Role of Ribosomal RNA Methylases in the Regulation of Ribosome Production in Mammalian Cells†

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ABSTRACT: The activity of rRNA methylases was stimulated by high-energy precursors of RNA (ribonucleoside triphosphates) and inhibited by degradation products of RNA (ribonucleotides and oligoribonucleotides). The response of methylases from rat Novikoff ascites tumor and liver to these metabolites was strikingly different. The highly active tumor enzymes responded preferentially to inhibition by catabolic metabolites, whereas the less active liver enzymes responded exclusively to stimulation by anabolic metabolites. When the activity of rRNA methylases was assayed in response to increasing concentration of *S*-adenosylmethionine, the tumor enzymes responded with a hyperbolic substrate dependence curve and the liver enzymes with a sigmoidal curve. In the presence of an inhibitory dinucleotide, ApA, the tumor enzymes responded with a sigmoidal curve; in the presence of a stimulator, adenosine 5′-triphosphate, the liver enzymes responded with a hyperbolic substrate concentration curve. When normal rats were subject to a series of treatments by thioacetamide, a hepatocarcinogen, the liver nucleolar rRNA methylases became responsive to inhibition by ApA and relatively unresponsive to stimulation by adenosine 5′-triphosphate. When tumor-bearing rats were treated with polyinosinate:polycytidylate, an antitumor agent, the tumor nucleolar rRNA methylases became unresponsive to inhibition by ApA and more responsive to stimulation by adenosine 5′-triphosphate. A correlation was noted between increased methylation efficiency in vivo and increased stability of nucleolar RNA during incubation in vitro, or vice versa. These results are interpreted to indicate that rRNA methylases are regulated by cellular metabolites during the nucleolar biosynthesis of ribosomes and that rRNA methylases may provide a favorable site for selective action by cancer chemotherapeutic agents.

The bulk of evidence supports the view that the growth rate of mammalian cells can be correlated with the rate of ribosome production. During the transition from resting to growing state, an acceleration of ribosome production can always be observed prior to the commencement of DNA synthesis resulting from growth stimuli (Novi and Baserga, 1972; Luck and Hamilton, 1972; Johnson et al., 1974). Conversely, the suppression of ribosome production in actively growing cells leads to the cessation of growth (Toniolo et al., 1973; Sturani et al., 1973; Liau et al., 1975). The mechanism by which the production of ribosomes is accelerated or decelerated under different growing conditions of mammalian cells remains obscure. A major factor in the regulation of ribosome production apparently operates through control of maturation processes. This control mechanism is primarily concerned with the efficiency of ribosome processing. Thus, most of 45S pre-rRNA is efficiently processed to yield functional ribosomes in a growing state, whereas considerable amounts of 45S pre-rRNA are ultimately degraded in a resting state. Factors most concerned with this control mechanism are protein synthesis (Rizzo and Webb, 1969; Cooper and Gibson, 1971; Pederson and Kumar, 1971; Cooper, 1973) and the methylation of pre-rRNA (Vaughan et al., 1967; Luck and Hamilton, 1975). The essentiality of the methylation of pre-rRNA is obvious, since methylation is confined to the sequences conserved and destined to become ribosomes (Maden and Salim, 1974). It has been shown that 45S pre-rRNA synthesized without methylation underwent total degradation (Vaughan et al., 1967), and that the inhibition of the methylation of pre-rRNA led to the disruption of ribosome production (Ascione and Vande Woude, 1969; Liau et al., 1975). Thus, the methylation of pre-rRNA in some way protects the rRNA sequences from being degraded during maturation processes.

In this paper, we report the different characteristics of nucleolar rRNA methylases of rapidly growing Novikoff ascites tumor cells and resting hepatic cells and the correlation of the activity of rRNA methylases with the stability of nucleolar RNA. Evidence is presented to indicate that rRNA methylases are subject to regulation by RNA metabolites and that the behaviors of enzymes from tumor cells and hepatic cells in response to regulatory effectors are distinctly different. Furthermore, the behaviors of tumor enzymes can be made to mimic those of liver enzymes by an antitumor agent, poly(I)-(C),[1] and the behaviors of liver enzymes can be made to mimic those of tumor enzymes by a hepatocarcinogen, thioacetamide. These findings demonstrate that rRNA methylases, and therefore the methylation of pre-rRNA, play a significant role in regulating the production of ribosomes.

Materials and Methods

S-Adenosyl-L-[*methyl*-^{3}H]methionine (5 Ci/mmol) and [5-^{3}H]uridine 5′-triphosphate (12.6 Ci/mmol) were obtained from Amersham-Searle. [5-^{3}H]Orotic acid (10 Ci/mmol) was obtained from Schwarz BioResearch. *S*-Adenosyl-L-methionine, ribonucleoside monophosphates and triphosphates, ApA, CpG, CpC, and polyinosinate were obtained from P-L Biochemicals, Inc. Thioacetamide was obtained from Mann Research Laboratories, Inc., and sodium tripolyphosphate from

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[1] The abbreviations used are: NMP, ribonucleoside monophosphates; NTP, ribonucleoside triphosphates; NmpNp, 2′-*O*-methylated dinucleotides; poly(I)(C), polyinosinate:polycytidylate; *S*-Ado-Met, *S*-adenosylmethionine; DEAE, diethylaminoethyl; SE, standard error; EDTA, (ethylenedinitrilo)tetraacetic acid; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol.

