	3.3	6.3	21.1	8.6	60.7	0.3	0.3	17.7	0.1	81.7	pppGpUp[b]
14	31.7	0.7	2.5	62.3	2.8						GpGpCp
15	21.0	44.3	0.9	26.9	6.9	12.7	2.5	0.3	17.0	67.6	ApApGpCp, GpApApCp[b]
16	0.4	4.3	33.0	53.8	8.5						GpGpUp
17	24.6	25.8	1.1	43.9	4.6	22.4	1.8	1.2	25.1	49.4	ApGpGpCp[b]
18	1.6	23.9	25.0	44.8	4.7	0.1	1.4	1.3	44.5	52.8	GpGpApUp
19	25.6	3.6	3.2	64.4	3.2						GpGpGpCp
20	0.8	1.5	26.3	68.3	3.1						GpGpGpUp
21	1.0	23.5	0.8	70.2	4.5	2.2	18.3	4.0	62.0	13.3	GpGpGpAp[b]
22	7.3	24.4	9.7	51.1	7.5	8.6	2.1	10.5	36.0	42.7	(ApGp,Gp,Gp)Up, (Gp,Gp,ApApGp)Cp[b]
23	1.7	30.6	16.5	46.2	5.3	1.2	2.9	5.5	41.3	59.1	GpGpGpApApUp
24	13.2	26.0	1.9	55.6	3.4	3.6	5.2	2.6	47.2	41.0	(Gp,Gp,Gp,ApGp)ApCp[b]

[a] Fractions of the DEAE-cellulose chromatography of the pancreatic ribonuclease digest of ^{32}P-labeled 5S RNA were subjected to alkaline hydrolysis or T1 ribonuclease digestion as described in Materials and Methods (section G). The data pertaining to peaks 1 to 8, 11, 12, 14, and 16 are derived from the previously published sequence analysis of 18 and 28S HeLa cell RNA (Amaldi and Attardi, 1968). [b] These assignments required additional tests (see text and Table V).

TABLE V: Analysis of Components from Peaks 15 and 17 after Special Treatment.

	Peak 15		Peak 17	
	T1 Digestion, DEAE-Sephadex[a]		*E. coli* Alkaline Phosphatase, Then SVP[b]	
Alkali Digestion Products	Mononucleotide Peak, cpm (%)	Trinucleotide Peak, cpm (%)	Enzymatic Digestion Products	cpm (%)
2′,3′-Cp	50.8	15.5	5′-Cp	20.5
2′,3′-Ap	2.9	61.7	5′-Ap	0.9
2′,3′-Up	6.2	2.5	5′-Up	<0.05
2′,3′-Gp	40.0	20.2	5′-Gp	39.8
			Orthophosphate	38.5

[a] Material from peak 15 of the DEAE-cellulose chromatographic pattern was dried down and digested with T1 ribonuclease, and the digest run on DEAE-Sephadex A-25 (see Materials and Methods (section G)). A mono- and a trinucleotide peak were eluted; the material from each peak was desalted on Bio-Gel P-2, incubated 16 hr in 0.5 N NaOH at 30°, and chromatographed on Dowex 1-X8. [b] Material from peak 17 was dried down, treated with *E. coli* alkaline phosphatase, phenol extracted, then treated with snake venom phosphodiesterase (SVP); see Materials and Methods (section G); the resulting enzymatic digest was finally run on Dowex 1-X8.

28S RNA (Amaldi and Attardi, 1968); the relevant data are included for the sake of completeness. Two of the other peaks, 19 and 20, could be identified unambiguously as GpGpGpCp and GpGpGpUp, on the basis of the alkali-digestion data. The determination of the sequences of oligonucleotides in peaks 9, 18, and 23 (as ApGpCp, GpGpApUp, and GpGpGpApApUp, respectively) required the additional analysis of the T1 ribonuclease digestion products. It should be recalled that digestion with T1 ribonuclease splits bonds following Gp residues, releasing 3′-guanylic acid and oligonucleotides ending in Gp; any oligonucleotides produced, as well as any 5′-terminal nucleoside di-, tri-, and tetraphosphates, are eluted with 1 N HCl from Dowex 1-X8.

Peaks 10 and 13 did not correspond to any optical density marker. After alkaline hydrolysis or T1 RNase digestion of material from peak 10, about twice as much radioactivity was eluted with 1 N HCl as was found in uridylic acid; these observations suggested pGpUp as the possible main component of peak 10. Material of peak 13 gave a ratio of radioactivity in 1 N HCl eluate to that in uridylic acid of about 3:1 after alkaline hydrolysis, and of about 4.5:1 after T1 RNase digestion; taking into account the effect of the contamination of peak 13 by adjacent peaks, these results seemed compatible with the identification of this peak as pppGpUp. These tentative assignments of pGpUp and pppGpUp as the main components of peak 10 and 13, respectively, were confirmed by further analysis of the 5′-terminal groups of the 5S RNA molecule (see Results, section D).

Material from peak 15 had the approximate base composition 1 Cp, 2 Ap, 1 Gp upon alkali digestion. When a T1 RNase digest of this material was run on DEAE-Sephadex in urea, with a linear NaCl gradient, a mononucleotide and a trinucleotide peak were obtained, containing 26 and 72% of the radioactivity, respectively. The mononucleotide peak had Cp and Gp in the approximate ratio 1.3:1, while the trinucleotide peak had Ap, Gp, and Cp in the ratios 4:1.3:1. This led to the conclusion that peak 15 was a mixture of the two tetranucleotides GpApApCp and ApApGpCp. The apparent slight excess of GpApApCp over ApApGpCp, which is suggested by the base composition data (Tables IV and V), may be due to a partial separation of the two components in the combined peak (fractions corresponding to the left side of peak 15 were used for the analysis).

Material from peak 17, when subjected to alkali and T1 RNase digestion, gave results consistent with either one or both sequences ApGpGpCp or GpApGpCp. In order to resolve this ambiguity, peak 17 material was first treated with *E. coli* alkaline phosphatase to remove the terminal phosphate, then digested with snake venom phosphodiesterase to liberate 5′-mononucleotides, and finally run on Dowex 1-X8. The results indicated that Ap is the only 5′-terminal nucleotide in the oligonucleotides of peak 17 (Table V). (The higher than expected yield of orthophosphate may be due to a slight nucleolytic activity of the alkaline phosphatase or to incomplete removal of the latter by phenol extraction.) This allowed the unambiguous assignment of ApGpGpCp to these oligonucleotides.

Material from peak 21, both after alkali and T1 RNase digestion, gave a nucleotide composition corresponding to approximately 3 Gp, 1 Ap, thus consistent with the sequence GpGpGpAp. Since ApU (but not ApC) is eluted in the adenylic acid region on Dowex 1-X8 (formate) chromatography (as shown by direct experiments utilizing ^{32}P-labeled, alkaline phosphatase treated ApCp and ApUp) there was the possibility of the existence of a 3′-terminal uridine nucleoside in the oligonucleotides of peak 21. However, phosphatase treatment of the radioactive material eluted in the adenylic acid region after alkali digestion–Dowex 1-X8 chromatography of peak 21 components released substantially all of the label as orthophosphate (eluted after the adenylic acid peak and before the uridylic acid peak in Dowex 1-X8 chromatography). Therefore, the conclusion was reached that peak 21 results from nonspecific action of pancreatic RNase at a bond following Ap (see Discussion).

