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Interrelationships between Synthesis and Methylation of Ribosomal RNA in Isolated Novikoff Tumor Nucleoli†

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ABSTRACT: Nucleoli isolated from Novikoff hepatoma cells of the rat were previously shown to carry out synthesis of predominantly ribosomal precursor RNA and methylation of this RNA *in vitro.* In order to develop *in vitro* systems for further detailed study of these processes and their interrelationships, isolated nucleoli were incubated in a complete RNA-synthesizing medium using [5-^{3}H]cytidine 5′-triphosphate or *S*-adenosyl[*methyl*-^{3}H]methionine to measure the activities of RNA synthesis and methylation, respectively, under the same reaction conditions. Methylation of the *ribose* of the nascent ribosomal precursor RNA predominated. It occurred in close coordination with the transcriptional step by RNA polymerase as shown by the kinetic data, the analysis of labeled RNA in sucrose gradients, the inhibition by increased ionic strength or actinomycin D, and the release of labeled nucleotides by a 3′-exonuclease, venom phosphodiesterase. Methylation of the RNA *bases* occurred more slowly, continued longer after transcription ceased, and appeared to follow later in the processing of the RNA. Certain divalent cations (Mg^{2+}, Mn^{2+}, and Ca^{2+} at higher concentrations, and Zn^{2+} and Cu^{2+}) inhibited both RNA synthesis and methylation to similar extents. RNase inhibitors (bentonite and dextran sulfate) at low concentration inhibited methylation while stimulating RNA synthesis, and pyrophosphate greatly decreased RNA synthesis with relatively little effect on methylation. These results indicated that RNA polymerase and ribosomal RNA methylases can function independently despite their close relationship. An exogenous substrate for the nucleolar rRNA methylases was found: nuclear RNA prepared from Novikoff hepatoma cells, cultured in the absence of methionine, served as a good substrate for methylation of both ribose and bases. Other exogenous RNAs, including cytoplasmic ribosomal RNA from these methionine-starved cells, nucleolar RNA from normal cells, and wheat germ ribosomal RNA were almost devoid of methyl-acceptor activity. A description of these parameters helps establish isolated nucleoli as a suitable system for further study of interaction of RNA polymerase, methylases, and nucleases in control of synthesis of ribosomal RNA.

Methylation of ribosomal RNA has been previously studied by labeling intact cells with methyl-labeled methionine and by subsequently isolating fractions of nucleolar RNA (Greenberg and Penman, 1966; Wagner *et al.,* 1967; Zimmerman and Holler, 1967; Weinberg *et al.,* 1967; Muramatsu and Fujisawa, 1968). These studies indicated that incorporation of methyl groups occurred concomitantly with or very soon after the synthesis of nucleolar 45S preribosomal RNA, rather than directly into smaller intermediates or mature ribosomal RNA, and that all the methyl groups incorporated in 45S RNA were conserved in the ribosomal RNA products during the processing of this precursor. Vaughan *et al.* (1967) have demonstrated that the formation of ribosomes was completely abolished during methionine starvation of the cultured cells, although 45S RNA continued to be synthesized. Vande Woude *et al.* (1970) have indicated that the selective inhibition of ribosomal RNA methylation was the primary cause which led to the disruption of host-cell metabolism during infection by foot-and-mouth disease virus. These facts indicate that methylation of preribosomal RNA is essential for some steps during processing and maturation of ribosomes, and that ribosomal RNA methylases may be among the key factors in the regulation of cell metabolism.

We have sought to examine these critical processes in

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finer detail by use of subcellular enzyme systems. In previous reports (Liau *et al.*, 1965, 1970, 1973), isolated nucleoli from Novikoff hepatoma cells of the rat have been shown to carry out DNA-dependent synthesis of RNA which was predominantly preribosomal RNA, and to carry out methylation of preribosomal RNA. The methylation of ribosomal RNA has also been detected in isolated rat liver nucleoli by Culp and Brown (1970) and in isolated rat liver nuclei by Al-Arif and Sporn (1972). In our present study, efforts were directed toward determining the interrelationships between RNA polymerase and ribosomal RNA methylases during synthesis of preribosomal RNA. Isolated nucleoli were incubated under identical conditions for measuring RNA synthesis by incorporation of [^{3}H]CMP from [^{3}H]CTP, and for measuring RNA methylation by incorporation of labeled methyl groups from *S*-adenosyl[*methyl*-^{3}H]methionine ([^{3}H]-*S*-Ado-Met[1]) into the bases and into the ribose of preribosomal RNA. Experiments were performed to study a number of activating or inhibiting factors which might differentially affect the rates of RNA synthesis and RNA methylation, thereby permitting closer analysis of the sequence and interdependence of these activities and offering methods of selective control as aids in elucidating function of the methylation steps.

In a previous report (Liau *et al.*, 1973), the patterns of *in vitro* methylation of the ribose moieties in preribosomal RNA of isolated nucleoli were determined and found to correspond to those resulting from methylation of Novikoff tumor ribosomal RNAs *in vivo* by Lane and Tamaoki (1969) and by Egawa *et al.* (1971).

Materials and Methods

S-Adenosyl-L-[*methyl*-^{3}H]methionine (1 Ci/mmol) and [5-^{3}H]cytidine 5′-triphosphate (8.1 Ci/mmol) were obtained from Schwarz/Mann; ribonucleoside triphosphates, *S*-adenosylmethionine, and polyinosinate (potassium salt, mol wt >100,000) from P-L Biochemicals, Inc.; phosphoenolpyruvate and pyruvate kinase (EC 2.7.1.40) from Sigma Chemical Co.; actinomycin D from Merck and Co., Inc.; calf thymus DNA from Mann Research Laboratories, Inc.; polyvinyl sulfate from K & K Laboratories, Inc.; dextran sulfate 200 (mol wt 2×10^6) from Pharmacia Fine Chemicals, Inc.; Sarcosyl NL 97 from Geigy Industrial Corp.; wheat germ ribosomal RNA and Russel's viper venom phosphodiesterase from Calbiochem; and electrophoretically purified deoxyribonuclease I (EC 3.1.4.5) from Worthington Biochemical Corp. Calf thymus histone was a gift from Dr. L. S. Hnilica.

