A SHORT LIVED POLYPEPTIDE COMPONENT OF ONE OF TWO DISCRETE FUNCTIONAL POOLS OF HEPATIC NUCLEAR α-AMANITIN RESISTANT RNA POLYMERASES

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SUMMARY

Poly d(A-T) addition to isolated nuclei results in increased RNA polymerase activity which is additive to and does not inhibit or compete with transcription of the endogenous template. Levels of cycloheximide administered *in vivo*, which are adequate to inhibit protein synthesis, yield hepatic nuclei with levels of α-amanitin resistant RNA polymerase activity which are unimpaired when assayed with exogenous poly d(A-T) template and markedly lowered when acting upon the endogenous DNA template. Thus (1), functionally discrete pools of α-amanitin resistant RNA polymerase activity co-exist in hepatic nuclei; and (2) a rapidly turning over polypeptide seems to regulate the rate of ribosomal RNA synthesis by determining the proportion of the existant RNA polymerase I molecules which at any moment in time are transcribing the nucleolar DNA template.

INTRODUCTION

Rat liver cells, like other eukaryotic cells, contain three classes of nuclear DNA dependent RNA polymerases (1,2) and reportedly one cytoplasmic polymerase (3). Polymerase I is classically considered to be localized in the nucleolus, where it catalyzes the synthesis of ribosomal precursor RNA (4), is insensitive to the fungal toxin α-amanitin, and is active at low ionic strength (1,5). Polymerase II is located in the nucleoplasm, where it presumably catalyzes the transcriptive synthesis of mRNA (4), is totally inhibitable by α-amanitin and is active at high ionic strength (1,3,5). Polymerase III is of uncertain function, is resistant to α-amanitin and is active at low to moderate ionic strength (1,4,6).

Recent work has shown that the intracellular rate of nucleolar RNA synthesis is subject to rapid modulation. Amino acid or glucose deprivation, or α-amanitin or cycloheximide treatment rapidly decrease ribosomal RNA synthesis in cell suspensions (7) and *in vivo* (8-10). Reciprocally, ribosomal RNA synthesis increases *in vivo* within a few hours following hormonal treatment (11-14), and the inception of liver regeneration (15). It has been known for some time that nuclei contain some easily extractable RNA polymerase (16). In

studying the regulation of ribosomal RNA biosynthesis, this laboratory has become interested in whether there exists in the nucleus a reserve of polymerase I functionally unsaturated with DNA template that can be drawn on to rapidly increase the rate of biosynthesis of the 45S ribosomal precursor RNA. If this were the case, it would be important to determine whether the proportion of polymerase actively transcribing the template could be regulated independently of the total polymerase.

Measurement of polymerase activity in isolated rat liver nuclei and nucleoli in the presence of actinomycin D has indicated the presence of RNA polymerase activity that is available for transcribing exogenous polydeoxycytidylate or polydeoxyadenylate-thymidylate [poly d(A-T)] templates (17,18). This polymerase is active at low ionic strength in the presence of α-amanitin and, therefore, operationally shares the properties attributed to polymerase I. Experiments to date had not determined whether this represented species of polymerase I which had been transcribing the endogenous template but which were dissociated therefrom by the synthetic template and/or actinomycin D, or whether this represented a separate nucleolar-bound form of polymerase which was present but not actively transcribing the endogenous template. The present studies indicate that, in the presence of α-amanitin, RNA synthesis evoked by the addition of exogenous poly d(A-T) does not diminish ongoing endogenous template directed RNA synthesis. Furthermore, in isolated nuclei, incorporation of uridine into RNA directed by exogenous poly d(A-T) and by the endogenous nuclear DNA are additive.

Cycloheximide, a potent inhibitor of eukaryotic protein synthesis when administered *in vivo*, had been shown to cause a rapid progressive diminution of nucleolar polymerase I activity. The rate at which this enzyme activity decreases suggested the presence of a catalytically essential polypeptide with an estimated biological half-life of 1.3 hours (18). We now report that in the intact nucleus, this polypeptide component is necessary only for the α-amanitin resistant RNA polymerase transcribing endogenous template, and that the nuclear RNA polymerase available to function on exogenous template is unaffected by administration of cycloheximide *in vivo*. These findings indicate the presence of an intranuclear pool of template-free α-amanitin insensitive RNA polymerase; its intranuclear redistribution allowing its binding to and transcription of the nucleolar genome may permit rapid control over the rate of rRNA synthesis; furthermore, the rapidly turning over polypeptide component may participate in the intranuclear partition of RNA polymerase I.