Peak 22, both after alkali and T1 RNase digestion, appeared to be heterogeneous in composition, with approximately

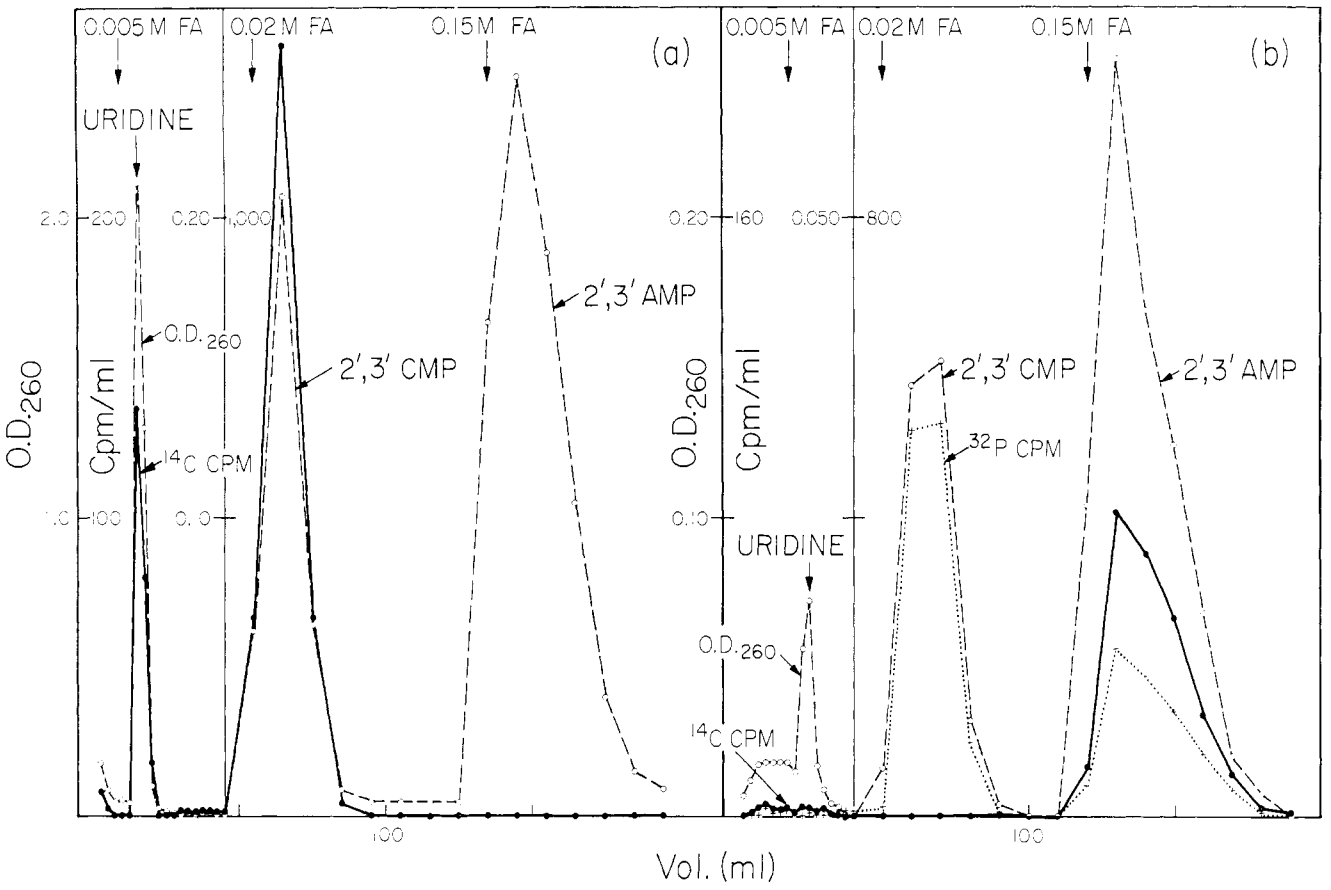

FIGURE 7: Separation on Dowex 1-X8 of nucleosides from mononucleotides in an alkaline digest of 5S RNA extracted from HeLa cells labeled with [2-^{14}C]uridine (a) or doubly labeled with [8-^{14}C]adenosine and [^{32}P]orthophosphate (b). Only the elution pattern of the nucleosides and the first two mononucleotides is shown. FA, formic acid; (O----O) OD_{260}, (●——●) counts per minute of ^{14}C, and (+....+) counts per minute of ^{32}P.

equimolar amounts of components terminated with Up and Cp. A comparison of the proportion of the individual nucleotide components released by alkali and T1 RNase treatment revealed that about 3 Ap and 2 Gp (taking the terminal Up or Cp as unity) were not eluted in the mononucleotide region upon Dowex 1-X8 chromatography of the T1 RNase digest; this suggested the presence of ApApGp and ApGp among the T1 RNase digestion products. The average length of oligonucleotides in peak 22, estimated from the proportion of pyrimidine nucleotides after alkali and T1 RNase digestion, was 5.9 and 5.2, respectively (mean of 5.5), hinting at the possible presence of a pentanucleotide and a hexanucleotide in the peak. This was confirmed by DEAE-Sephadex chromatography in urea of peak 22 material; two peaks of almost equal size were resolved here, one in the pentanucleotide region, and the other in the hexanucleotide region (estimated with reference to the position of a known trinucleotide run in parallel, on the basis of the salt concentration required for elution). This information, together with the approximate 3:1 ratio of Gp to 3'-terminal pyrimidine nucleotides in the two components, permitted the partial identification of the sequences as (ApGp,Gp,Gp)Up and (ApApGp,Gp,Gp)Cp.

Peak 24 material after alkali digestion gave a nucleotide composition corresponding to approximately 1 Cp, 2 Ap, and 4 Gp, suggesting the presence of a heptanucleotide. After T1 RNase digestion, about 41% of the radioactivity was eluted with 1 N HCl, 47% with Gp, and the rest was distributed among the other mononucleotides. DEAE-Sephadex chromatography of the T1 RNase digest gave a mononucleotide and a dinucleotide peak, without any indication of a trinucleotide peak, thereby tending to exclude the presence of the partial sequences ApApCp and ApApGp (the latter was also incompatible with the T1 RNase digestion data, in view of the low release of Cp). These results suggested the occurrence of ApGp and ApCp in the component of peak 24, therefore permitting the partial identification as (ApGp,Gp,Gp,Gp)-ApCp. The low recovery of radioactivity in the 1 N HCl eluate after T1 RNase digestion, relative to that which would be expected on the basis of this assignment, remains unexplained.