Bentonite was purified by the procedure of Petermann and Pavlovec (1963) with the initial inclusion of 2 mM EDTA.

Preparation and Incubation of Nucleoli. Novikoff ascites hepatoma cells were grown in 140–160-g Sprague-Dawley rats from Sprague-Dawley farms, Madison, Wis. The preparation of nucleoli was as described previously (Liau *et al.*, 1972). Isolated nucleoli were incubated under the same set of reaction conditions using [^{3}H]CTP for the assay of RNA polymerase and [^{3}H]-*S*-Ado-Met for the assay of RNA methylases. The standard reaction mixture in a total volume of 0.25 ml contained: 0.25 M sucrose; 0.05 M Tris-chloride (pH 7.8); 2 mM ATP; 1 mM each of GTP, UTP, and CTP; 5 mM phosphoenolpyruvate; 2 μg of pyruvate kinase; 10 μM *S*-Ado-Met; 1 mM $MgCl_2$; 0.2 mM EDTA; 0.04 M NH_4F; and an amount of nucleoli containing 50–100 μg of DNA (from 0.5 to 1.0 ml of packed cells). For the study of RNA synthesis, 1 mM CTP was replaced by 0.2 mM [^{3}H]CTP (sp act. 123 Ci/mol), and for RNA methylation, 10 μM *S*-Ado-Met was replaced by 10 μM [*methyl*-^{3}H]-*S*-Ado-Met (sp act. 1 Ci/mmol). The incubation was allowed to proceed at 30° for 15 min, unless otherwise stated.

The reaction was stopped by immersing the tubes in an ice bath, followed by the addition of 5 ml of cold 0.4 N $HClO_4$–1% sodium pyrophosphate. After 10 min in the ice bath, the precipitate was sedimented by centrifugation and washed once more with cold $HClO_4$–pyrophosphate. Sodium nucleates were extracted with 1 ml of 2 M NaCl at 100° for 30 min (Hurlbert and Potter, 1952). Before heating, the suspension was carefully neutralized with 0.5 M Tris-Cl (pH 7.4), using Phenol Red as indicator. Nucleic acids were reprecipitated from the NaCl extract by the addition of 0.1 vol of 4 N $HClO_4$ and 10 ml of cold $HClO_4$–pyrophosphate. The precipitate was then treated with 0.7 ml of 0.3 M KOH at 37° overnight to hydrolyze RNA, and the DNA was precipitated by adjusting the hydrolysate to 0.4 N $HClO_4$. The acid-soluble supernatant was taken for the analysis of RNA content, radioactivity, and separation of methylated nucleotides. The precipitate was subjected to hydrolysis in hot 0.4 N $HClO_4$, and the amount of DNA was determined to establish the relative amounts of nucleoli present.

Preparation of RNA by Phenol Deproteinization. After incubation of isolated nucleoli, either with [^{3}H]CTP or [^{3}H]-*S*-Ado-Met, 2 ml of 0.14 M NaCl–0.05 M sodium acetate (pH 5.1)–1% Sarcosyl NL 97 was added, and the mixture was shaken with an equal volume of buffer-saturated phenol containing 0.1% 8-hydroxyquinoline at room temperature (25°) for 30 min. The aqueous phase was separated by centrifugation and withdrawn. The remaining interphase and phenol were reextracted with 2 ml of fresh buffer–Sarcosyl solution by shaking at 60° for 10 min. Nucleic acids were precipitated from the combined aqueous extracts by the addition of 2 vol of ethanol. High molecular weight RNA was freed from DNA and low molecular weight RNA by precipitation once in 2 M NaCl at 4° overnight. The analysis of RNA on a sucrose gradient was conducted as previously described (Liau *et al.*, 1970).

Nuclear and cytoplasmic ribosomal RNA from Novikoff hepatoma cells cultured in the absence of methionine[2] were prepared by similar procedures from purified nuclei and ribosomes.

Analytical Procedures. RNA was determined by a modified orcinol reaction (Hurlbert *et al.*, 1954) and DNA by Burton's (1956) diphenylamine procedure. The separation of mononucleotides and dinucleotides on DEAE-cellulose columns was as previously described (Liau *et al.*, 1973). Radioactivity in aqueous solution was determined in a water-miscible fluor (Liau *et al.*, 1972) by scintillation counting in a Nuclear-Chicago Mark I instrument with correction for efficiency.

Results

Under conditions specified under Materials and Methods, the activity of isolated nucleoli from Novikoff hepato-

[1] Abbreviations used are: *S*-Ado-Met, *S*-adenosyl-L-methionine; [^{3}H]-*S*-Ado-Met, [*methyl*-^{3}H]-*S*-Ado-Met.

[2] Novikoff hepatoma cells cultured for 24 hr in the absence of methionine were kindly supplied by Dr. T. W. Sneider of the Department of Pharmacology, Baylor College of Medicine.