TABLE I: Characteristics of Isolated Nucleoli.[a]

Sources of Nucleoli	RNA/DNA	RNA Synthesis (nmol of UMP/mg of DNA)	RNA Methylation (pmol/mg of DNA)		Ratio of RNA Methylation (−NTP) to RNA Synthesis
			+NTP	−NTP	
Tumor	1.46 ± 0.05	7.11 ± 1.07	482 ± 23	256	0.036
Tumor, poly(I)C)	0.61 ± 0.05	0.95 ± 0.30	149 ± 10	110	0.116
Liver	0.65 ± 0.05	0.83 ± 0.12	135 ± 8	94	0.113
Liver, thioacetamide	1.81 ± 0.17	1.46 ± 0.43	157 ± 14	86	0.059

[a] Nucleoli were prepared from untreated Novikoff ascites hepatoma cells, from hepatoma cells pretreated in the rat with poly(I)(C), from normal livers, and from livers of rats pretreated with thioacetamide. The administrations of poly(I)(C) to tumor-bearing rats and of thioacetamide to normal rats were described under Methods. Aliquots of nucleolar preparations were taken for the determination of RNA and DNA content. Aliquots of nucleoli were incubated in the complete RNA-synthesizing medium for the measurement of RNA synthesis and RNA methylation. The complete RNA-synthesizing medium in a total volume of 0.25 ml contained: 0.25 M sucrose, 0.05 M Tris-Cl (pH 7.8), 1 mM $MgCl_2$, 0.2 mM EDTA, 0.04 M NH_4F, 0.4 mM ATP, 0.2 mM each of GTP, CTP, and UTP, 15 μM *S*-Ado-Met, and an amount of nucleoli containing approximately 50 μg of DNA. −NTP designates the incubation medium without ribonucleoside triphosphates. For the measurement of RNA synthesis, 0.2 mM of UTP was replaced by 0.2 mM of [^{3}H]UTP (sp act. = 0.2 Ci/mmol); for the measurement of RNA methylation, 15 μM of *S*-Ado-Met was replaced by 15 μM [^{3}H]CH_3-*S*-Ado-Met (sp act. = 0.8 Ci/mmol). The incubation and assay were as described previously (Liau and Hurlbert, 1975a). The data are the average of 3–7 determinations expressed as means ± SE.

Alfa Products. Bentonite was purified by the procedure of Petermann and Pavlovec (1963). Novikoff ascites hepatoma cells were grown in 150–175 g Sprague-Dawley rats. The cells were harvested for experiments on the 5th day after transplantation.

Preparation of Oligonucleotides. The preparation of alkali-stable dinucleotides was as previously described (Liau et al., 1973). For the preparation of oligoinosinates, poly(I) was hydrolyzed in 0.3 M KOH for 0.5 or 1 h at 37 °C. Alkaline hydrolysis for 0.5 h yielded primarily tri- to heptainosinate and longer oligonucleotides that were recovered as a mixture, and alkaline hydrolysis for 1 h yielded primarily di- to tetrainosinates. The fractionation of oligoinosinates was achieved by column chromatography on DEAE-cellulose according to Tener (1967). The recovery of oligoinosinates was as previously described (Liau et al., 1973). The fractions thought to be di- or triinosinate were further confirmed by co-chromatography on DEAE-cellulose columns with alkali-stable 2′-*O*-[^{3}H] methyl dinucleotides.

Preparation and Incubation of Nucleoli. The treatment of rats bearing Novikoff ascites hepatoma cells with poly(I)(C) was as previously described (Liau et al., 1975) by administering intraperitoneally a single dose, 5 mg/kg, for 16 h. The treatment of normal rats with thioacetamide was as described by Steele and Busch (1961) by consecutive intraperitoneal injections for 9 days with a daily dosage of 50 mg/kg. The preparation and incubation of nucleoli were as previously described (Liau et al., 1975; Liau and Hurlbert, 1975a)

Incubation of Prelabeled Nucleoli. Normal rats or thioacetamide-treated rats, as mentioned above, were given, by intraperitoneal injection, 0.25 mCi/rat of [^{3}H]orotic acid dissolved in isotonic saline solution. Rats were sacrificed 3 h later. A small aliquot of liver homogenate was analyzed for the incorporation of [^{3}H]orotic acid into nuclear and cytoplasmic RNA according to previous procedures (Liau et al., 1973). The rest of the liver homogenate was processed for the preparation of nucleoli. Half of each nucleolar preparation was suspended in 0.25 M sucrose, 5 mM $MgCl_2$, 0.04 M NH_4F, 0.05 M Tris, pH 7.4, and 1 mg/ml of bentonite, and incubated at 30 °C for 15 min. The other half was suspended in the same solution but was left at 0 °C. Nucleoli, incubated and nonincubated, were recovered by centrifugation at 900*g* for 10 min. The extraction of RNA by phenol deproteinization and analysis of RNA on sucrose gradients were as previously described (Liau et al., 1973; Liau and Hurlbert, 1975a).