D. Identification of 3' and 5' Ends of 5S RNA. Identification of the 3' end of the 5S RNA molecule was performed with RNA extracted from cells exposed for 48 hr to [2-^{14}C]uridine or [8-^{14}C]adenosine. When an alkaline digest of the [2-^{14}C]-uridine-labeled 5S RNA was run on Dowex 1-X8 (formate form), about 1.5% of the total radioactivity was eluted with 0.005 M formic acid, almost all in coincidence with the uridine marker (Figure 7a); in agreement with this latter finding, at least 95% of the radioactivity in the 0.005 M formic acid eluate was recovered as uracil after perchloric acid hydrolysis–Dowex 50 (Cl^-) chromatography (Figure 8a). When 5S RNA

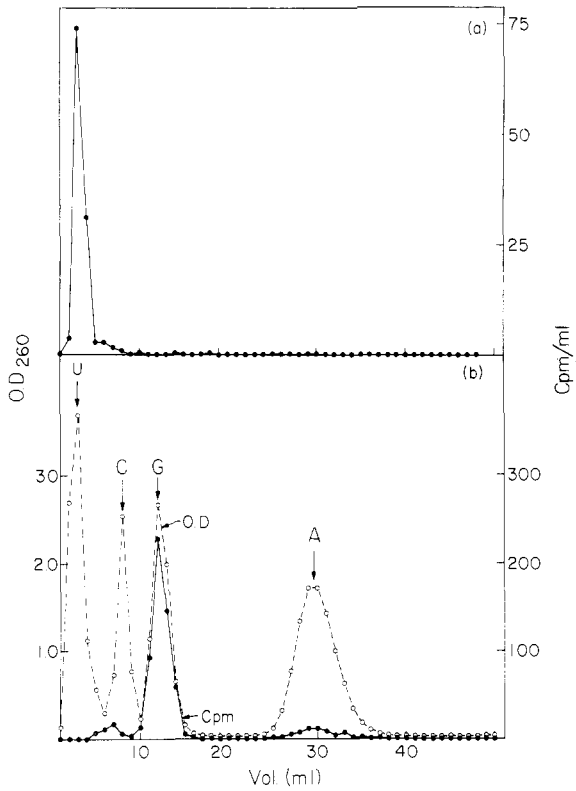

FIGURE 8: Separation on Dowex 50 (Cl^-) of the bases released by perchloric acid treatment of the material eluted with 0.005 M formic acid and 1 N HCl on Dowex 1-X8 chromatography of 5S RNA alkaline digests. (a) The material eluted with 0.005 M formic acid on Dowex 1-X8 chromatography of alkali-digested 5S RNA from HeLa labeled with [2-^{14}C]uridine was hydrolyzed with perchloric acid in the presence of carrier RNA, then chromatographed on Dowex 50 (Cl^-) as described in Materials and Methods (section G). (b) The material eluted with 1 N HCl on Dowex 1-X8 chromatography of alkali-digested 5S RNA from HeLa cells labeled with [8-^{14}C]adenosine was treated as in part a. (O----O) OD_{260} and (●—●) cpm ^{14}C.

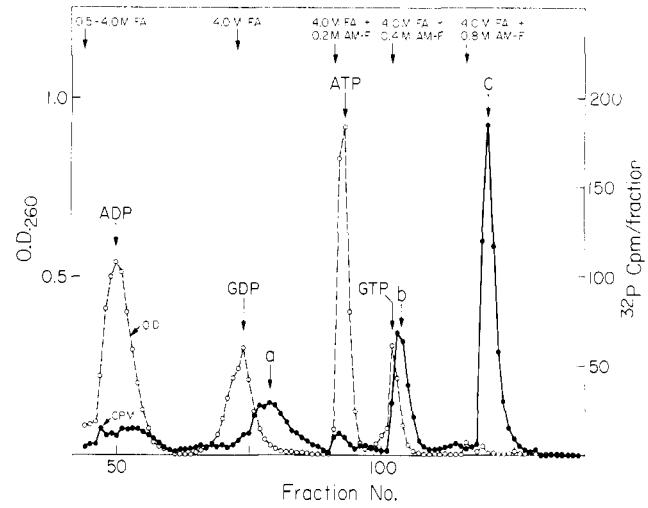

FIGURE 9: Elution profile after 2′,3′-guanylic acid in a Dowex 1-X8 chromatography of 5S RNA extensively digested with alkali. HeLa 5S RNA from cells labeled with [^{32}P]orthophosphate was digested for 36 hr in 0.5 N NaOH and chromatographed on Dowex 1-X8; after elution of 2′,3′-guanylic acid, elution with high concentrations of formic acid–ammonium formate was carried out as described in Materials and Methods (section G). AM-F, ammonium formate. (O- - - -O) OD_{260} and (●—●) cpm ^{32}P.

doubly labeled with [8-^{14}C]adenosine and [^{32}P]orthophosphate was alkali digested and chromatographed on Dowex 1-X8, only a very small amount of the ^{14}C (0.20%) and ^{32}P (0.06%) label was eluted with 0.005 M formic acid, without any indication of definite peaks (Figure 7b). These results pointed to uridine as the 3′-terminal nucleoside of 5S RNA from HeLa cells.

After Dowex 1-X8 chromatography of an alkaline digest of ^{32}P-labeled 5S RNA, about 3.3% of the radioactivity was eluted with 1 N HCl. This eluate was expected to contain, besides any purine-rich alkali-resistant oligonucleotides (Lane and Butler, 1959), any nucleoside di-, tri-, and tetraphosphate pertaining to the 5′ end of the molecule. A calculation showed that only 1.7% of the total ^{32}P radioactivity could be accounted for by 5′ ends if these were in the form of nucleoside 3′,5′-diphosphates (assuming a length of 120 nucleotides for HeLa 5S RNA, as in 5S RNA from KB cells (Forget and Weissman, 1967a) and from *E. coli* (Sanger *et al.*, 1967)); the likely existence of pGp and pppGp at the 5′ ends of the 5S RNA molecule had been indicated by the results of DEAE-cellulose chromatography of 5S RNA pancreatic RNase digests (Results, section C). After Dowex 1-X8 chromatography of [8-^{14}C]adenosine-labeled 5S RNA, about 2.1% of the radioactivity was eluted with 1 N HCl; base analysis of this material revealed 84% of the radioactivity to be associated with guanine and 10% with adenine (Figure 8b and Table VI). By contrast, less than 0.1% of the radioactivity was found in the 1 N HCl eluate when 5S RNA labeled with [2-^{14}C]uridine was used (Table VI).