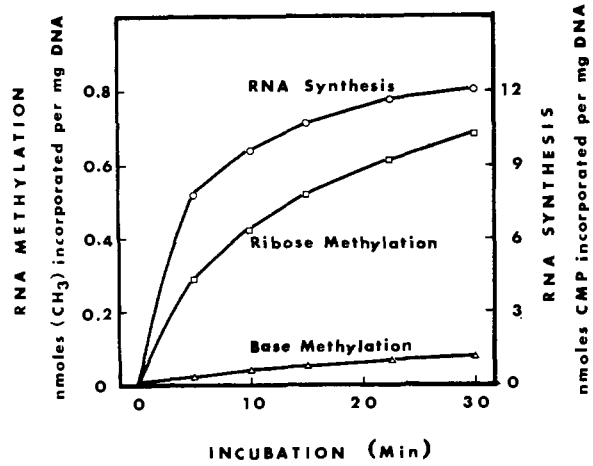

FIGURE 1: Time course of RNA synthesis and RNA methylation in isolated nucleoli. Isolated nucleoli were incubated at 30° for the indicated periods of time in the standard reaction mixture, either with [^{3}H]CTP to measure RNA synthesis, or with [*methyl*-^{3}H]-*S*-Ado-Met to measure RNA methylation. As described under Materials and Methods, nucleates were extracted with hot 2 M NaCl, the RNA was hydrolyzed with 0.3 N KOH, and the DNA was precipitated with cold $HClO_4$. Incorporation of [^{3}H]CMP from CTP was determined on a portion of the hydrolysate; the rest of the methylated RNA was further fractionated into mononucleotide and dinucleotide fractions on DEAE-cellulose columns for determination of the incorporation of methyl-^{3}H groups into RNA bases and RNA ribose, respectively. The DNA content of the precipitate was determined, and the results are expressed in terms of the amount of nucleoli containing 1 mg of this DNA.

TABLE I: Extractability of Nucleolar RNA Labeled *in Vitro*.[a]

% Sarcosyl NL 97	% Extractability of Nucleic Acids			
	Labeled RNA		Total	
	[^{3}H]CMP	[^{14}C]CH_3	RNA	DNA
0	14.6	13.8	46	1
0.3	35.2	31.6	87	17
1	87.0	82.5	97	84

[a] Isolated nucleoli were incubated simultaneously with [^{3}H]CTP and [^{14}C]*S*-Ado-Met in the standard reaction mixture. The reaction was terminated by the addition of 5 ml of cold 0.25 M sucrose–5 mM $MgCl_2$–0.01 M Tris-acetate (pH 7), and nucleoli were sedimented by centrifugation at 900*g* for 10 min. The nucleolar pellet was suspended by homogenization in 0.14 M NaCl–0.05 M sodium acetate (pH 5.1) containing various amounts of Sarcosyl NL 97 as indicated. Nucleic acids were extracted into the aqueous phase by phenol deproteinization and hydrolyzed in hot 0.4 N perchloric acid for the determination of nucleic acid content and radioactivity. The nucleic acids remaining in the phenol interphase were washed with ethanol and cold 0.4 N perchloric acid, and then extracted in hot 0.4 N perchloric acid for the determination of nucleic acid content and radioactivity.

ma cells for RNA synthesis was in the range of 10–13 nmol of [^{3}H]CMP, and the activity for RNA methylation was in the range of 0.5–0.6 nmol of methyl-^{3}H groups, incorporated into preribosomal RNA, in an amount of nucleoli containing 1 mg of DNA. Methylation of transfer RNA (Liau *et al.*, 1970, 1972) was not measured in these experiments because no acceptor tRNA was added; previous studies (Liau *et al.*, 1970, 1973) have shown that the methylated endogenous RNA is made up almost entirely of material larger than tRNA.

Stimulation and Inhibition by Divalent Cations. Since nucleoli contained bound Mg^{2+} resulting from isolation in sucrose solutions containing magnesium ion, RNA synthesis and methylation took place readily in a reaction mixture without the further addition of magnesium ion. Addition of magnesium ion in the range 0.5–2.5 mM was optimal for both RNA synthesis and RNA methylation. Higher concentrations of magnesium ion resulted in some precipitation of nucleoli during incubation, accompanied by decreased synthesis and methylation of nucleolar RNA. The addition of various other divalent cations (Mn^{2+}, Ca^{2+}, Zn^{2+}, or Cu^{2+}), together with optimal 1 mM Mg^{2+}, was inhibitory. Both RNA synthesis and RNA methylation were inhibited by these divalent cations to similar extents at various concentrations. At 2.5 mM, Mn^{2+} and Ca^{2+} inhibited both reactions by 30% and Zn^{2+} and Cu^{2+} inhibited reactions by 88 and 95%, respectively. Some precipitation of nucleoli was observed at the higher concentrations of these divalent cations.

Time Course of Ribose Methylation and Base Methylation. Figure 1 shows the kinetics of RNA synthesis and methylation of ribose and bases of endogenous preribosomal RNA. For these studies, RNA was labeled either with [^{3}H]CTP or [^{3}H]-*S*-Ado-Met at 30° for the period indicated. The alkaline hydrolysates of methylated RNA were fractionated into mononucleotides and dinucleotides on DEAE-cellulose columns, as described previously (Liau *et al.*, 1973). The labeled methyl content of the mononucleotide fraction represents methylation of bases, and the labeled methyl content of the dinucleotide fraction represents methylation of ribose. The shape of the curves indicates that the maximum extent of nucleotide incorporation (RNA synthesis) was attained sooner than the maximum extent of ribose methylation; both rates diminished rapidly during the incubation. Methylation of bases proceeded even more slowly and was maintained at the same rate during the time of incubation. While longer incubations might have revealed more about the kinetic relationships, they were not practical because of degradation reactions. These results with isolated nucleoli indicate (but do not by themselves prove) that modification of rRNA by methylation, particularly methylation of bases, is a later process than transcription.