Analytical Procedures. Determinations of RNA, DNA, and radioactivity were as previously described (Liau and Hurlbert, 1975a).

Results

Characteristics of Isolated Nucleoli. Table I summarizes RNA contents and activities of RNA polymerase and rRNA methylases of nucleoli prepared from four sources: rat Novikoff ascites tumor cells, tumor cells from rats treated with poly(I)-(C), normal rat livers, and livers from rats treated with thioacetamide. RNA content of nucleoli represents the balance of nucleolar activities including synthesis and processing of pre-rRNA and wastage of rRNA. The amount of labeled nucleotide incorporated into RNA is a measure of the relative amount of RNA polymerase engaged in transcription and, therefore, the relative amount of endogenous nascent pre-rRNA. We have previously demonstrated that methyl groups were incorporated in vitro both into the acceptor sites on nascent pre-rRNA generated in vivo and those elongated in vitro (Liau and Hurlbert, 1975a). Methylation occurring when nucleoli were incubated in the absence of ribonucleoside triphosphates is a measure of the former acceptor sites, and methylation occurring when nucleoli were incubated in the presence of ribonucleoside triphosphates is a measure of the total acceptor sites. The ratio "RNA methylation (-NTP)/RNA synthesis" is, therefore, an indication of the efficiency of RNA methylation taking place in vivo. A lower ratio indicates a higher efficiency of RNA methylation in vivo. The RNA synthesizing activity of tumor nucleoli was 8.6 times that of liver nucleoli, and the efficiency of RNA methylation in tumor cells was greater than that in livers by a factor of 3.1. The accumulation of RNA in tumor nucleoli was only 2.2 times that in liver nucleoli, indicating that the rate of processing may be higher in tumor nucleoli and/or the wastage of rRNA may be greater in liver nucleoli. Poly(I)(C) is an antitumor agent that inhibits Novikoff tumor nucleolar rRNA methylases and causes great reduction of nucleolar RNA content (Liau *et al.*, 1975). The characteristics of nucleoli isolated from poly(I)-(C)-treated tumor cells were found to resemble closely those of liver nucleoli with respect to the RNA content and the activities of RNA polymerase and rRNA methylases. Thioace-

TABLE II: Effectiveness of Regulatory Effectors on the Activity of rRNA Methylases.[a]

Inhibitory Effectors, 1 mM	% Inhibition of the Tumor Enzymes
IMP, NMP, N(2′,3′)P[b]	15–27
$(Ip)_2$, ApA, CpG, CpC, NmpNp	39–45
$(Ip)_3$	70
$(Ip)_4$	65
$(Ip)_5$	42
$(Ip)_6$	32
$(Ip)_7$	19
$(Ip)_8$ and above	0

Stimulatory Effectors, 1 mM	% Stimulation of the Liver Enzymes
NTP	87–105
P-P_i	65
P-P-P_i	81

[a] Nucleoli were prepared from tumor cells and livers. The incubation was carried out in a medium described in Table I but without ribonucleoside triphosphates and nonlabeled *S*-Ado-Met. [^{3}H]CH_3-*S*-Ado-Met was employed at 1–2 μM (sp act. = 5 Ci/mmol). The effect of each nucleotide was tested singly except for NmpNp which was a mixture of 2′-*O*-methylated dinucleotides. [b] N stands for adenosine, guanosine, cytidine, or uridine. $(Ip)_2$ is IpIp.