In order to analyze the nature of the components not eluted with the mononucleotides, a system of elution with higher concentrations of formic acid and ammonium formate (as detailed in Materials and Methods, section G) was applied to the Dowex 1-X8 after elution of 2′,3′-guanylic acid. The pattern obtained (Figure 9) showed, in addition to some radioactive material eluted with increasing concentration of formic acid before GDP (with a broad peak in the region of ADP), three well-defined labeled peaks: the first (a), rather broad, eluted with 4.0 M formic acid after GDP; the second (b) eluted with 0.4 M ammonium formate in 4.0 M formic acid immediately after GTP; and the third one (c) eluted with 0.8 M ammonium formate in 4.0 M formic acid. The radioactive material eluted before GDP was found in variable amounts in different runs (from 0.5 to 1% of the total ^{32}P label), and could be substantially reduced (by as much as 70%) by prolonging the time of digestion with alkali to 36 hr. The 5S RNA extracted from cells labeled with [8-^{14}C]-adenosine showed a considerable amount of radioactivity (0.8–1.3%) eluted in this region; base analysis (Table VI) revealed a high proportion of adenine in these components. On the other hand, a negligible amount of radioactivity (<0.05%, Table VI) was found in this region of the chromatographic pattern when 5S RNA from [2-^{14}C]uridine-labeled cells was used. These results suggest that the components eluted before GDP consist mainly of purine-rich (in particular, adenine-rich) alkali-resistant oligonucleotides (Lane and Butler, 1959). A series of experiments were carried out to investigate the nature of the components a, b, and c. Peak a

TABLE VI: Analysis of Peaks Eluted after Mononucleotides in the Dowex 1-X8 Chromatographic Patterns of 5S RNA Alkaline Hydrolysates.

Fraction	RNA Doubly Labeled with [^{32}P]Orthophosphate and [8-^{14}C]Adenosine			RNA Labeled with [8-^{14}C]-Adenosine	RNA Labeled with [2-^{14}C]-Uridine	RNA Labeled with [8-^{14}C]-Adenosine	Probable Composition of main component(s)
	Molar Ratio of Phosphate to Guanine[c]	% ^{32}P Released by Alkaline Phosphatase as Orthophosphate	% ^{14}C Released by Alkaline Phosphatase as Nucleosides	% of Total cpm		% cpm in Bases	
Total material eluted with 1 N HCl[a]				2.13	<0.1	G, 83 A, 10	
Material eluted with 0.5–4.0 M formic acid before GDP[b]				0.8–1.3	0.05	G, 14 A, 69	Alkali-resistant nucleotides
a	1.6			0.33	nd	G, 87 A, 7	pGp
b	2.6	>95	>95	0.31	nd	G, 93 A, nd	ppGp
c	4.1	>95	>95	0.61	nd	G, 95 A, nd	pppGp

[a] HeLa 5S RNA was isolated from cells grown for two generations in the presence of [8-^{14}C]adenosine or [2-^{14}C]uridine, digested with alkali, and chromatographed on Dowex 1-X8 columns, formate system (Amaldi and Attardi, 1968). The material eluted with 1 N HCl was lyophilized and hydrolyzed with perchloric acid, and the bases were separated by Dowex 50 (Cl^-) system, as shown in Figure 5. [b] Samples of 5S RNA from HeLa cells labeled with [8-^{14}C]adenosine and [^{32}P]orthophosphate were digested with alkali and chromatographed on Dowex 1-X8, as in *a;* after elution of the 2′,3′-guanylic acid peak, a system of elution with higher concentrations of formic acid–ammonium formate was applied, as detailed in Materials and Methods (section G) (Figure 9). Aliquots of individual components in the Dowex 1-X8 chromatographic pattern were lyophilized and analyzed for base composition by perchloric acid treatment and Dowex 50 (Cl^-) chromatography, as in Figure 5. Alkaline phosphatase treatment of material from peak b and c was carried out after lyophilization, as outlined in Materials and Methods (section G); the resulting digest was then chromatographed on Dowex 1-X8, as in *a.* [c] Determined by taking as a reference the ratio of ^{32}P to ^{14}C counts per minute in 2′,3′-guanylic acid.

material was subjected to a second alkali digestion and then chromatographed on a Dowex 1-X2 column; more than 90% of the radioactivity was eluted as two partially resolved peaks between the GDP and UDP markers, in the position expected for 2′- and 3′-pGp (Takanami, 1967). This indicated pGp as the main component of peak a. In agreement with this identification, base analysis of the material from peak a isolated from 5S RNA labeled with [8-^{14}C]adenosine showed that 87% of the label was associated with guanine, and 7% with adenine (Table VI); on the contrary, peak a was not labeled when [2-^{14}C]uridine was used as a precursor. As in the case of peak a, peaks b and c were also found to be labeled when [8-^{14}C]adenosine was used as a precursor, but not when [2-^{14}C]uridine was used; base analysis again indicated that the label was in guanine (Table VI). Alkaline phosphatase treatment of material from these two peaks doubly labeled with [8-^{14}C]adenosine and [^{32}P]orthophosphate caused the release of more than 95% of the ^{32}P radioactivity as orthophosphate and of more than 95% of the ^{14}C radioactivity as nucleosides. These results indicated that none of the phosphates in these components were involved in internucleotide linkages. The molar ratios of phosphate to guanine in the peaks a, b, and c were found to be 1.6, 2.6, and 4.1, respectively, to be compared with the values of 2, 3, and 4 expected for pGp, ppGp, and pppGp. The position of elution of peak b immediately after GTP, analogous to the elution of pGp after GDP, is in agreement with the identification of peak b as ppGp.

Additional evidence in favor of the proposed identification of the 5′-terminal groups of 5S RNA was provided by an analysis by DEAE-Sephadex chromatography of the products larger than mononucleotides produced by alkali digestion of ^{32}P-labeled 5S RNA. As shown in Figure 10, an appreciable amount of radioactivity was eluted in the general region of the marker di-, tri-, and tetranucleotides (provided by a pancreatic ribonuclease digest of [2-^{14}C]uridine-labeled 5S RNA). Of the radioactivity which eluted in correspondence with the dinucleotides (peak I), after second alkali treatment–Dowex 1-X8 chromatography, about 50% was recovered as