Differential Extractability of Newly Labeled RNA. The nucleolar RNA labeled *in vitro* with radioactive CTP or *S*-Ado-Met represents a fraction of RNA which is demonstrably different from the bulk of nonlabeled RNA. Table I shows that the RNA labeled in these experiments was primarily extractable only with vigorous treatment by phenol (high temperature and concentration of detergent) as contrasted to less vigorous treatments which only release molecules of the transfer and ribosomal size classes bound as ribonucleoprotein particles. The release of nucleolar RNA doubly labeled with [^{3}H]CTP and [^{14}C]-*S*-Ado-Met *in vitro* into the aqueous phase upon phenol deproteinization was examined at several concentrations of detergent. The majority of labeled RNA (83–87%) could only be released into the aqueous phase at 1% Sarcosyl NL 97. This latter extraction condition also caused the bulk of DNA (84%) to be released into the aqueous phase. Thus, the extractability of labeled RNA corresponds better with the extractability of DNA than of RNA, suggesting that the labeled RNA studied in these experiments represents primarily nascent RNA associated with the DNA.

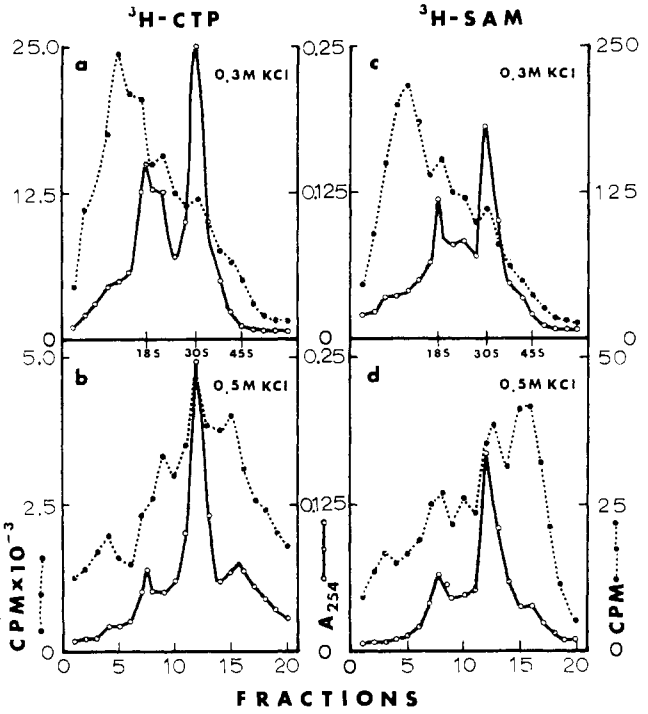

FIGURE 2: Sucrose gradient centrifugation patterns of RNA labeled by [^{3}H]CTP or [*methyl*-^{3}H]-*S*-adenosylmethionine: the effect of ionic strength. Isolated nucleoli were incubated in the complete RNA synthesizing medium with [^{3}H]CTP (2.4 mCi/μmol) or [^{3}H]-*S*-Ado-Met (1 mCi/μmol) in the presence of the indicated concentrations of KCl. RNA was prepared by extraction with phenol, as described under Materials and Methods, and centrifuged on 5 ml of a 10–40% sucrose gradient in 0.1 M NaCl–1 mM EDTA–0.01 M sodium acetate (pH 5.1) at 45,000 rpm for 4.5 hr. After fractionation, the RNA was precipitated with cold 5% trichloroacetic acid and collected and counted on Millipore filters. In this figure the abbreviation for *S*-adenosyl-L-methionine is SAM.

TABLE II: Conditions Which Show Preferential Inhibition of Ribosomal RNA Methylase Activity.[a]

Variations during Incubation	Enzyme Act. % of Control Act. RNA Synthesis	rRNA Methylation
Bentonite, 0.9 mg/ml	109	63
Dextran sulfate, 0.05 mg/ml	137	55
0.1 mg/ml	84	28
Polyvinyl sulfate, 0.1 mg/ml	122	38
Polyinosinate, 0.2 mg/ml	94	52
Calf thymus DNA, 0.4 mg/ml	195	67
Deoxycholate, 5 mg/ml	89	16
Preincubation	102	59

[a] Isolated nucleoli were incubated in the standard reaction mixture (control) or in the presence of additional chemicals, as indicated, or in the standard reaction mixture after preincubation in isotonic sucrose medium. In the tubes with calf thymus DNA, 10 mM dithiothreitol was included; this was necessary for the observed stimulation of RNA synthesis. The addition of dithiothreitol alone, however, did not have a significant effect either on RNA synthesis (104% of the control) or on RNA methylation (102% of the control). Preincubation of nucleoli was conducted at 30° for 15 min in 0.25 M sucrose–5 mM $MgCl_2$–0.05 M NH_4F–0.05 M Tris-Cl (pH 7.8); nucleoli were then sedimented by centrifugation and incubated in the standard reaction mixture. RNA synthesis and ribosomal RNA methylases were measured by incorporation of [^{3}H]CMP from [^{3}H]CTP and methyl-^{3}H from [*methyl*-^{3}H]-*S*-Ado-Met, respectively.

Analyses of RNA Species by Sedimentation in Sucrose Gradients and Effects of Ionic Strength. The sedimentation patterns of RNA *newly labeled* under various conditions also showed considerable difference from the sedimentation patterns of *nonlabeled* nucleolar RNA (Figure 2). Under all circumstances, however, the patterns of RNA labeled with [^{3}H]CTP resembled closely the patterns of RNA labeled with [^{3}H]-*S*-Ado-Met. For instance, RNA patterns labeled with [^{3}H]CTP resembled those labeled with [^{3}H]-*S*-Ado-Met under the influence of increased ionic strength as shown in Figure 2. The activities of nucleolar RNA polymerase and RNA methylases were quite responsive to the variation of ionic strength. In the presence of 0.3 M KCl, both RNA synthesis and RNA methylation were inhibited about 50%, and in the presence of 0.5 M KCl, about 85%. NH_4Cl and $(NH_4)_2SO_4$ produced similar results at comparable ionic strength. The labeling patterns in the presence of 0.3 M KCl, with most material sedimenting at less than 18 S, result from synthesis or methylation of higher molecular weight RNA followed by degradation by ribonucleases. The same pattern of lower S-value peaks was obtained when nucleoli were incubated in the absence of bentonite under standard incubation conditions, namely without added KCl (data not shown).