MATERIALS AND METHODS

Animals: Male Sprague-Dawley rats, weighing 100-250 gms, were maintained

on Purina Lab Chow ad libitum. Cortisone sensitive lymphosarcoma P1798 was maintained by subcutaneous transplant in six to ten week old female Balb/c mice.

Isolation of nuclei: Nuclei were isolated from rat liver by a slight modification of the method of Busch (19), with homogenization in and centrifugation through 2.3 M sucrose - 3 mM $MgCl_2$. Nuclei from 5 grams of liver were suspended in 1 ml of 0.34 M sucrose.

Ten to forteen day old tumors were excised, cleansed of blood and connective tissue and homogenized in 5 volumes of cold 10 mM tris pH 6.5-3 mM $MgCl_2$ by hand in a Potter-Elvehjem homogenizer with 5 strokes of a loose fitting pestle. The homogenate was filtered through cheesecloth, 2.5 volumes of 0.88 M sucrose-23.5 mM $MgCl_2$ were added and the suspension centrifuged at 1000 x g for 5 minutes. The supernatant was discarded, the nuclear pellet suspended in one volume of 2.0 M sucrose - 3 mM $MgCl_2$, layered over 9-10 volumes of 2.0 M sucrose - 3 mM $MgCl_2$, and centrifuged in a swinging bucket rotor for one hour at 48000 x g. The sedimented nuclei were suspended in one volume of 0.34 M sucrose.

Measurement of nuclear RNA polymerase: Since the present study employs isolated nuclei in the presence of inhibitory concentrations of α-amanitin, the cytoplasmic and nuclear polymerase II activities are eliminated. Although polymerase III activity cannot be completely inhibited by α-amanitin, nuclei have been reported to contain 5-10 times more polymerase I than polymerase III activity (20). The standard reaction mixture contained 2.9 μmoles Tris-HCl, pH 7.8; 0.385 nmoles $MgCl_2$; 31 μmoles $HSCH_2CH_2OH$; 4.5 ngrams α-amanitin (Henley & Co.); 76.9 μmoles ATP (Sigma Chemical Co.); 7.7 μmoles UTP (Boehringer Co.); 7.7 μmoles CTP (Boehringer Co.); 5.8 μmoles GTP (Sigma Chemical Co.); and 0.062 μcuries of either 8-^{14}C-GTP (37.6 c/mole in 30% ethanol) or 2-^{14}C-UTP (40.7 c/mole in 50% ethanol) (Schwarz/Mann) in a total volume of 50 μl. Reaction was initiated by adding 25 μl of the nuclear suspension to 25 μl of a mixture of all components of the reaction at 37°C. Actinomycin D (Nutritional Biochemical Co.) or Poly d(A-T) (Miles Laboratories Co.), when present, were added at the concentrations indicated in the figures. The reaction was stopped at the times indicated by adding 5 ml of 7.5% Cl_3CCOOH containing 30 mM $Na_4P_2O_7$. The contents were collected by filtration, washed extensively with 5% Cl_3CCOOH containing 30 mM $Na_4P_2O_7$ and 60% ethanol. Radioactivity was estimated in a liquid scintillation counter. Zero time control values were obtained and subtracted from all data presented. All values are the average of duplicate determinations. DNA was determined by the method of Burton (21).

RESULTS AND DISCUSSION

Previous studies indicated the existence of species of RNA polymerase in isolated nucleoli which could transcribe exogenous polydeoxynucleotide templates in vitro (17,18). Figure 1 demonstrates the detectability and progressive saturation by poly d(A-T) of a comparable activity in whole nuclei when 11.5 μg/ml actinomycin D is added to inhibit transcription of the endogenous template. All measurements are made at the low ionic strength conditions favorable for polymerase I (5) and in the presence of 0.11 μg/ml of α-amanitin which we have verified is sufficient to inhibit the activity of nucleoplasmic polymerase II by at least 75-80% (22).