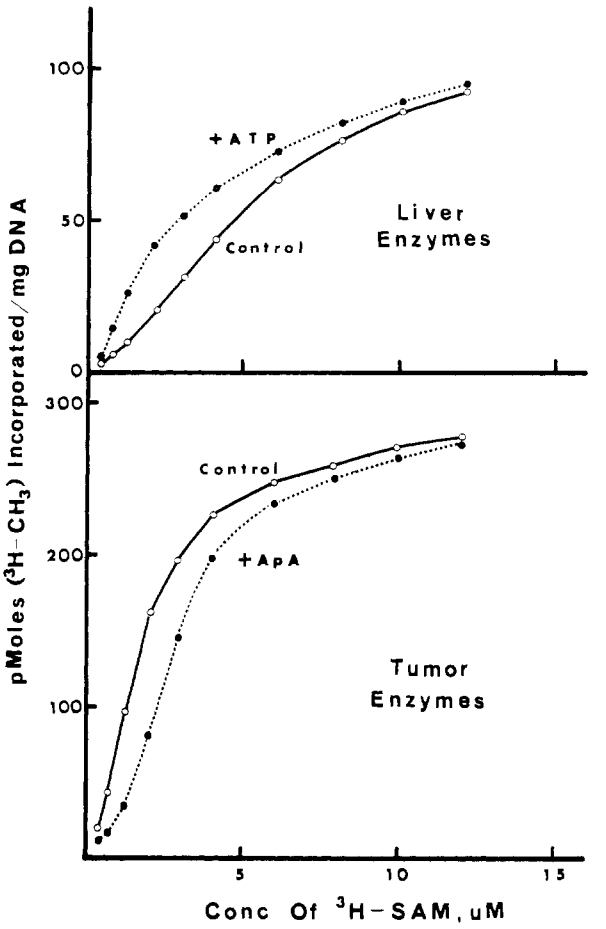

FIGURE 1: Activity of nucleolar rRNA methylases as a function of *S*-Ado-Met concentration. Nucleoli were prepared from tumor cells and livers and incubated for 10 min in the medium described in Table II with various concentrations of [^{3}H]CH_3-*S*-Ado-Met (^{3}H-SAM), as indicated. Tumor nucleoli were also incubated in the presence of 1 mM ApA, and liver nucleoli in the presence of 1 mM ATP with the same variations of [^{3}H]CH_3-*S*-Ado-Met.

tamide is a weak hepatocarcinogen that greatly enhances the accumulation of RNA in liver nucleoli (Steele and Busch, 1961; Gonzalez-Mujica and Mathias, 1973). The treatment of normal rats with thioacetamide promoted a 1.8-fold increase in RNA polymerase activity in isolated nucleoli, a 1.9-fold increase in the efficiency of RNA methylation in vivo, and a 2.8-fold increase in RNA content. The greater increase in RNA content, compared to the increase in the activity of RNA polymerase, indicates that the processing rate of pre-rRNA may be reduced and/or the wastage of rRNA normally taking place in liver nucleoli may be reduced. (Further evidence on this point, the retention of high-molecular-weight precursor RNA in thioacetamide-treated liver nucleoli, is shown later in Figure 4.)

Modulation of the Activity of rRNA Methylases by RNA Metabolites. The activity of rRNA methylases was significantly affected by the presence of nucleotide metabolites, added singly, during incubation in the medium without ribonucleoside triphosphates. As presented in Table II, mononucleotides and oligonucleotides inhibited the activity of tumor rRNA methylases, and ribonucleoside triphosphates stimulated the activity of liver rRNA methylases. The inhibition of tumor enzymes by oligonucleotides of inosine was dependent upon the chain length. Trinucleotide and tetranucleotide were the most inhibitory, and the inhibitory activity diminished with decreasing or increasing chain length. Oligonucleotides with chain length of eight and above had no inhibitory effect. The degree of inhibition was not significantly different among different mononucleotides, whether 5′-phosphates or 2′(3′)-phosphates. The stimulation of liver rRNA methylases was effected to the same degree by four common ribonucleoside triphosphates, and to a lesser extent by pyrophosphate and tripolyphosphate, indicating that the polyphosphate group may be the responsible portion of the molecules. As will be described later, the responses of tumor enzymes and liver enzymes to these regulatory effectors are distinctly different.

The inhibitory or stimulatory activities of nucleotide metabolites on rRNA methylases were dependent upon *S*-Ado-Met concentration and pH during the incubation. One of the characteristics that serves to distinguish the activities of tumor and liver enzymes is the response to substrate concentrations as shown in Figure 1. When the activity of rRNA methylases was assayed as a function of increasing [^{3}H]methyl-*S*-Ado-Met concentrations; the tumor enzymes responded with a typical Michaelis–Menten curve and the liver enzymes with a sigmoidal dependence curve, deviating from Michaelis–Menten kinetics. The presence of an inhibitory effector, ApA, caused the tumor enzymes to respond with a sigmoidal curve like the liver enzymes; the presence of a stimulatory effector, ATP, caused the liver enzymes to respond with a hyperbolic curve like the tumor enzymes. The apparent K_m values for *S*-Ado-Met estimated from Lineweaver–Burk plots (not shown) are 2.17 μM for the tumor enzymes and 4.5 μM for the liver enzymes, which is an approximate value since the plot does not yield a linear relationship. The K_m value for the tumor enzymes is raised to approximately 3.3 μM in the presence of 1 mM ApA, and that of the liver enzymes is lowered to 3.45 μM in the presence of 1 mM ATP. Apparently the rRNA methylases have an extremely high affinity for *S*-Ado-Met. The actual K_m values may be lower than the values presented here, since *S*-Ado-Met is also significantly utilized by protein methylases and to a very limited extent by DNA methylase;