During incubation in the presence of 0.5 M KCl or other salts of similar ionic strength (which is generally known to inhibit ribonuclease action and to inhibit initiation by RNA polymerase) the profile of *endogenous* RNA is unaltered from the zero-time control, and an incorporation of labeled CMP predominantly into 10–20S peaks at short time intervals (5 min) is followed by a shift predominantly into 30–45S peaks at later intervals (15 min) (Liau *et al.*, 1968, and unpublished observations). Accumulation of labeled RNA in high molecular weight regions is evident in Figure 2 with the methyl label as well as the CMP label.

Since the patterns of RNA labeled either with [^{3}H]CTP or [^{3}H]-*S*-Ado-Met under various conditions of synthesis and degradation resembled closely each other, RNA molecules newly incorporating [^{3}H]CMP appear by this criterion to be the same molecules which accept methyl-^{3}H groups. The appearance of *polydispersed* patterns of nucleolar RNA labeled with [^{3}H]-*S*-Ado-Met, even under conditions when nuclease activities were nearly completely inhibited by the presence of 0.5 M KCl, suggests that methylation occurs on short nascent (growing) molecules before the precursor molecule is completed. Thus, the extractability and sedimentation patterns of nucleolar RNA labeled either with [^{3}H]CTP or [^{3}H]-*S*-Ado-Met are almost indistinguishable.

Effects of Ribonuclease Inhibitors and Other Chemicals Preferentially Inhibiting RNA Methylases. As shown in Table II, ribosomal RNA methylases could be inhibited by some agents without inhibition of the RNA polymerase activity. The addition of ribonuclease inhibitors (bentonite, polyvinyl sulfate, or dextran sulfate), which improves the recovery of labeled preribosomal RNA and which also stimulates apparent RNA polymerase activity at certain low concentrations, was inhibitory to methylase activity.

The addition of calf thymus DNA stimulated RNA synthesis and inhibited RNA methylation. Stimulation of RNA synthesis by exogenous DNA has been previously reported by Miller and Hurlbert (1969) to be caused by the presence in isolated nucleoli of a pool of "free" RNA polymerase which transcribed the *exogenous* DNA; therefore, the RNA formed would not be expected to provide additional substrate for the methylases. Deoxycholate produced the greatest depression of methylation with little decrease of RNA synthesis. Polyinosinate also depressed methylation more than synthesis of RNA. In a separate study (Liau *et al.*, 1973) poly(I) has been shown to inhibit methylases of both tRNA and rRNA by specific and selective interactions with the methylation enzymes. Preincubation of isolated nucleoli in isotonic sucrose medium (0.25 M sucrose–5 mM $MgCl_2$–0.04 M NH_4F–0.05 M Tris-acetate, pH 7.8) also depressed methylation more than synthesis of RNA. The activity of RNA polymerase then certainly is not dependent upon concomitant activity of ribosomal RNA methylases.

Methylation of Exogenous RNAs. Proof that ribosomal RNA methylases could act independently of RNA polymerase was obtained by providing exogenous methyl-acceptor RNA. Nuclear RNA, prepared from Novikoff hepatoma cells cultured in the absence of methionine, was added during the incubation of nucleoli. A 2.3- to 2.4-fold stimulation was obtained when 30 μg of presumably methyl-deficient nuclear RNA was added to nucleolar preparations containing 40–90 μg of DNA. Although we have no analytical proof that this nuclear RNA is methyl deficient, Vaughan *et al.* (1967) have indicated that undermethylated RNA accumulated in the nucleoli of methionine-starved HeLa cells. Also, a tRNA preparation from these Novikoff hepatoma cells cultured in the absence of methionine showed about 45% of the methyl-accepting activity of *Escherichia coli* B tRNA, while a tRNA preparation from normal cells had but 1.5% of the methyl-accepting activity of *E. coli* B tRNA, when assayed on a tRNA methylase preparation of Novikoff ascites cells as described previously (Liau *et al.*, 1972). DNA from these cells has also been shown by Sneider (1971) to be undermethylated. About half of this stimulated methylation by exogenous RNA was bound to and cosedimented with the nucleoli. In a separate experiment, analyses of the extent of ribose methylation stimulated in the presence of methyl-deficient nuclear RNA showed that 84% of the methylated RNA in the supernatant fraction (900*g*, 10-min centrifugation of the incubated mixture) and 87% of the methylated RNA in the pellet were due to the methylation of ribose, and the rest was due to the methylation of bases. This pattern of distribution is typical of rRNA (Tamaoki and Lane, 1968). Therefore, the additional methylation in the presence of exogenous undermethylated nuclear RNA is indeed catalyzed by ribosomal RNA methylases. We assume at present that the exogenous RNA is the recipient of the methyl groups, although this point has not been studied thoroughly. The cosedimentation of part of the methylated exogenous RNA with the nucleoli presumably occurs because of association with the ribosomal RNA methylases, which are very tightly bound to the nucleolar body and are not readily extractable (Liau *et al.*, 1972).

Cytoplasmic rRNA (30 μg) prepared from methionine-starved cells stimulated only 10% over the control activity. In similar experiments, RNA (50 μg) prepared from normal nucleoli stimulated 3–6%, and wheat germ rRNA (50 μg) stimulated only 4–9% above the control activity. These stimulations are minimally significant; addition of any RNA may be expected to increase recovery of endogenous RNA by competing for the action of ribonucleases. The failure to stimulate tumor nucleolar RNA methylation by wheat germ rRNA is in contrast to a marked stimulation of ribose methylation by this type of RNA in isolated rat liver nuclei (Al-Arif and Sporn, 1972). Treatment of wheat germ RNA by several denaturing agents and incubation under a variety of experimental conditions failed to cause any significant improvement of methyl-accepting activity.