It was then explored whether the nuclear polymerase which transcribes the added poly d(A-T) is enzyme which was displaced from the endogenous DNA template by actinomycin D and/or the poly d(A-T), or whether this represents free RNA polymerase not otherwise engaged in transcription. Employing isolated hepatic nuclei, the incorporation of ^{14}C-UTP into polynucleotide was measured with

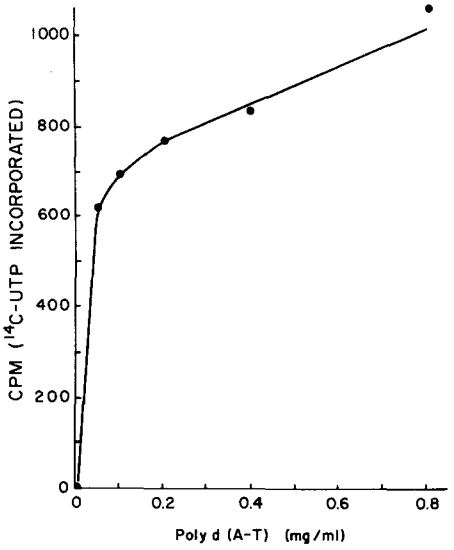

FIGURE 1: POLY d(A-T) STIMULATION OF EXOGENOUS TEMPLATE DEPENDENT RNA SYNTHESIS. Nuclei were prepared from 2 rats. The reaction contained 11.5 µg actinomycin D and the indicated amounts of poly d(A-T). Reaction proceeded for 10_5minutes. Data are plotted as ^{14}C-UTP counts per minute incorporated per 10^5 counts per minute in the reaction. A zero time blank and an incubation without poly d(A-T) (61 cpm) were subtracted from all determinations. Each reaction contained 0.191 mg nuclear DNA.

endogenous template alone, with exogenous poly d(A-T) template added to functional endogenous template and with the exogenous poly d(A-T) template in the presence of actinomycin D to inhibit the endogenous template. Actinomycin D, which is known to bind to dG residues in DNA, does not seem to cause release of polymerase from the template, as the total incorporation in the presence of poly d(A-T) (without actinomycin D) was found to be the sum of the incorporation specified by the endogenous template per se, and the exogenous poly d(A-T) directed synthesis measured in the presence of actinomycin D (Figure 2).

In order to determine if this phenomenon is unique to rat liver, similar studies were made employing nuclei from lymphosarcoma P1798. Figure 3 depicts the stimulation of ^{14}C-UTP incorporation by added poly d(A-T), and the additivity of the polyribonucleotide synthesis dependent upon the endogenous and exogenous templates, thus again indicating that the exogenous template is transcribed by a pool of polymerase molecules not transcribing the endogenous template. To evaluate whether these compartments of RNA polymerase were in equilibrium, studies were made to determine the degree to which added poly d(A-T) might competitively displace polymerase from endogenous template and thus affect the incorporation of ^{14}C-GTP for which the endogenous template, but not the

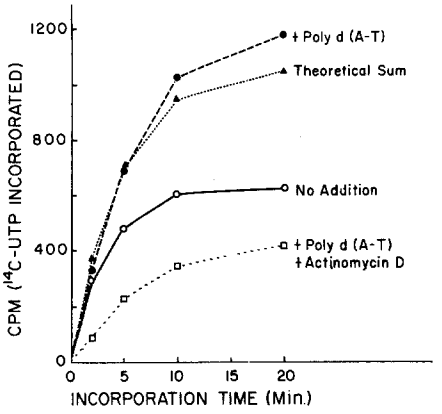

FIGURE 2: ADDITIVITY OF ENDOGENOUS AND EXOGENOUS TEMPLATE DEPENDENT REACTION IN RAT LIVER NUCLEI. Nuclei were isolated from untreated rats. The control reaction is the standard reaction mixture of Materials and Methods. Poly d(A-T) and actinomycin D, when added, were present at 87 µg/ml, and 19.2 µg/ml respectively. Control incorporations containing actinomycin D but no poly d(A-T) (not shown) were subtracted from the values of the incubation with both poly d(A-T) and actinomycin D (squares) to correct for incomplete inhibition of synthesis on the endogenous template. This blank was 50% to 28% of the gross cpm incorporated on poly d(A-T). Inhibition of endogenous template activity was 68% to 77%. Each reaction contained 0.144 mg of nuclear DNA. Data plotted are ^{14}C-UTP cpm incorporated per 8.5 x 10^4 cpm input into the reaction, and the reaction was terminated at the times indicated. "Theoretical sum" is the sum of the incorporation in the control reaction and the reaction supplemented with poly d(A-T) and actinomycin D.