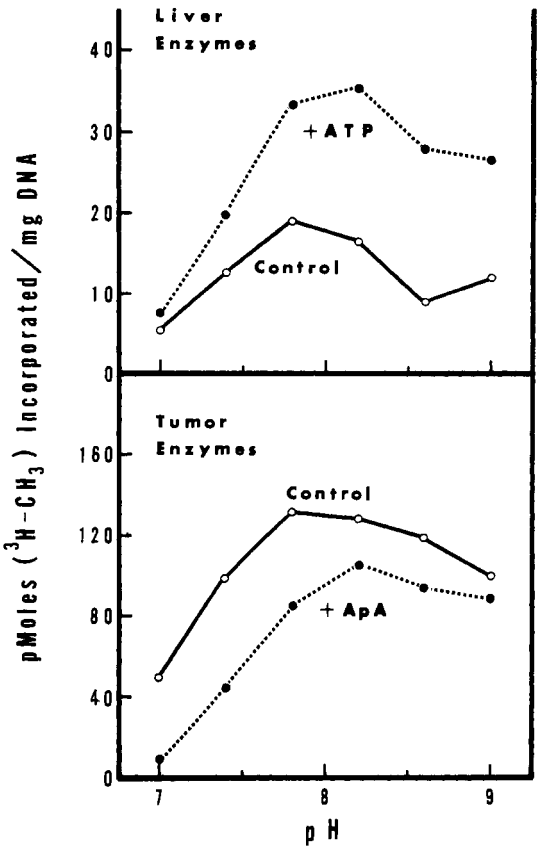

FIGURE 2: Effect of pH on the effectiveness of regulatory effectors on the activity of rRNA methylases. Preparation and incubation of nucleoli were the same as described in Table II except that pH's were varied as indicated. Nucleoli were incubated with subsaturating concentrations of [^{3}H]CH$_3$-*S*-Ado-Met, 1.24 μM for tumor nucleoli and 2.08 μM for liver nucleoli.

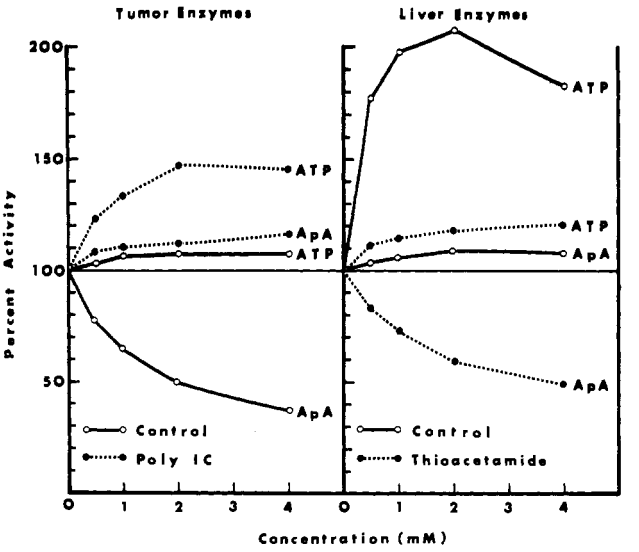

FIGURE 3: Responsiveness of nucleolar rRNA methylases toward regulatory effectors. Nucleoli were prepared from tumor cells (tumor enzymes-control), from tumor cells treated with poly(I)(C) in the rat (tumor enzymes-poly(IC), from normal livers (liver enzymes-control), or from livers of rats treated with thioacetamide (liver enzymes-thioacetamide). These were incubated with 1.24 μM [^{3}H]CH$_3$-*S*-Ado-Met in the medium described in Table II, in the presence of varied amounts of ApA or ATP as indicated. The control activities were 110, 22, 12, and 30 pmol of methyl groups incorporated per mg of DNA for tumor nucleoli, poly(I)(C)-treated tumor nucleoli, liver nucleoli, and thioacetamide-treated liver nucleoli, respectively.

in addition, *S*-Ado-Met may be degraded in these complex systems. It appears that the action of these regulatory effectors on rRNA methylases is primarily at concentrations of *S*-Ado-Met between 1-6 μM. Beyond these ranges of *S*-Ado-Met, these effectors seem to exert very little effect on the activity of rRNA methylases.

Figure 2 shows that the stimulation of the liver rRNA methylases by ATP was greater at pH above 7.8, and the inhibition of the tumor enzymes by ApA was greater at pH below 7.8.

Different Response of Novikoff Tumor and Liver Nucleolar rRNA Methylases to Regulatory Effectors. Figure 3 shows that the rRNA methylases associated with isolated tumor nucleoli were inhibited up to 63% by 4 mM ApA but were stimulated less than 10% by 1-4 mM ATP. Conversely, the liver enzymes were stimulated up to 108% by 2 mM ATP but were not inhibited by the inhibitory effectors. As a matter of fact, all inhibitory effectors tested on the liver enzymes showed slight stimulation of the activity, due perhaps to the inhibition of ribonuclease activity.

The responses of nucleolar rRNA methylases to these regulatory effectors were found to be altered by treatments affecting nucleolar metabolic activities. Figure 3 also shows that the treatment of tumor-bearing rats with poly(I)(C) caused the tumor enzymes to respond to these effectors like the liver enzymes, and the treatment of normal rats with thioacetamide caused the liver enzymes to respond like the tumor enzymes.

Demonstration of the Link between the Activity of rRNA Methylases and the Stability of Nucleolar RNA. The stability of nucleolar RNA was studied by incubation of isolated nucleoli, prelabeled with [^{3}H]orotic acid in vivo, in the presence of bentonite and 0.04 M NH_4F to reduce as much as possible nonspecific ribonuclease activities (Liau et al., 1970; Lazarus and Sporn, 1967). The degradation of RNA under these conditions, therefore, represents mainly the activities of enzymes that are responsible for the specific processing of pre-rRNA or the wastage of rRNA. We have previously shown that nucleolar RNA of tumor cells was more stable than nucleolar RNA of the tumor cells pretreated with poly(I)(C) (Liau et al., 1975) and present here observations on liver nucleolar RNA.