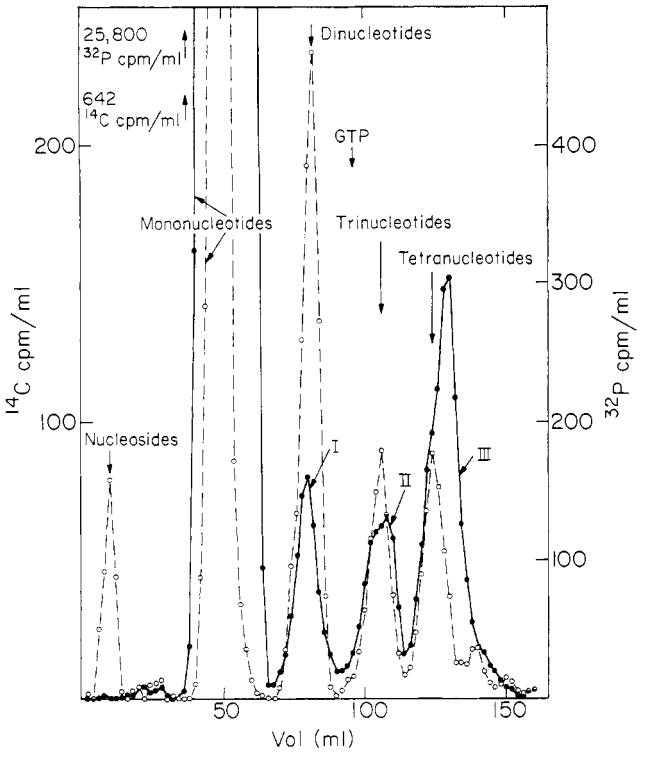

FIGURE 10: Pattern of DEAE-Sephadex chromatography of a mixture of an alkaline digest of ^{32}P-labeled 5S RNA and a pancreatic RNase digest of [2-^{14}C]uridine-labeled 5S RNA. ^{32}P-labeled 5S RNA was digested with 0.5 N KOH at 30° for 16 hr and neutralized with perchloric acid; after removal of the potassium perchlorate by centrifugation, the hydrolysate was mixed with a pancreatic RNase digest of [2-^{14}C]uridine-labeled 5S RNA (1 mg of total RNA, 200 μg of pancreatic RNase, in 0.20 ml of 0.1 M Tris buffer, pH 7.4, 4 hr at 37°), then brought to 7 M urea–0.02 M Tris buffer (pH 7.4) and charged onto a column of DEAE-Sephadex A-25. Conditions of elution and analysis of the various components eluted are as described in Materials and Methods (section G). (O——O) cpm ^{14}C and (●——●) ^{32}cpm P.

adenylic and guanylic acids, and the rest, presumably represented by alkali-resistant dinucleotides, was eluted in the region preceding GDP (see Figure 9). Of the ^{32}P label eluted in the region of trinucleotides from DEAE-Sephadex (peak II), about 20% was recovered after a second alkali treatment as guanylic acid, 56% was eluted in the position of the Dowex 1-X8 corresponding to pGp (immediately following the GDP marker, see Figure 9), and the rest was spread in the region preceding GDP. The elution of nucleoside diphosphates (pXp) with the trinucleotide peak in the DEAE-cellulose chromatographic pattern has been previously reported by Takanami (1966). The last peak of ^{32}P radioactivity in the DEAE-Sephadex pattern (peak III), which was more than twice the size of either peak I or II, was eluted after the tetranucleotide marker in the position previously reported for the elution of pppGp (Roblin, 1968) and pppAp (Takanami, 1966). The small shoulder on the leading edge hinted at the presence of an additional component; it seemed likely that this component was ppGp; in fact the possible elution of ppXp components just prior to pppXp had been suggested by Takanami (1966). The presence of ppGp and pppGp in peak III was confirmed by the analysis of the distribution of ^{32}P radioactivity of this peak after a second alkali treatment and Dowex 1-X8 chromatography; 24% of the label was recovered in the position of elution previously found for ppGp (Figure 9), while 66% was eluted in correspondence with pppGp.

E. Frequency of Nucleotide Sequences in 5S RNA. More than 95% of the original radioactivity associated with 5S RNA was recovered in the DEAE-cellulose chromatographic pattern. The distribution of nucleotides among partial sequences released by pancreatic ribonuclease digestion was calculated from the proportion of ^{32}P radioactivity pertaining to the individual components in the DEAE-cellulose pattern (Table VII). Very similar values for the distribution of nucleotides among partial sequences (up to trinucleotides) were obtained from the ratio of the frequencies of each nucleotide sequence in the 5S RNA to that in the 28S RNA (determined from the specific activity data) and from the distribution of nucleotides among partial sequences in the latter RNA (Amaldi and Attardi, 1968; Jeanteur *et al.*, 1968).

Table VII shows also the average number of each type of sequence per molecule, calculated on the basis of a 5S RNA 120 nucleotides long. An inspection of Table VII shows a reasonably good agreement between the average numbers of different sequences per molecule found in the present work and the corresponding ones previously reported for KB cell 5S RNA (Forget and Weissman, 1967b). The lower than expected molar yield of Cp and Up in the 5S RNA pancreatic RNase digest was attributed by the authors to the losses of 2′,3′-cyclic nucleoside monophosphates. It appears from Table VII that the molar yield of a considerable number of the larger RNase digestion products of HeLa 5S RNA is reproducibly lower than unity, to an extent which may not be accounted for by artifacts of the method. In most cases, deviations in the same direction have been reported for the corresponding sequences in KB cell 5S RNA (Table VII).

The available data strongly suggest that the two unresolved components of peak 22 correspond to ApGpGpGpUp and GpGpApApGpCp isolated from KB cell 5S RNA. The component of peak 24, for which the tentative assignment of (ApGp,Gp,Gp,Gp)ApCp was made, probably corresponds to the GpGpGpApGpApCp identified in the pancreatic ribonuclease digest from KB cell 5S RNA. Two components not reported in the analysis of KB cell 5S RNA were detected in the present work among the oligonucleotides released by pancreatic RNase from HeLa 5S RNA. One of these components, GpGpGpAp, present in a molar yield of 0.33, appears to be due to a nonspecific RNase attack on a bond following Ap (Beers, 1960). The presence of the other component, ApGpCp, in a molar yield of 0.81 is presumably indicative of the existence of more than one sequence of 5S RNA in HeLa cells (see Discussion).

It appears from Table VIII that uridine accounts for nearly all the 3′ termini of the 5S molecules. A small amount of purine nucleosides was actually found at the 3′ end, presumably as a result of some breakage of the 5S chains very near the end (see Discussion). The three groups found at the 5′ end of the 5S molecule, pGp, ppGp, and pppGp, were recovered, after alkali digestion and Dowex 1-X8 chromatography, in an overall amount corresponding to 0.76–0.87 mole/mole of 5S RNA (Table VIII). The recovery figures for pGp and pppGp were rather close to those obtained for the presumptive corresponding RNase digestion products

TABLE VII: Molar Yields of Pancreatic RNase Digestion Products of 5S RNA from HeLa Cells.[a]

