The Rapid Turnover of RNA Polymerase of Rat Liver Nucleolus, and of Its Messenger RNA

(cycloheximide/actinomycin D/polymerase I)

FU-LI YU AND PHILIP FEIGELSON

The Institute of Cancer Research and the Department of Biochemistry, College of Physicians and Surgeons, Columbia University, New York, N.Y. 10032

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ABSTRACT Turnover rates of the components of systems for RNA synthesis of rat-liver nucleus, nucleolus, and nucleoplasm were investigated. Cycloheximide administered *in vivo* selectively diminished nucleolar RNA synthesis *in vitro*. In contrast to the relatively stable nucleoplasmic RNA polymerase, nucleolar RNA polymerase (polymerase I) from rat liver decays rapidly upon cycloheximide administration, following pseudo-first order kinetics with a half-life of about 1.3 hr. Cycloheximide elicits this effect not through direct interaction with nucleolar RNA polymerase itself, nor by alteration of template function, but rather by inhibition of *de novo* synthesis of one or more of the protein components of nucleolar RNA polymerase. Similarly, when actinomycin-D was administered *in vivo* to inhibit RNA synthesis, the rate of decay of nucleolar RNA polymerase, assayed in the presence of exogenous poly d(A-T) template, was similar to that observed after cycloheximide administration. Thus, the messenger RNA(s) that codes for one or more of the catalytically essential polypeptide components of this enzyme turn over very rapidly with a half-life considerably shorter than 1.3 hr. The rapidity of turnover of both the enzyme protein and its messenger RNA(s) renders nucleolar RNA polymerase highly responsive to altered transcriptional, translational, or post-translational modulation. The marked differences in turnover rates of nucleolar and nucleoplasmic RNA polymerases indicate that at least certain of the protomeric components of nucleolar RNA polymerase I are distinct from those of nucleoplasmic RNA polymerases II and III.

The biochemical mechanisms underlying regulation of gene transcription during hormone action and development, and in response to environmental or physiological stimuli, have been actively investigated in our own and many other laboratories. In addition to messenger RNA (mRNA), which codes for the primary structure of proteins, transfer RNA, and ribosomal RNA are also products of gene transcription. Regulation of the transcriptional synthesis of ribosomal RNA is particularly amenable to study, as it constitutes 80% of hepatic RNA and is synthesized *in vitro* by a reasonably well understood biosynthetic pathway in conveniently isolated nucleoli whereupon it matures, becoming cytoplasmic 18S and 28S ribosomal RNA (1-4).

Recently, we demonstrated that within a few hours after administration of cortisone *in vivo* there is a dramatic increase in ribosomal RNA synthesis, as measured either *in vivo* (5-7) or *in vitro* in isolated nucleoli (3). Furthermore, this increase was due, not to increased availability of the DNA template coding for ribosomal RNA, but rather to an augmentation in the activity of the nucleolar RNA polymerase

per se (3). These findings suggested that nucleolar RNA polymerase (polymerase I) may rapidly turn over and may be hormonally inducible. Several previously unexplained phenomena seemed compatible with this inference: (i) whereas the mean half-life ($t_{1/2}$) of the activity of nucleolar RNA polymerase from whole normal liver is about 12 hr, after partial hepatectomy the increased RNA polymerase activity (presumably largely nucleolar) decays with a maximum $t_{1/2}$ of 2.5-3 hr (8); (ii) inhibition of protein synthesis, either with puromycin or by amino-acid deprivation, results in selective decreases in ribosomal RNA synthesis (9-13); (iii) *in vivo* administration of α -amanitin, a specific inhibitor of RNA polymerase II (14-16), results in a rapid decrease in ribosomal RNA synthesis (17), suggesting participation of rapidly turning over mRNA and protein in maintaining polymerase I levels.