TABLE III: Conditions Which Show Preferential Inhibition of RNA Polymerase Activity.[a]

Variations during Incubation	Enzyme Act. % of Control Act.	
	RNA Synthesis	rRNA Methylation
Minus nucleotides	13	53
Actinomycin D, 10 μg/ml	10	65
Calf thymus histone, 0.04 mg/ml	17	63
Sodium pyrophosphate, 10 mM	41	96
20 mM	11	82
EDTA, 10 mM	69	90
Pretreatment with DNase, 50 μg/ml	45	62
200 μg/ml	26	43

[a] Isolated nucleoli were incubated in the standard reaction mixture (control) or with the omissions, additions, or treatments indicated. Pretreatment of isolated nucleoli with DNase was carried out by incubation of nucleoli containing about 100 μg of DNA in 1 ml of isotonic sucrose medium with the indicated amounts of DNase in an ice bath for 10 min. Nucleoli were then sedimented at 900*g* for 10 min and washed three times with cold isotonic sucrose medium. An aliquot was withdrawn for the determination of DNA. It showed removal of 58 and 78% of DNA at the DNase concentrations of 50 and 200 μg/ml, respectively. The remainder was incubated for the assay of enzyme activities, as described in Table II.

Effects of RNA Polymerase Inhibition and Timing of Methylation. Under certain conditions methylation of nucleolar RNA took place to a considerable extent, despite drastically limited synthesis of RNA, as measured by the incorporation of [^{3}H]CMP. Table III shows that the omission of nucleotides or the addition of actinomycin D, histone, pyrophosphate, or EDTA preferentially inhibited nucleotide incorporation with much less effect on incorporation of methyl groups into nucleolar RNA. Pretreatment of isolated nucleoli with deoxyribonuclease inhibited both RNA synthesis and RNA methylation; however, the synthesis of RNA was inhibited to a greater extent than the methylation of RNA. (The differential effects of pyrophosphate and EDTA may result from the complexing of divalent cations; it appears that Mg^{2+} is not essential for activity of the methylases.)

The most effective RNA polymerase inhibitors, the omission of full complement of nucleotides, or the inclusion of actinomycin D, reduced incorporation of [^{3}H]CMP by 87–90%, but reduced methylation by only 35–47%. Methylation occurring in the presence of these inhibitors was likely caused partly by the incorporation of methyl groups into en-

TABLE IV: Effect of Unlabeled *S*-Ado-Met and Actinomycin D on the Timing of Methylation of Nucleolar RNA.[a]

	nmol of Methyl-^{3}H Incorp. per mg of DNA		
	0–30 min[b]	7.5–30 min[b]	15–30 min[b]
Control			
Ribose methylation	0.692	0.164	0.055
Base methylation	0.072	0.034	0.025
Plus actinomycin D, 10 μg/ml			
Ribose methylation	0.421	0.061	0.028
Base methylation	0.094	0.033	0.025

[a] Isolated nucleoli were preincubated at 30° without (control) or with 10 μg/ml of actinomycin D (plus actinomycin D) in the complete standard reaction mixture containing 8 μM unlabeled *S*-Ado-Met (2 nmol/tube). Two nanomoles of [*methyl*-^{3}H]-*S*-Ado-Met were added per tube either at time zero, 7.5 min, or 15 min after preincubation. Incubation was then allowed to continue up to 30 min. The activity was analyzed as described in Figure 1. [b] Time period of presence of [^{3}H]-*S*-Ado-Met.

dogenous nascent RNA chains which had not yet been methylated *in vivo*, and partly by methylation of the small amounts of RNA synthesized *in vitro*. These endogenous nascent methyl-accepting sites are judged from the data above to contribute a significant fraction of the total methyl groups incorporated when nucleoli were incubated in the complete RNA synthesizing medium.

As shown in Table IV, timing of ribosomal RNA methylation in relation to RNA transcription was studied by further examining the effect of actinomycin D. The objective was to determine the extent of methyl group incorporation at later times, after synthesis of new RNA had completely ceased. The addition of actinomycin D did not immediately stop all transcription, as shown in Table IV; other experiments showed that after 7.5 min of incubation in the presence of actinomycin D, essentially no further incorporation (0.2% of the control activity) of [^{3}H]CMP could be detected. Addition of ^{3}H-labeled *S*-Ado-Met was made at intervals after beginning incubation of nucleoli in the presence of unlabeled *S*-Ado-Met; a parallel series was run with actinomycin D added. When the labeled *S*-Ado-Met was added to the control series after the first 7.5-min period, during which both RNA synthesis and methylation (unlabeled) were occurring, an additional incorporation of 164 pmol of methyl groups into *ribose* (about 24% of the zero time control) occurred. In the presence of actinomycin D, the corresponding value (61 pmol) represented an inhibition of 63%, indicating that much of the later methylation activity is dependent on RNA synthesis *in vitro*. Conversely, incorporation of methyl-^{3}H groups into *bases* was not affected by the presence of actinomycin D.