Peak	Digestion Products	Distribution of Nucleotides among Digestion Products (%)		Yield (M)[b]	Yield (M) Reported for KB Cell 5S RNA[c]
		Mean	Range		
1	Cp	15.2	15.1–15.4	18.3	13.2[d]
2	Up	11.7	11.3–12.0	14.1	10.1[d]
3	ApCp	8.9	8.5–9.2	5.3	4.8
4	ApUp	2.3	2.2–2.5	1.4	1.3
5	GpCp	7.4	7.2–7.5	4.4	4.2
6	GpUp	3.7	3.6–3.7	2.2	2.2
7	ApApCp	0.12	0.06–0.16	<0.1	
8	ApApUp	0.05	0.03–0.07	<0.1	
9	ApGpCp	2.0	1.80–2.2	0.81	
10	pGpUp	0.70	0.67–0.73	0.28	0.5
11	GpApUp	5.7	5.6–5.8	2.3	1.9
12	ApGpUp	3.1	3.08–3.1	1.2	1.1
13	pppGpUp	1.63	1.53–1.73	0.39	
14	GpGpCp	3.1	2.9–3.3	1.2	0.94
15	ApApGpCp + GpApApCp	7.4	7.0–7.6	2.2	1.03 0.95
16	GpGpUp	3.5	3.3–3.7	1.4	1.15
17	ApGpGpCp	3.6	3.5–3.6	1.1	0.73
18	GpGpApUp	3.6	3.4–3.8	1.1	1.07
19	GpGpGpCp	2.0	1.8–2.2	0.60	0.76
20	GpGpGpUp	2.1	2.0–2.3	0.64	1.01
21	GpGpGpAp	1.09	1.06–1.12	0.33	
22[e]	(ApGp,Gp,Gp)Up + (Gp,Gp,ApApGp)Cp	4.6	4.1–5.1	1.0	0.59 0.97
23	GpGpGpApApUp	2.7	2.3–3.1	0.55	0.8
24[f]	(Gp,Gp,Gp,ApGp)ApCp	3.7	3.5–3.9	0.63	0.57

[a] This table summarizes the data obtained in four different experiments concerning the frequency of partial nucleotide sequences released from 5S RNA by pancreatic RNase digestion, as calculated from the distribution of radioactivity. These experiments involved the use of two different preparations of ^{32}P 5S RNA. [b] A length of 120 nucleotides for HeLa 5S RNA has been assumed in this calculation (Forget and Weissman, 1967). [c] From Forget and Weissman (1967). [d] The lower than expected values were due to the use of relatively low RNase concentrations, resulting in production of 2′,3′-cyclic nucleoside monophosphates, which were not identified in the electrophoresis pattern. [e] Corresponding presumably to sequences ApGpGpGpUp and GpGpApApGpCp in KB cells. [f] Corresponding presumably to the sequence GpGpGpApGpApCp in KB cells.

pGpUp and pppGpUp (peaks 10 and 13) in the DEAE-cellulose chromatographic pattern. The failure to detect ppGpUp in the latter pattern is presumably due to its being hidden under GpApUp or ApGpUp (both recovered in higher than expected yield).

Discussion

The 5S RNA utilized in the present work appeared by several criteria to be substantially free from any contamination by known RNA species. The resolution obtained by Sephadex G-100 fractionation, and the results of reconstruction experiments, excluded the presence in the 5S peak of any appreciable amount of high molecular weight rRNA or degradation products thereof, or of 4S RNA; this conclusion was confirmed by the absence in 5S RNA of pseudouridylic acid or methylated nucleotides, which are known components of those RNA species in HeLa cells (Brown and Attardi, 1965; Amaldi and Attardi, 1968). On the basis of the known content of pseudouridylic acid and methyl groups in rRNA and 4S RNA it can be estimated that the total possible contamination of the 5S preparations by these species was less than 2%. Regarding the possible contamination of 5S by short mRNA chains, the use of a cold chase after the long-term labeling of HeLa cells with an RNA precursor was expected to reduce considerably (from two to eight times) the specific activity of any labile messenger fraction (Penman *et al.*, 1963; Attardi and Attardi, 1967). In order to directly test the possible presence of mRNA in the 5S preparations, RNA was isolated from polysomes of cells subjected to a 45-min ^{32}P pulse. A small amount of labeled RNA (about 4% of the labeled high molecular weight RNA of polysomes) was eluted from the Sephadex G-100 as a fairly sharp peak, in close correspondence with the 5S optical density profile; this RNA had a base composition which was rather similar to that of fully labeled 5S RNA. The possibility that this

TABLE VIII: Molar Yields of 3′- and 5′-Terminal Groups from HeLa 5S RNA.[a]

3′ End			5′ End			
	RNA Labeled with [2-^{14}C]-Uridine	RNA Labeled with [8-^{14}C]-Adenosine		RNA Labeled with [^{32}P]-Orthophosphate		RNA Labeled with [8-^{14}C]-Adenosine
Component	Yield (M)		Component	% of Total cpm	Yield (M)	Yield (M)
Uridine	0.94–0.99		pGp	0.37	0.22	0.22
Adenosine + guanosine		0.07–0.09	ppGp	0.46	0.18	0.22
			pppGp	1.19	0.36	0.43
				Total	0.76	0.87

[a] The molar yield of 3′-terminal groups was determined from the proportion of radioactivity eluted with 0.005 M formic acid on Dowex 1-X8 chromatography of an alkaline digest of 5S RNA isolated from cells labeled with either [2-^{14}C]uridine or [8-^{14}C]-adenosine (see Figure 7), by assuming a length of 120 nucleotides for the 5S RNA molecule. As reference values for these calculations, the amount of radioactivity and mole per cent of uridylic acid in 5S in the former case, and the amount of radioactivity and mole per cent of adenylic acid (lower value) or of guanylic acid (higher value), in the latter case, were used. (After long labeling with [8-^{14}C]adenosine, adenylic acid was found to have 1.4 times the specific activity of guanylic acid.) The molar yield of the 5′-terminal groups was determined from the proportion of total radioactivity eluted in peaks a, b, and c (Figure 9) after chromatography on Dowex 1-X8 of an alkaline digest of 5S RNA labeled with [^{32}P]orthophosphate or [8-^{14}C]adenosine; in the latter case the amount of radioactivity and mole per cent of guanylic acid were used as reference values.

pulse-labeled "5S" material represents some precursor of tRNA (Burdon and Clason, 1969), is practically excluded by the difference in base composition from "4S" labeled in a short pulse, and especially by the fact that the RNA was extracted from polysomes which would be expected to contain only mature tRNA. It seems possible that the "5S" material labeled after a 45-min pulse represents newly synthesized 5S rRNA. This would imply that under the conditions used in this experiment labeled 5S RNA arrives in the polysomes prior to the appearance of 28S rRNA, a conclusion which is at variance with the results reported by Knight and Darnell (1967). No explanation can at present be given for this discrepancy, although the metabolic imbalance introduced by the phosphate starvation preceding labeling may possibly account for it. The possibility that the pulse-labeled "5S" material represents mRNA seems unlikely both because of the difference in base composition from mRNA and because of the lack of indication of degradation of mRNA which would result in tailing of the high molecular weight peak. If the entire pulse-labeled "5S" material were represented by mRNA, it can be estimated that the contamination of 5S RNA by mRNA in long-term labeled cells would correspond to 10%; this figure would have to be reduced by a factor of 2–8 if the contaminating mRNA had the same half-life as the total mRNA population.

The analysis of the sedimentation properties of 5S RNA in 0.1 M NaCl or under denaturing conditions showed the substantial homogeneity in size and the intactness of the RNA molecules used in the present work.