Pretreatment of rats with thioacetamide, as described under Methods, increased the incorporation of [^{3}H]orotic acid into liver nucleolar RNA by 687%, nuclear RNA by 197%, but cytoplasmic RNA by only 4% over the control liver. Thus, the increased RNA products are primarily retained within the nucleus. A similar result was previously reported by Kleinfeld (1966). During incubation of labeled nucleoli, the specific activity of normal liver nucleolar RNA decreased 8.6%, whereas the specific activity of thioacetamide-treated nucleolar RNA remained the same, indicating that most of the processed or degraded products remained acid-insoluble. As shown in Figure 4, when normal liver nucleoli were incubated in vitro, a large proportion of the labeled RNA was found to sediment below 18S, and the labeled RNA in 18S region exceeded that in 28S region; these results indicate that considerable amounts of labeled rRNA sequences underwent endonucleolytic degradation. When nucleoli from thioacetamide-treated livers were incubated, the majority of the RNA was maintained at higher sedimentation values (above 18S) characteristic of undegraded nucleolar RNA. Thus, in the case of both liver and Novikoff tumor nucleoli, an increased activity of rRNA methylases in vivo is associated with an increased stability of

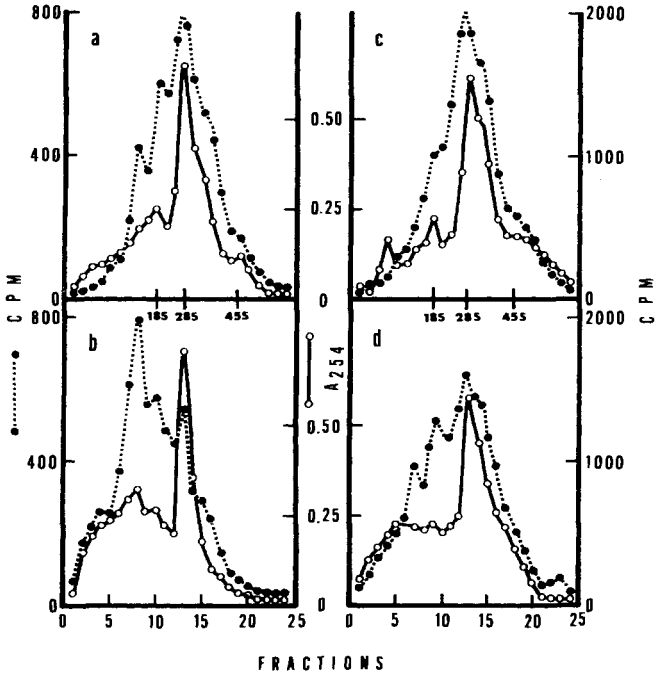

FIGURE 4: Stability of nucleolar RNA from livers and thioacetamide-treated livers. Nucleolar RNA was labeled in vivo for 3 h by intraperitoneal injection of 0.25 mCi of [^{3}H]orotic acid into each control rat or thioacetamide-treated rat. Nucleoli were prepared from control livers and thioacetamide-treated livers. One-half of each nucleolar preparation was incubated in 0.25 M sucrose, 5 mM $MgCl_2$, 0.04 M NH_4F, 0.05 M Tris-Cl (pH 7.4), and 1 mg/ml of bentonite at 30 °C for 15 min. The other half was set aside as nonincubated control. RNA was prepared by extraction with phenol and precipitation in 2 M NaCl, as previously described (Liau et al., 1975), and centrifuged on 5 ml of 10–40% sucrose gradients in 0.1 M LiCl, 0.01 M EDTA, 0.01 M Tris-Cl (pH 7.4), and 0.2% Sarcosyl NL 97 at 45 000 rpm for 4.5 h in an SW 50 rotor. Gradients were fractionated with an ISCO automatic fractionator collecting 0.2 ml/fraction. RNA was precipitated with cold 5% trichloroacetic acid in the presence of 0.1 mg of albumin as carrier, collected, and counted on Millipore filters. Panels a and b are the nonincubated and incubated nucleolar RNA from liver, respectively; panels c and d are the nonincubated and incubated nucleolar RNA from thioacetamide-treated liver, respectively.