Relative Rate of Hydrolysis by Phosphodiesterase of Nucleolar RNA Labeled in Vitro. Another approach to establish the interrelationships between RNA synthesis and RNA methylation is to determine the relative rate of release of the labeled nucleotides from RNA by a 3′-exonuclease. The nucleolar RNA was doubly labeled with

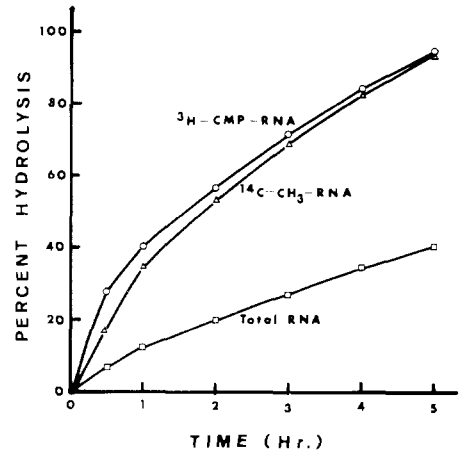

FIGURE 3: Hydrolysis by phosphodiesterase of nucleolar RNA labeled *in vitro;* relative rate of release of [^{3}H]CMP and methyl-^{14}C-labeled nucleotides. The nucleolar RNA was doubly labeled with [^{3}H]CMP and methyl-^{14}C groups by incubation of isolated nucleoli simultaneously with [^{3}H]CTP and [^{14}C]-*S*-Ado-Met in the standard reaction mixture. The labeled RNA was purified by phenol deproteinization. The hydrolysis of RNA by phosphodiesterase was carried out at 37° in a volume of 0.65 ml which contained: 0.01 M Tris-Cl (pH 9.4), 1 mM $MgCl_2$, 2 mg/ml of albumin, 0.15–0.17 mg of nucleolar RNA, and 15 units of Russel's viper venom phosphodiesterase. At the times indicated, 0.1-ml aliquots were withdrawn, and 0.4 N perchloric acid soluble nucleotides and 0.4 N perchloric acid insoluble residues were separately analyzed for RNA content and radioactivity. At the 2-hr time interval the acid-soluble nucleotides were further fractionated on a DEAE-cellulose column as described in Figure 1 to check whether the hydrolysis was exclusively done by exonuclease. The results showed that 92–94% of the radioactivity was eluted in the mononucleotide fraction, and the rest was distributed among di- and trinucleotide fractions, thereby excluding the possibility that the hydrolysis was caused by any contaminating endonuclease.

[^{3}H]CMP and methyl-^{14}C groups by incubation of isolated nucleoli simultaneously with [^{3}H]CTP and [^{14}C]-*S*-Ado-Met. The labeled RNA was purified by phenol deproteinization and 2 M NaCl precipitation, and then submitted to hydrolysis by snake venom phosphodiesterase. As shown in Figure 3, the release of both [^{3}H]CMP and methyl-^{14}C-labeled nucleotides was faster than the release of nonlabeled nucleotides, indicating the presence of a higher proportion of labeled nucleotides at the 3′ ends of polynucleotide chains. The relative rate of the release of [^{3}H]CMP was evidently faster than the release of methyl-^{14}C-labeled nucleotides, particularly during the first 0.5 hr of hydrolysis. However, the rate of the release of both labeled nucleotides approached a similar proportion beyond 3 hr of hydrolysis. These results are interpreted as an indication that the labeling of nucleolar RNA with [^{3}H]CTP *in vitro* is primarily a limited extension of the nonlabeled nascent chains formed *in vivo*. Whereas there is lower distribution of methyl-^{14}C-labeled nucleotides at the very end of labeled RNA as compared to the distribution of [^{3}H]CMP, the majority of methyl-^{14}C-labeled nucleotides are either intermingled with or distributed proximal to the distribution of [^{3}H]CMP along the labeled portion of the nascent chains. If methylation is not taking place close to the point of transcription, the release of methyl-^{14}C-labeled nucleotides is expected to lag considerably behind the release of [^{3}H]CMP, or commence only after the complete release of [^{3}H]CMP. This is exactly the case found on the release of base-methylated nucleotides. When RNA labeled with [^{3}*H*]-*S*-Ado-Met in the absence of ribonucleoside triphosphates was subjected to the same treatment, the release of base-methylated nu-

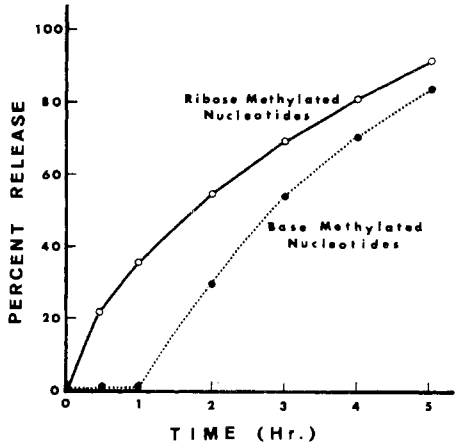

FIGURE 4: Hydrolysis by phosphodiesterase of nucleolar RNA methylated *in vitro;* relative rate of release of ribose and base-methylated nucleotides. The nucleolar RNA was labeled with [^{3}H]-*S*-Ado-Met in the absence of ribonucleoside triphosphates and subjected to the same treatments as described in Figure 3. The acid-insoluble residue was hydrolyzed in 0.3 N KOH at 37° for 18 hr. The alkaline hydrolysate was then fractionated on a DEAE-cellulose column as described in Figure 1 for the determination of radioactivity distributed between ribose and base-methylated nucleotides.

cleotides was not detectable during the first hour of hydrolysis, during which 30% of ribose-methylated nucleotides had been hydrolyzed as shown in Figure 4. These and previous data thus suggest that the methylation of ribose must be taking place soon after the sites have been generated by RNA polymerase and the methylation of bases is a process not tightly coupled to the transcription.

Discussion

These enzyme studies on isolated subcellular organelles indicate that ribose methylation takes place on newly transcribed nascent preribosomal RNA in nucleoli. These results thus independently confirm the interpretations of Greenberg and Penman (1966) who studied the problem by labeling of intact cells and isolation of nucleolar RNA fractions. In addition, our results show that the polymerase and methylases act independently, not in concerted fashion, and that methylation of the *bases* of nucleolar ribosomal precursor RNA occurs later than the ribose methylation.