HeLa cell 5S RNA was shown in the present work not to contain any detectable methylation of the bases or of the ribose moieties. The low level of labeling of this RNA species which was found after long-term exposure of the cells to [*methyl*-^{14}C]methionine, in agreement with a previous report (Knight and Darnell, 1967), was shown here to be completely accounted for by the labeling of purine rings. The lack of methyl groups in HeLa 5S RNA is in agreement with what has been reported for 5S RNA from other sources (Galibert *et al.*, 1965; Comb and Zehavi-Willner, 1967). The absence of methyl groups and of pseudouridylic acid represents a definite structural difference between 5S RNA and the high molecular weight rRNA, the significance of which is at present unknown.

The results obtained on the partial sequence distribution after pancreatic RNase digestion in HeLa cell 5S RNA are generally in good agreement with the published sequence data on 5S RNA from KB cells, another cell line of human origin (Forget and Weissman, 1967b). The oligonucleotides of peak 22, which were only partially identified in the present work, presumably correspond to ApGpGpGpUp and GpGpApApGpCp found in KB 5S RNA; similarly, the component of peak 24 is probably GpGpGpApGpApCp. There are, however, some differences which have been found in the partial sequence data between HeLa and KB 5S RNA. Two components have been detected among the pancreatic RNase digestion products from HeLa 5S RNA which were absent or present only in trace amount in the analysis performed with KB material. One of these components, GpGpGpAp, very likely results from a nonspecific action of pancreatic RNase on a bond following Ap; such nonspecific action of pancreatic RNase has been previously described (Beers, 1960), and may occur to a limited extent when drastic conditions of RNase digestion are used (in order to avoid the accumulation of digestion products containing 2′,3′-cyclic phosphates). It is significant in this respect that a region very susceptible to T1 RNase action has been found in KB

cell 5S RNA, and this region indeed contains the sequence GpGpGpAp (see the heptanucleotide GpGpGpApGpApCp in the paper on KB 5S RNA and the presumptive corresponding oligonucleotide 24 in HeLa 5S RNA (Table VII)). It may not be a coincidence that, as will be discussed below, the sequence GpGpGpApGpApCp was recovered, both from HeLa and KB cell 5S RNA, with a molar yield considerably lower than unity. However, it should be noticed that no evidence was found of the presence of the fragment GpApCp (resulting from the splitting of the heptanucleotide) in the expected position in the DEAE-cellulose chromatographic pattern, either overlapping or immediately preceding peak 9 (Amaldi and Attardi, 1968). On the other hand, it is not excluded that GpGpGpAp may arise from nonspecific splitting of the sequence of GpGpGpApApUp, which is also recovered in molar yield lower than unity, while ApUp is consistently found in a yield significantly higher than unity (1.4).

The other component found among the pancreatic RNase digestion products of HeLa 5S RNA, but present only in trace amount in the analysis of KB 5S RNA (Forget and Weissman, 1967a), is the sequence ApGpCp. The possibility that this digestion product derives from contaminating rRNA or 4S RNA seems to be excluded on the basis of the purity of the 5S preparations utilized in the present work, as judged by the stringent criteria discussed above. Even for mRNA, the presence of small amounts of which is difficult to exclude, a maximum estimate of the contamination would be 5%; this could not possibly account for the amount of ApGpCp recovered, especially when contrasted to the absence of ApApUp and ApApCp, which would be expected to be found in digests of RNA species with high AU content, as mRNA. The possibility that the occurrence of ApGpCp is related to the presence of the unusual digestion product discussed above, GpGpGpAp, in the sense that they both derive from nonspecific cleavage of a hypothetical oligonucleotide, GpGpGpApApGpCp, seems to be excluded by the large difference in molar yield between the two products, and by the fact that the only peak in the DEAE-cellulose pattern containing heptanucleotides (peak 24) was found to consist of oligonucleotides lacking the sequence ApGpCp; furthermore, the difference from unity (0.37) of the molar yield for peak 24 is much too small to account for the appearance of 0.81 mole of ApGpCp. Another possibility to consider is that ApGpCp derives from nonspecific splitting of GpGpApApGpCp (which is the presumptive sequence of the hexanucleotide in peak 22, and which is the only digestion product containing ApGpCp recovered in a molar yield lower than unity). This does not seem to be likely, since no evidence of the presence of the other half of the sequence, GpGpAp, was found; this fragment would be expected to be eluted after GpGpUp, by analogy with GpGpGpAp, which is eluted after GpGpGpUp, and should be easily detected because of its amount (identical with that of peak 9).

A more likely explanation for the occurrence of ApGpCp seems to be the presence of alternate sequences in HeLa 5S RNA, resulting from one or more base changes in one or more of the 5S cistrons. To limit attention to the single base changes which could bring about the appearance of this pancreatic digestion product, there are two possibilities: (1) ApGpCp derives from a different trinucleotide in the 5S chain by a change in one of its bases. This can be excluded by a comparison of the molar yields of the various digestion products for HeLa 5S with those reported for KB cells; this comparison does not show any decrease in the relevant dinucleotides (ApCp, ApUp, GpCp), trinucleotides (ApGpUp, GpGpCp), and tetranucleotides (ApGpGpCp), as expected if the base change discussed here had occurred. The pentanucleotide ApGpGpGpUp which was recovered in molar yield lower than unity is also ruled out as the main source of ApGpCp by the absence of the required concomitant increase in GpUp; however, a change of ApGpGpGpUp to ApGpCpGpUp may contribute some of the ApGpCp.

(2) ApGpCp derives from a purine to pyrimidine change of the base immediately preceding this trinucleotide in the 5S chain. A detailed analysis of the complete sequence of KB 5S RNA and of the partial sequence data obtained in the present work, together with a consideration of the molar yields found for the different RNase digestion products, suggests that the only sequence which could produce by a single mutation the sequence PypApGpCp is the presumptive sequence of peak 22, GpGpApApGpCp; the occurrence of two alternative forms of this sequence, with the first Ap being substituted by Up or Cp, would account at the same time for the molar yield remarkably lower than unity obtained for GpGpApApGpCp, for the appearance of the new ApGpCp peak, and, finally, for the molar yields higher than unity of GpGpCp and GpGpUp. In this respect, it should be noticed that GpGpUp was recovered in molar yield greater than unity also in chromatographic patterns where it was completely resolved from the adjacent peaks. The recovery of GpGpApApGpCp estimated on the basis of the ratio of Cp to Up in the alkaline and T1 digests of material from peak 22 was about 0.45 mole/mole of 5S RNA; splitting of the missing 0.55 mole would account for about 70% of the ApGpCp found in the present work. In agreement with the interpretation proposed above, KB 5S RNA, which yielded almost no ApGpCp after pancreatic ribonuclease digestion, released the sequence GpGpApApGpCp in a molar yield close to unity, and, correspondingly, yielded the expected amount of ApApGp after T1 ribonuclease digestion. Therefore, the alternate sequence which apparently occurs in HeLa cells, as suggested by the present results, is not present in appreciable amounts in KB cells.