To indirectly measure the turnover rate of hepatic RNA polymerase I, we used cycloheximide (Acti-Dione), a potent inhibitor of protein synthesis in animals (18, 19). Treatment with this antibiotic selectively diminishes rates of ribosomal RNA synthesis *in vivo* in several eukaryotic cells, including *Neurospora crassa* (20), yeast (21, 22), chlorella (23), *Xenopus laevis* (24), HeLa cells (25), and rat liver (2, 26). We have considered the following mechanisms as possibly underlying the diminution of rates of ribosomal RNA synthesis by this inhibitor of protein biosynthesis: (i) repression at the DNA level, by a direct or a negative feedback mechanism, thereby preventing transcription of the ribosomal RNA genome (23, 25); (ii) direct interaction of this antibiotic with nucleolar RNA polymerase, which is responsible for synthesis of ribosomal precursor RNA rendering it inactive either at initiation [as in rifamycin interaction with bacterial RNA polymerase (27)] or during elongation [as in α -amanitin inhibition of mammalian nucleoplasmic RNA polymerase II (14-16)]; or (iii) inhibition of synthesis of nucleolar polymerase by cycloheximide. The last possibility presupposes that one or more of the protein components of nucleolar RNA polymerase is metabolically unstable, i.e., is rapidly turning over, and requires uninterrupted protein synthesis to maintain the normal enzyme concentration (28). To distinguish between these alternative mechanisms, we used a technique recently developed in this laboratory (3) that enables us to differentiate whether altered rates of synthesis of ribosomal precursor RNA by nucleolar RNA polymerase is due to modulated repression of the genes coding for ribosomal RNA or to

TABLE 1. Cycloheximide inhibition of synthesis of rat hepatic protein *in vivo*

Group	Cycloheximide	Time	Acid-soluble fraction*		Protein†	
			(cpm/g liver)	(%)	(cpm/g liver)	(%)
1	—	20 min	4,175	+112	144,675	-98
2	+	20 min	8,850		3,600	
3	—	4 hr	3,088	+427	186,525	-99
4	+	4 hr	16,269		2,400	

5 μ Ci/100 g body weight of L-[1- 14 C] leucine (New England Nuclear Corp., 34.1 Ci/mol) was given intraperitoneally 20 min before they were killed to male Sprague-Dawley rats (150–200 g body weight) that were starved overnight before use. Cycloheximide (3 mg/100 g body weight) was given intraperitoneally for 20 min or 4 hr before the animals were killed.

* Acid-soluble fraction was isolated as described (40).

† The proteins were isolated and their radioactivities were determined as described (41).

altered activity *per se* of nucleolar RNA polymerase. Our findings indicate that diminution of rates of ribosomal RNA synthesis *in vivo* by cycloheximide is associated with decreased concentrations of nucleolar RNA polymerase I and that this enzyme, in contrast to the relatively stable nucleoplasmic polymerase, turns over rapidly, with a biologic half-life of about 1.3 hr. Parallel experiments with actinomycin D indicate that the mRNA coding for the rapidly turning over component of nucleolar RNA polymerase is itself short-lived with a $t_{1/2}$ considerably less than 1.3 hr.

MATERIALS AND METHODS

Male Sprague-Dawley rats (about 200-g body weight) were starved overnight before use. Cycloheximide (Acti-Dione,

TABLE 2. Effect of cycloheximide *in vivo* upon RNA polymerase activities of isolated hepatic nuclear, nucleolar, and extranucleolar components

Fraction	Cycloheximide* (hr)	RNA polymerase activity†			
		[3 H]UTP	%	[14 C]GTP	U/G ratio
Nuclear	0	89 $\pm$ 9	100	548 $\pm$ 33	100
	2	80 $\pm$ 47	90	344 $\pm$ 159	63
	4	69 $\pm$ 25	78	222 $\pm$ 67	41
Nucleolar	0	149 $\pm$ 56	100	891 $\pm$ 204	100
	2	69 $\pm$ 74	46	271 $\pm$ 21	30
	4	51 $\pm$ 15	34	129 $\pm$ 25	15
Extra-nucleolar	0	86 $\pm$ 11	100	117 $\pm$ 40	100
	2	81 $\pm$ 23	94	86 $\pm$ 6	74
	4	92 $\pm$ 35	107	83 $\pm$ 10	71

* Cycloheximide (3 mg/100 g body weight) was given intraperitoneally, and the animals were killed at the indicated times thereafter.