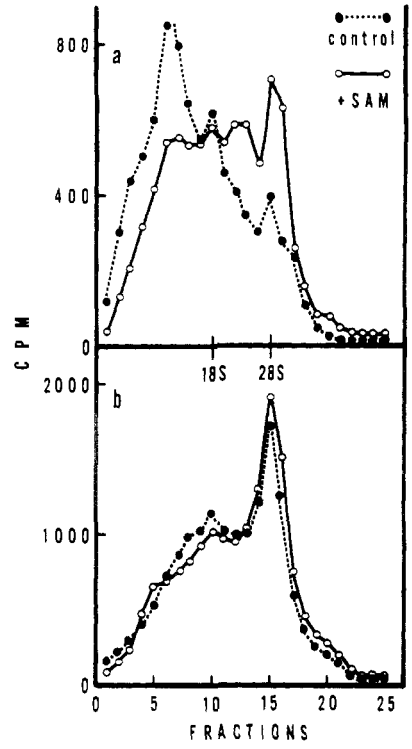

FIGURE 5: Effect of *S*-adenosylmethionine on the stability of nucleolar RNA from livers and thioacetamide-treated livers. Pretreatment of rats with thioacetamide and labeling of nucleolar RNA with [^{3}H]orotic acid were as described in Figure 4. Nucleolar preparation was suspended in 0.25 M sucrose, 5 mM $MgCl_2$, 0.04 M NH_4F, 0.05 M Tris (pH 7.4), and divided into two equal parts. To one part, the nucleolar preparation was allowed to interact with 15 μM of *S*-Ado-Met (+SAM) at 0 °C for 10 min. Before incubation at 30 °C for 15 min, bentonite was added to a concentration of 1 mg/ml. The preparation of RNA and resolution of RNA on sucrose gradients were as described in Figure 4, except that 5–20% sucrose gradient was used and that centrifugation was at 45 000 rpm for 3 h. Panel a is the control liver nucleolar RNA and panel b is the thioacetamide-treated liver nucleolar RNA. Only radioactive profiles are shown in the figure.

nucleolar RNA, or vice versa. Addition of *S*-Ado-Met during incubation of labeled nucleoli significantly protected labeled liver nucleolar RNA from being degraded as shown in Figure 5, whereas the effect of added *S*-Ado-Met on the stability of labeled nucleolar RNA from thioacetamide treated liver nucleoli was not significant.

Discussion

The production of ribosomes evidently is regulated by multiple mechanisms, both at the transcriptional (Pogo, 1972; Yu and Feigelson, 1973; Cooke and Kay, 1973) and the post-transcriptional (Vaughan et al., 1967; Rizzo and Webb, 1969; Cooper and Gibson, 1971; Pederson and Kumar, 1971; Luck and Hamilton, 1972, 1975; Cooper, 1973; Smith et al., 1974) levels. Some evidence indicates that the control operating at the transcriptional level is not efficiently regulated (Vaughan et al., 1967), but rather that controls operating at the post-transcriptional level respond more promptly and efficiently to factors that promote or reduce the growth rate (Vaughan et al., 1967; Rizzo and Webb, 1969; Cooper and Gibson, 1971; Pederson and Kumar, 1971; Cooper, 1973; Luck and Hamilton, 1975). However, as pointed out by Warner and Udem (1972), a mechanism disrupting one of the post-transcriptional processes will ultimately affect the other ribosome-producing processes.

The study of enzyme regulation is usually best conducted with purified enzyme preparations. At present, however, we do not have convenient substrates to permit purification of rRNA methylases, which no doubt include several methylases with different specificity for nucleosides or nucleotide sequences. We must therefore rely on the complex rRNA methylation system present in isolated nucleoli. Use of isolated nucleoli may have some benefits over purified enzyme preparations in that the methylation enzymes can be maintained in their native state and in their normal intimate association with other nucleolar components, especially the rRNA cistrons, RNA polymerases, specific nucleases, and other proteins of various functions, and the results are more likely to reflect what is actually taking place in vivo. The fact that none of the heterologous RNA's serve as substrates for nucleolar rRNA methylases (Liau and Hurlbert, 1975a) and that the methylation patterns obtained in isolated nucleoli resemble closely the methylation patterns of rRNA taking place in vivo (Liau et al., 1973; Liau and Hurlbert, 1975b) indicates the efficacy and specificity of the nucleolar rRNA methylation system.

Our results thus far provide subcellular evidence that rRNA methylases are involved in the regulation of ribosome production. The evidence is based on the responsiveness of these enzymes to regulation by anabolic and catabolic metabolites of RNA, different characteristics of the enzymes from rapidly growing tumor cells and resting hepatic cells, and the reversal

of enzyme characteristics by agents that inhibit or promote nucleolar activities. The response of nucleolar rRNA methylases from rat Novikoff ascites tumor and liver to anabolic and catabolic metabolites of RNA was strikingly different. The highly active tumor enzymes responded preferentially to inhibition by catabolic metabolites, whereas the less active liver enzymes responded exclusively to stimulation by anabolic metabolites. We tentatively intrepret these findings as an indication that the tumor and liver enzymes in the intact cells are controlled in their activity by the influence of these or similar metabolic effectors. The tumor enzymes appear to be constantly activated in vivo by an excess of stimulatory over inhibitory effectors, thus responding preferentially to inhibitory effectors in vitro. The liver enzymes appear to be constantly suppressed by an excess of inhibitory effectors in vivo, thus responding exclusively to stimulatory effectors in vitro. After exposure of tumor cells to poly(I)(C), the activity of the tumor enzymes is modulated to assume a less active form like the liver enzymes, and after treatment of normal rats with thioacetamide, the activity of the liver enzymes is modulated to assume a more active form like the tumor enzymes. We have no information regarding the precise mechanism involved in this modulation of enzyme activity in vivo, which persists during isolation of nucleoli. It is unlikely that effective amounts of low-molecular-weight effectors (such as ATP and oligonucleotides) would be retained with isolated nucleoli. Thus, the mechanism seems more likely to involve changes in organization of enzyme complexes rather than the immediate response of classical "allosteric" enzymes to effector concentrations.