Aside from the occurrence of ApGpCp in the pancreatic RNase digest of HeLa 5S RNA, another type of evidence suggests the existence of multiple species of 5S RNA in human cells. This evidence is provided by the deviations from unity of the molar yields of GpGpUp (previously discussed) and of all the larger oligonucleotides. Deviations in the same direction were reproducibly observed in the various DEAE-cellulose chromatographic runs of different HeLa 5S RNA preparations; this fact and the fact that similar deviations have also been reported for the molar yields of several corresponding pancreatic RNase digestion products of KB cell 5S RNA, obtained under different conditions of RNase digestion and oligonucleotide fractionation (GpGpGpCp, ApGpGpGpUp, GpGpGpApApUp, and GpGpGpApGpApCp), strongly suggest that these discrepancies may reflect the existence of alternate sequences at various sites in the molecule. This need not be a surprising conclusion, since in *E. coli* strains several forms of 5S molecules differing from one another in one or two bases have been iden-

tified (Sanger *et al.*, 1967; Brownlee *et al.*, 1968). On the contrary, it is somewhat surprising that this heterogeneity is not more pronounced, as expected from the great gene redundancy for 5S RNA in animal cells (more than 50,000 cistrons in *Xenopus laevis* (Brown and Weber, 1968); about 7500 cistrons in HeLa cells (Hatlen and Attardi, in preparation)). The relatively low degree of variability found for human 5S RNA may reflect the great restrictions imposed upon evolutionary base sequence changes by the structural requirements for 5S function. In agreement with this idea is the recent observation that the T1 and pancreatic ribonuclease fingerprints of 5S RNA from two mouse cell lines are identical with or very similar to those of KB cell 5S RNA (Williamson and Brownlee, 1969).

The 3′-terminal nucleoside in HeLa cell 5S RNA is uridine, as has been previously found in KB cell 5S RNA; from the data obtained in the present work it is not possible to say whether two forms of 3′-terminal sequences ($CpUpUpU_{OH}$ and $CpUpU_{OH}$) exist also in HeLa 5S RNA. On the other hand, clear evidence was obtained for the existence of three forms of the 5′-terminal sequences, pGp, ppGp, and pppGp, which, taken together, account for 76–87% of the 5′ end of the molecule. This is the first instance where the presence of pppXp and ppXp at the 5′ terminus of a naturally occurring RNA, other than viral, has been reported. In the published sequence work on KB cell 5S RNA, a puzzling half-molar yield of the 5′-terminal fragment after digestion with pancreatic RNase (pGpUp) or T1 RNase (pGp) was reported; it seems possible that the alternative forms of the 5′ terminus, ppGp and pppGp, also exist in KB 5S RNA, but that they were not recognized. The presence of β- and γ-phosphates at the 5′ terminus of some 5S RNA molecules may be due to incomplete removal of phosphate from the 5′-triphosphate terminus of the nascent 5S molecules; alternatively, their absence may result from degradation occurring in the preparation of 5S RNA. The RNA made *in vitro* with *E. coli* RNA polymerase (Bremer *et al.*, 1965; Maitra and Hurwitz, 1965) or Qβ RNA polymerase (Banerjee *et al.*, 1967) is known to have pppGp or pppAp at its 5′ ends. Among the naturally occurring RNA species, before the present observation, only viral RNA had been reported to contain nucleoside 5′-triphosphate termini (Takanami, 1966; Roblin, 1968; Glitz, 1968). Whether the general absence of 5′-triphosphates in nonviral RNA is due to *in vivo* enzymatic removal of the terminal phosphates or to the derivation of the RNA molecules by cleavage of longer precursors is not known. Recently, evidence has been presented for the occurrence in *E. coli* of precursors of 5S RNA containing an extra sequence (one to four nucleotides long) at their 5′ end, which is clipped off after incorporation of the 5S molecule into the structure of ribosomal precursor particles (43S particles) (Monier *et al.*, 1969); the presence of ppGp and pppGp at the 5′ end of the 5S molecules in HeLa cells implies that in these cells there are no precursors of 5S RNA with extra sequences at the 5′ end. The fact that all 5S RNA analyzed in the present work has been isolated from mature ribosomal particles suggests that the presence of a di- or triphosphate at the 5′ end does not interfere with the functional role of 5S RNA; actually, an interesting possibility is that the addition or removal of terminal phosphates may have a regulatory role. The presence of 5′-di- and -triphosphate groups in 5S molecules indicates that transcription of 5S RNA from the multiple 5S cistrons on the DNA must occur in the form of discrete units and not as polycistronic RNA chains secondarily cut into 5S size. The existence of ppGp and pppGp at the 5′ end of the major part of the 5S molecules would furthermore restrict any possibility of derivation of 5S RNA from the 45S RNA precursor to one copy per molecule, in correspondence with the 5′-terminal segment; this result, being incompatible with the large excess of 5S sites over 45S sites in HeLa DNA (Hatlen and Attardi, in preparation), brings further evidence against the existence of any relationship between 45S RNA and 5S RNA, in agreement with kinetic data (Knight and Darnell, 1967), and RNA–DNA hybridization data (Brown and Weber, 1968; Brown and Dawid, 1968).

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A Model for the Role of Metal Ions in the Enzyme-Catalyzed Hydrolysis of Polyphosphates*

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ABSTRACT: Several new compounds containing pyrophosphate linkages and other oxygen and nitrogen ligands have been synthesized and purified. It was thought that the presence of these other ligand groups might induce metal ions to bind to the penultimate monoanionic phosphate position while leaving the terminal dianionic phosphate position unbound. This mode of binding would result in a metal ion–ligand complex resembling an unsymmetrical diester of pyrophosphoric acid. As previous work had indicated that such diesters hydrolyze extremely rapidly, it was hoped that the rates of pyrophosphate hydrolysis of the newly synthesized compounds would be greatly accelerated by metal ions, thus providing an attractive model for the binding of metal ions to substrates on the surface of polyphosphatases. The effects of a large number of metal ions on the hydrolysis rates of these compounds were studied and in no case was significantly large catalysis found, even though ^{31}P nuclear magnetic resonance studies revealed that the desired mode of binding could be at least partially obtained. These findings were taken as strong evidence against the validity of the proposed model.

Enzymes catalyzing polyphosphate hydrolysis have quite generally been shown to require divalent metal ions, but the precise catalytic role of the metal ions has not been established. Metal ion effects on nonenzymatic polyphosphate hydrolysis have been extensively studied (Schneider and Brintzinger, 1964; Miller and Westheimer, 1966; Tetas and Lowenstein, 1963; Hofstetter and Martell, 1959) and the catalysis observed is not large, the acceleration in the great majority of cases being less than a factor of 10. It is true that in the presence of solid metal salts, especially hydroxides, very fast hydrolyses have been observed (Torralba, 1960; Bamann, 1939; Bamann *et al.*, 1954), but these results have not been amenable to mechanistic interpretation.

That metal ions by themselves do not greatly accelerate polyphosphate hydrolysis does not necessarily prevent metal ions bound to enzymes from acting as efficient catalysts. However, it is evident that a mode of action not readily

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