† Values given are mean $\pm$ SE of pmol of nucleoside monophosphate incorporated per mg DNA of duplicate determinations in each of three replicate experiments.

Mann Research Lab.) (3 mg/100 g of body weight) and actinomycin D (Nutritional Biochemicals Corp.) (200 μ g/100 g of body weight) were injected intraperitoneally at the indicated times before the rats were killed. L-[1- 14 C]leucine (New England Nuclear Corp., 34.1 Ci/mol) (5 μ Ci/100 g of body weight) was also administered intraperitoneally 20 min before the rats were killed.

The procedures for isolation of rat-liver nuclei, nucleoli, and nucleoplasm have been described (3). RNA polymerase activity was assayed *in vitro*, with a low ionic strength-Mg $^{+2}$ -rich reaction mixture (29) of 0.6–0.9 ml total volume containing: 100 mM Tris-HCl pH 8.2, 5 mM MgCl $_2$, and 0.2 mM each of ATP, CTP, UTP, and GTP; 0.1 μ Ci [3 H]GTP (Schwarz/Mann, Div. of Becton and Dickson Corp., 39 Ci/mol) and 2 μ Ci [5 H]UTP (New England Nuclear Corp., 22.2 Ci/mmol), singly or together, and 20 mM 2-mercaptoethanol were included, as indicated. The reaction was initiated by addition of 0.1 ml nuclear, nucleolar, or nucleoplasmic suspension in 0.34 M sucrose, derived from 0.1, 0.6, and 0.1 g of liver, respectively, and incubated for 15 min at 37° with shaking. In appropriate experiments, 10 μ g of actinomycin-D in 50 μ l and 40 μ g poly d(A-T) (Miles Laboratories Corp.) in 100 μ l were added to the incubation medium at zero time. The reaction was stopped by transfer to chilled ice followed by immediate addition of 5 ml of cold 10% trichloroacetic acid containing 1% sodium pyrophosphate. The acid-soluble material was collected on Millipore filters (24 mm, Type HA), which were then washed three times with 10 ml of 5% cold trichloroacetic acid containing 1% sodium pyrophosphate and once with 3 ml of 60% ethanol. The filters were air-dried in liquid scintillation vials and counted in 10 ml of Bray's solution (30). The specific activity of RNA polymerase was expressed as pmol of labeled [3 H]UMP or [14 C]GMP incorporated per mg DNA. DNA concentrations were determined as described by Burton (31).

RESULTS AND DISCUSSION

For evaluation of rates of synthesis and degradation of nucleolar RNA polymerase, protein synthesis was inhibited

FIG. 1. Rate of decay of nucleolar RNA polymerase after *in vivo* administration of cycloheximide. See Table 2 for experimental conditions.

TABLE 3. Effect of cycloheximide injected *in vivo* on activity of rat-liver nucleolar RNA polymerase measured *in vitro* with endogenous and exogenous template

Exp.	Cyclo-hemimide*	Actino-mycin-D	Poly d(A-T)	[³ H]UTP	%	[¹⁴ C]GTP	%	U/G
1	—	—	—	493		1987		0.25
	+	—	—	105	—79	435	—78	0.24
	—	+	+	333		144	(—93)	2.3
	+	+	+	80	—76	62	(—86)	1.3
2	—	—	—	156		689		0.22
	+	—	—	89	—43	254	—63	0.35
	—	+	+	216		132	(—81)	1.6
	+	+	+	133	—39	68	(—73)	2.0

Values are given as pmol of nucleoside monophosphate incorporated per mg DNA.