The proposed regulation of rRNA methylases, namely stimulation by RNA polymerase substrates and inhibition by products of RNA degradation, is in good agreement with the patterns of RNA metabolism in growing and resting cells. When resting cells are stimulated to growth, the initial event detectable before the onset of increased RNA synthesis is increased uptake and phosphorylation of RNA substrates (Cooke and Kay, 1973). Thus, when the metabolic pattern is programmed for growth, rRNA methylases are modulated to a more active state by the excess of RNA polymerase substrates over RNA degradation products. Conversely, when the metabolic pattern is programmed for nongrowth, rRNA methylases may assume a less active state as modulated by the excess of RNA degradative products. That the liver may indeed contain an excess of catabolic over anabolic metabolites is suggested by the observation of Lazarus and Sporn (1967). They noted a preponderance of endoribonclease I in liver nuclei and of exoribonuclease I in tumor nuclei; this would tend in liver nuclei to favor a relative accumulation of oligonucleotides and in tumor nuclei the depletion of oligonucleotides. The products of endoribonuclease I are oligonucleotides, which are degraded to 5′-mononucleotides by exoribonuclease I (Lazarus and Sporn, 1967). Since tumor cells and rapidly growing normal cells are enriched in enzyme activities of salvage pathway (Criss, 1973; Roux, 1973), the mononucleotides produced will be converted quickly to triphosphates (Hurlbert et al., 1973), which in turn maintain rRNA methylases in an active state.

The results presented in this paper and a previous paper (Liau et al., 1975) show a good correlation between the characteristics of rRNA methylases and the stability of nucleolar RNA. Nucleolar RNA was found more stable when rRNA methylases were in active state characteristic of the tumor enzymes and less stable when the enzymes were in less active state characteristic of the liver enzymes. We have further demonstrated that liver nucleolar RNA but not thioacetamide-treated liver nucleolar RNA could be stabilized during in vitro incubation by *S*-Ado-Met. It is tempting to speculate that rRNA methylases may have dual functions as methylases and endoribonucleases, and that the expression of either activity is controlled by *S*-Ado-Met. The affinity of the enzymes toward *S*-Ado-Met is, on the other hand, regulated by RNA metabolites, since RNA metabolites act primarily to influence the affinity of rRNA methylases toward *S*-Ado-Met at low substrate levels of *S*-Ado-Met. An enzyme with dual functions is not a unique suggestion. We find an example that an enzyme actually possesses dual activities as DNA methylase and restriction endonuclease (Vovis et al., 1974). We also find a precedent that the activity of ribonuclease H is actually modulated by *S*-Ado-Met (Stavrianopoulos and Chargaff, 1973). Evidence thus far presented is by no means conclusive that methylation and wastage of rRNA are being carried out by the same enzyme. It does provide a rationale for further exploration. It appears that the concentration of *S*-Ado-Met in the nucleus may have an important role in controlling the production of ribosomes. We have demonstrated that the activity of *S*-Ado-Met synthetase was detectable in isolated nucleoli, and that the activity of *S*-Ado-Met synthetase in tumor nucleoli was greater than that in liver nucleoli by approximately fourfold (Liau, Hunt, and Hurlbert, manuscript submitted to *Cancer Research*). *S*-Ado-Met synthetase associated with nucleoli is probably related specifically to the production of ribosomes, since Judes and Jacobs (1972) have demonstrated that intracellular pools of *S*-Ado-Met are compartmentalized. The enhanced activities of rRNA methylases and *S*-Ado-Met synthetase in tumor nucleoli may then be generally important factors contributing to the typical growth pattern of malignant cells.

In this and related papers we have provided enzymatic, subcellular, and intact tissue evidence and reasoning to support the postulate that the synthesis and processing of ribosomal RNA is controlled to a significant extent at the methylation step by the relative amounts of nucelotide substrates of RNA synthesis and degradation products of RNA. Evaluation of the extent of actual function, in vivo, of this potential regulatory process depends upon knowledge of the actual cellular concentrations of these inhibitory and simulatory metabolites, especially mono- and oligonucleotides and *S*-Ado-Met. It appears that 1 mM levels of cellular ATP are not unreasonable (Hommes et al., 1973). While 1–4 mM levels of mono- and oligonucleotides in the entire cell do not seem likely, it is conceivable that in the immediate vicinity of the nucleolar rRNA synthesizing apparatus such concentrations occur and fluctuate so as to provide the modulation postulated. Similarly, it can only be conjectured at present whether the concentrations of *S*-Ado-Met available to the rRNA methylases are appropriate for modulation. We believe however, that the proposed regulatory mechanism does provide a testable rationale for further understanding of the role of known metabolites in the control of ribosome biosynthesis. Furthermore, the present paper and the previous paper on the antitumor effect of poly(I)(C) (Liau et al., 1975) suggest that the activity of rRNA methylases plays a crucial role in perpetuating malignant growth and that inhibitors of rRNA methylases can be developed further to provide selective antitumor effects.

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