The specificity of ribosomal RNA methylases appears to be more restricted than base methylases of transfer RNA, which are capable of acting on heterologous tRNA (Srinivasan and Borek, 1963). Thus far, the nuclear RNA of Novikoff hepatoma cells cultured in the absence of methionine is the only RNA showing methyl-acceptor activity in our system. Although Culp and Brown (1970) showed increased methyl-acceptor activity when *E. coli* rRNA was added to rat liver nucleoli, and Al-Arif and Sporn (1972) showed that wheat germ rRNA stimulated methylation of rRNA ribose when added to rat liver nuclei, these substrates have repeatedly been devoid of activity with the Novikoff nucleolar system.

Several features of the work reported here appear puzzling and deserve further discussion. A significant incorporation of methyl groups into nucleolar RNA was readily observed when continued transcription of rRNA was minimal, thus leading to the proposed explanation that some nascent endogenous RNA remained unmethylated during preparation of the nucleoli. Yet purified nucleolar RNA was almost inactive in accepting methyl groups as an exogenous substrate. A dependence of methylation on continued transcription was indeed somewhat difficult to demonstrate. In at least partial explanation of these features, two facts should be pointed out. The first is that nucleolar RNA synthesis, under the best of conditions, is not very extensive and does not proceed *in vitro* beyond 15–20 min. Thus most of the new RNA synthesis *in vitro* occurs in the first few minutes, and it is difficult to distinguish it as a methyl acceptor from that which was already present. The second fact is that the rRNA methylases are tightly bound to the nucleoli; this suggests the possibility that they are not readily accessible to exogenous RNAs. It does appear that the very small amount of endogenous RNA is a much more efficient methyl acceptor than any exogenous RNA thus far tested; thus, the purified nucleolar RNA may not provide sufficient levels of accessible substrate to achieve a stimulation measurable in the presence of the endogenous incorporation.

As discussed, the methylation of rRNA bases occurs later in processing of the rRNA than the methylation of ribose. Some of the base methylation may occur later yet, outside the nucleoli. Zimmerman (1968) reported that the formation of *N*-dimethyladenine occurred after the completion of 45S RNA, and Salim and Maden (1973) showed that several base methylations occurred late in the maturation process. As reported elsewhere (Liau *et al.*, 1973), 42% of the total base methylation in isolated nucleoli was attributed to the methylation of adenylate. The analysis of methylated adenylates by acid hydrolysis and paper chromatography following the procedures described by Zimmerman (1968) revealed, however, that in our work, N^6-methyladenine was the only methylated adenylate. In another instance, the formation of N^6-dimethyladenosine in 16S rRNA of *E. coli* required the participation of protein components (Helser *et al.*, 1972). Thus, some of the methylation of bases may be regarded as occurring late and outside the nucleolus in the sequence of ribosome formation.

In related work reported separately (Liau, 1974) this nucleolar system has been used to explore the topography of the 45S preribosomal RNA in terms of the patterns of methylated bases and nucleotides. Correspondence of *in vitro* methylation patterns of 5′- and 3′-end regions of preribosomal nascent RNA with 18- and 28S ribosomal RNAs helps to demonstrate that the nucleolar system described here is capable of conducting processes similar to those occurring *in vivo*. We believe that the system may be further exploited to study details of ribosome biogenesis, both transcriptional and post-transcriptional modification which are not accessible by study of intact cells.

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Hydrodynamic Diameters of RNA Tumor Viruses. Studies by Laser Beat Frequency Light Scattering Spectroscopy of Avian Myeloblastosis and Rauscher Murine Leukemia Viruses†

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ABSTRACT: The diffusion constants of avian myeloblastosis virus (AMV) and murine leukemia virus (MuLV) (Rauscher) suspensions in buffer and in 30% sucrose were determined by laser beat frequency light scattering spectroscopy at a series of temperatures ranging from 5 to 25°. By the use of the Stokes–Einstein equation, the following hydrodynamic diameters are calculated at 20°: MuLV, 154 ± 3 nm in sucrose and 145 ± 7 nm in buffer; AMV, 144 ± 3 nm in sucrose and 138 ± 4 nm in buffer. While the diameters measured in buffer were temperature independent, the diameters measured in sucrose decreased by about 20% as the temperature was raised from 5 to 25°. The concentration of virus particles in the suspensions ranged from 10^7 to 10^9 particles/ml. The absolute particle concentrations are estimated within ±30% by determining the dilution needed to reach a concentration sufficiently low that the particle number fluctuation contribution was comparable to that of the interference scattering. Particle weights of 3.9×10^8 daltons for MuLV and 4.0×10^8 daltons for AMV were calculated from the diffusion constants and from our own experimentally determined sedimentation coefficients. From these particle weights and the hydrodynamic diameters of the viruses, we calculated the per cent of the hydrodynamic volume of the viruses which could be freely penetrated by water, *viz.*, 57% for AMV and 69% for MuLV.

Avian myeloblastosis virus (AMV)[1] and murine leukemia virus (MuLV) are widely used as typical representatives of oncornaviruses in investigations of the pathology of virus-induced animal tumors, the mode of infection, replication and transformation, and in the characterization of the viruses (Temin, 1971). While oncornaviruses have been etiologically associated with leukemias and sarcomas in mice, chickens, and other animals including some higher primates, their role in human cancer has not yet been unequivocally demonstrated. AMV and MuLV have been particularly useful since they are present at relatively high titers in the plasma of viremic animals and thus permit purification of sufficient quantities for physical and biochemical characterization.

Oncornaviruses are generally spherical in shape with diameter estimates ranging from 65 to 150 nm (Bader, 1969). As observed in electron micrographs, they contain an electron dense core positioned either centrally (C type particles)

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[1] Abbreviations used are: AMV, avian myeloblastosis virus; MuLV, murine leukemia virus.