* Cycloheximide (3 mg/100 g body weight) was given intraperitoneally 2 hr before the animals were killed.

in vivo with cycloheximide. Although, at the present time, its precise mechanism of action is uncertain (32–36), cycloheximide is an inhibitor of protein synthesis in eukaryotic cells, such as rat liver, which contain 80S ribosomes (19). Table 1 clearly shows that a single intraperitoneal injection of 3 mg of cycloheximide per 100 g of body weight rapidly and completely inhibited protein synthesis *in vivo* for at least 4 hr. Concurrent with its inhibition of incorporation of [¹⁴C]-leucine into hepatic protein, cycloheximide administration increased the amounts of labeled amino acid remaining in the acid-soluble amino-acid precursor pool, presumably due to its diminished use for protein synthesis.

In parallel experiments (Table 2), in which the antibiotic was administered *in vivo*, activity of nucleolar RNA polymerase was selectively diminished to one-third or less of the control value by 4 hr after administration of antibiotic. On the other hand, the activity of nucleoplasmic RNA polymerase barely decreased throughout this period of inhibited protein synthesis. This selective diminution of nucleolar RNA synthesis by cycloheximide, as herein measured *in vitro*, concurs with *in vivo* tracer studies in which ribosomal RNA synthesis was reduced upon inhibition of protein synthesis (2, 20–26). Table 2 also presents U/G incorporation ratios, which, in conformance with our earlier observations (3, 29), indicate that the nucleolar and nucleoplasmic fractions synthesize RNA *in vitro* the base compositions of which resemble those of ribosomal and messenger RNAs, respectively; these findings agree with the current concept that nucleoli are the sites of synthesis of guanine-rich ribosomal RNA (37–39) and the nucleoplasm is the site of synthesis of uracil-rich mRNA (1).

Fig. 1 depicts the decreasing activity of hepatic nucleolar RNA polymerase as a function of time after *in vivo* administration of cycloheximide; the activity decays with pseudo-first order kinetics manifesting a biologic half-life ($t_{1/2}$) for this RNA polymerase-chromatin system of about 1.3 hr. To determine whether the rapidly turning over, catalytically essential protein component(s) was intrinsic to the RNA polymerase itself or to the chromatin template being transcribed, we performed an experiment (3) that enables one to distinguish between altered template function and altered

RNA polymerase activity. Table 3 illustrates that the endogenous template function is virtually abolished by actinomycin-D (see [¹⁴C]GTP incorporation column). The activity *per se* of nucleolar RNA polymerase is then measured in the presence of saturation amounts of exogenous template poly d(A-T) (see [³H]UTP incorporation column); poly d(A-T) codes for poly(U, A) and is not inhibited by actinomycin-D (3). It is evident from Table 3 that synthesis of RNA by nucleolar polymerase, with either endogenous or exogenous template, was equally diminished by prior cycloheximide treatment *in vivo*. These results indicate that the cycloheximide-sensitive component is intrinsic to nucleolar RNA polymerase itself and not to the chromatin template.

A priori cycloheximide may reduce activity of nucleolar RNA polymerase either by interacting directly with the enzyme, rendering it inactive, or by inhibiting the synthesis of one or more catalytically essential protein component(s) of the enzyme, thereby decreasing the amount of active enzyme. The following experiments tested and excluded the first of these possibilities.

Cycloheximide, in various concentrations, was added directly to the reaction medium during transcription of the endogenous template by the native enzyme. Fig. 2 indicates

FIG. 2. *In vitro* effect of cycloheximide upon RNA polymerase activities of isolated nuclear (○), nucleolar (●), and nucleoplasmic (▲) fractions. The indicated amounts of cycloheximide were added at zero time to the incubation mixture (final volume of 0.7 ml). *In vitro* incorporation at 37° for 15 min was as described (3).