exogenous poly d(A-T) codes. Table I indicates that for both hepatic and lymphosarcoma nuclei, even high concentrations of poly d(A-T) do not decrease transcription of endogenous template throughout the course of the reaction. From these various results, we infer that hepatic and lymphoid nuclei contain pools of α-amanitin insensitive RNA polymerase, functionally independent under these in vitro conditions: one pool of polymerase is tightly bound to and transcribes the endogenous DNA-chromatin template and a distinct pool of enzyme is available to transcribe exogenous template.

We have previously reported a decrease of nuclear and nucleolar polymerase I activity after treatment in vivo with 30 mg/kg of cycloheximide (18). The time course of this decrease indicated that a rapidly turning over polypeptide with a half-life of 1.3 hours is involved in polymerase I activity. Due to questions recently raised (23), experiments were undertaken to determine whether a lower level of cycloheximide, still adequate to inhibit protein synthesis in vivo, would suffice to cause this effect and to explore the relationship between ongoing protein synthesis and the two functionally distinct pools of RNA polymerase I

activities. Figure 4a indicates that template bound α-amanitin insensitive nuclear RNA polymerase is inhibited by 55% 3 hours after administration of 5 mg cycloheximide per kg of body weight in the rat and by 70% with a dose of 30 mg/kg, as previously reported. Figure 4b also shows that within the same nuclei the α-amanitin insensitive pool of RNA polymerase which transcribes the exogenous template does not decrease at all. Therefore, within the intact nucleus, the cycloheximide sensitive component of polymerase I activity seems to be required for transcription of the endogenous template.

It is well appreciated that the degree and nature of intracellular RNA polymerase activity reflects the tissue level of catalytically active enzyme in conjunction with the availability of the DNA template it must transcribe. Regulation of polymerase activity may *a priori* be mediated either by controlling the level of functionally active enzyme or by altering the proportion of the enzyme actively transcribing its DNA template. The latter, in turn, reflects parameters which determine gene availability as well as factors which may act on the enzyme itself to influence its ability to bind with, or act upon, the DNA template.

At low ionic strength and in the presence of α-amanitin addition of poly d(A-T) template to hepatic nuclei leads to an increase in RNA polymerase activity. Two types of polymerase I-like activity seem to co-exist, that acting on the endogenous template and a reservoir of enzyme concurrently available to act upon exogenous template. Following poly d(A-T) addition to hepatic nuclei, the observed increase in RNA polymerase activity is additive to that due to transcription of the endogenous DNA template (Figures 2,3). Furthermore, no inhibition in the transcription of the endogenous template was observed when high levels of exogenous template are added and transcribed (Table I). It thus seems that there are discrete pools of polymerase I which are not in rapid equilibrium under these experimental conditions. A further inference is that within the nucleus reservoirs of polymerase I exist which are not, at any moment in time, acting upon their endogenous template. Alterations in intracellular conditions which would allow these polymerase I molecules to interact with their nucleolar DNA template could serve to rapidly modulate rates of ribosomal RNA synthesis.

These findings, in conjunction with the demonstration that following cycloheximide administration *in vivo* there is a decrease in α-amanitin resistant RNA polymerase activity acting upon the endogenous template with no alteration in the polymerase acting upon exogenously supplied polydeoxynucleotide template, may resolve a seeming discrepancy between experimental findings of several laboratories. Decreases in RNA polymerase I activity transcribing endogenous template have been reported in isolated nucleoli and nuclei following cyclo-