TABLE 4. Effect of cycloheximide added to the *in vitro* assay mixture of nucleolar RNA polymerase transcribing exogenous template

Cycloheximide (μ g)	Actinomycin-D	Poly d(A-T)	[3 H]UTP	%	[14 C]GTP	%
0	—	—	2681		7546	100
0	+	+	968	100	0	0
30	+	+	978	101	138	2
600	+	+	1157	120	46	1

Values are given as pmol of nucleoside monophosphate incorporated/mg DNA. In addition to other components (29), the incubation medium contained 20 mM 2-mercaptoethanol. The following were added to the incubation mixture at zero time: indicated amount of cycloheximide in 100 μ l of H₂O, 10 μ g actinomycin-D in 50 μ l of H₂O, and 40 μ g poly d(A-T) in 100 μ l of H₂O.

that even at very high concentrations this antibiotic fails to inhibit polymerase activity. However, these findings do not rule out the possibility that cycloheximide might interfere with the enzyme function only when the polymerase is unbound and requires the initiation step of RNA synthesis, in a manner similar to rifamycin inhibition of bacterial RNA polymerase (27). Therefore, the effect *in vivo* of cycloheximide on nucleolar RNA polymerase was evaluated with exogenous template under conditions under which the endogenous template was not being transcribed [i.e., in the presence of actinomycin-D (3)]. The results of this study (Table 4) again indicate no direct inhibition of RNA polymerase activity by cycloheximide *in vitro*. We, therefore, concluded that the diminution of activity of nucleolar RNA polymerase by cycloheximide *in vivo* is a consequence of inhibited *in vivo* synthesis of one or more essential protein components of the enzyme that are metabolically unstable and turn over rapidly with a half-life of about 1.3 hr.

The half-life of the mRNA that codes for nucleolar RNA polymerase was estimated by administration of actinomycin-D *in vivo* to block RNA synthesis; RNA polymerase activity *per se* was measured *in vitro* with exogenous template poly d(A-T) as a function of time after administration of actinomycin-D *in vivo*. Fig. 3 depicts a decay curve for RNA polymerase obtained under these conditions, that is similar to that obtained in cycloheximide-treated animals (Fig. 1). The absence of extended translational synthesis of polymerase after administration of actinomycin D indicates that the mRNA(s) that codes for the rapidly turning over protein component(s) of nucleolar RNA polymerase is itself turning over very rapidly with a half-life much shorter than 1.3 hr.

The present study provides the following evidence that one or more polypeptide components of nucleolar RNA polymerase, as well as the mRNA(s) that code for this component(s), are indeed among the most rapidly turning over macromolecules in liver. Isolated nucleoli are capable of template-dependent nucleotide incorporation *in vitro* with a U/G base ratio that corresponds to ribosomal precursor RNA. Cycloheximide has no direct effect *in vitro* on preformed RNA polymerase, whereas inhibition of protein synthesis mediated by cycloheximide *in vivo* results in a fall in nucleolar RNA polymerase activity, which is pseudo-first

FIG. 3. Rate of decay of nucleolar RNA polymerase after administration of actinomycin D *in vivo*. Actinomycin D (200 μ g/100 g body weight) was given intraperitoneally at the indicated times before the animals were killed. RNA polymerase activity of isolated nucleoli was assayed *in vitro* in the presence of actinomycin D and poly d(A-T) under conditions identical to those described in Table 3.

order with a $t_{1/2}$ of 1.3 hr. Similar half-lives are evident with both endogenous chromatin and exogenous synthetic deoxynucleotide templates. After actinomycin D administration *in vivo*, which blocks synthesis of the mRNAs coding for nucleolar polymerase, a comparable rate of decrease in nucleolar RNA polymerase activity is evident.

It remains unknown whether it is one or more of the protomeric units of nucleolar RNA polymerase that manifests this short half-life or whether all components of the molecule are rapidly synthesized and degraded coordinately. It is evident that the rapidly turning over polypeptide(s) is a component of the enzyme *per se* and not of the chromatin template. The short life-time of this polypeptide (28) and of its mRNA create the potential for rapid transcriptional, translational, and post-translational modulation of nucleolar RNA polymerase concentrations. These findings support the possibility that hormonal and developmental modulation of ribosomal RNA synthesis may be achieved not through derepression of the nucleolar genome that directly codes for ribosomal RNA but rather through derepression of the genome(s) that generate mRNA(s) coding for the synthesis of one or more of the catalytically essential polypeptide components of nucleolar RNA polymerase.

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