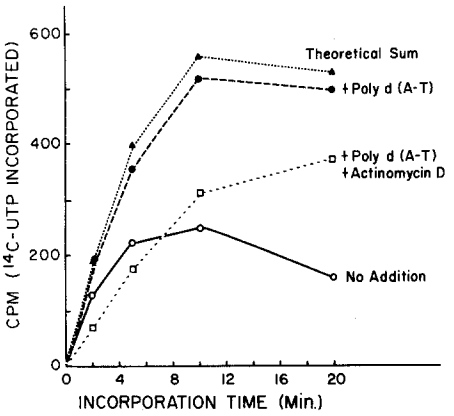

FIGURE 3: ADDITIVITY OF ENDOGENOUS AND EXOGENOUS TEMPLATE DEPENDENT REACTIONS OF LYMPHOSARCOMA P1798 NUCLEI. See the legend to Figure 2 regarding control incubations. Poly d(A-T) and actinomycin D, when added, were present at 0.23 mg/ml and 19.2 µg/ml respectively. Each reaction contained 0.135 mg nuclear DNA and was stopped at the time indicated. "Theoretical sum" is the sum of the incorporation in the control reaction and the reaction supplemented with poly d(A-T) and actinomycin D. Data plotted are ^{14}C-GTP cpm incorporated per 10^5 input cpm.

TABLE I

EFFECT OF ADDED POLY d(A-T) ON ENDOGENOUS TEMPLATE TRANSCRIPTION

	POLY d(A-T) µg/ml	INCORPORATION OF ^{14}C-GTP % OF CONTROL
RAT LIVER* NUCLEI	0	100
	35	96
	70	94
	140	100
LYMPHOSARCOMA P1798** NUCLEI	0	100
	40	107
	82	117
	186†	104

* Nuclei were prepared from the livers of two rats. Incorporation was for 10 minutes. Incorporation in the control was 1273 cpm ^{14}C-GTP/10^5 input cpm. Each reaction contained 0.159 mg nuclear DNA.

** Incorporation was for 10 minutes. Control incorporation was 366 cpm ^{14}C-GTP/10^5 input cpm. Each reaction contained 0.143 mg nuclear DNA.

† This reaction contained all the components for the standard reaction and 186 µg/ml poly d(A-T) in a final volume of 55 µl. Data are corrected for 10% dilution.

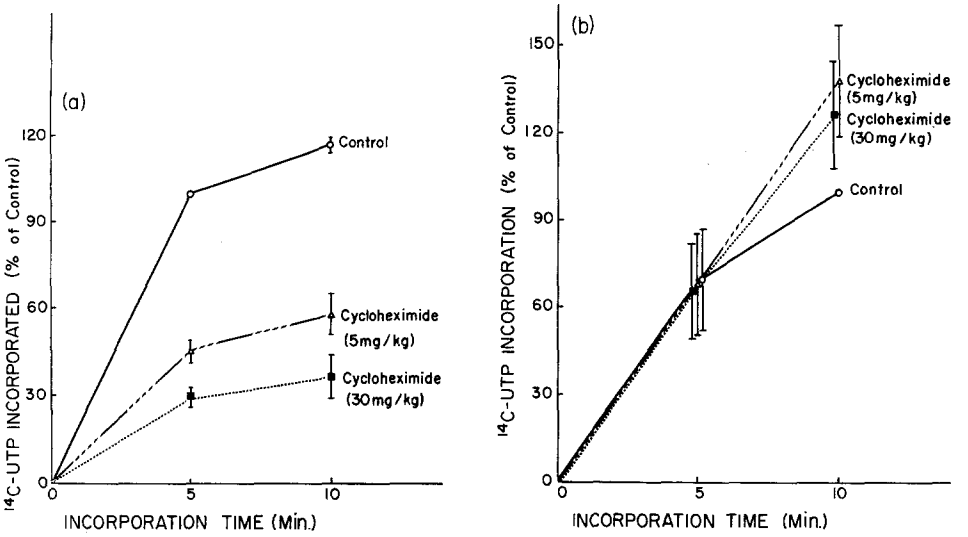

FIGURE 4: EFFECT OF CYCLOHEXIMIDE TREATMENT ON ENDOGENOUS AND EXOGENOUS TEMPLATE DEPENDENT REACTIONS. Rats were starved overnight prior to use. Control rats received 1 ml saline/100 gm body weight (solid line) by intraperitoneal injection. Cycloheximide treated rats received 1 ml of either 0.5 mg cycloheximide/ml saline per 100 gm body weight (5 mg/kg, triangles) or 3 mg cycloheximide/ml saline per 100 gm body weight (30 mg/kg, squares). Animals were killed 3 hours after injection. Values are per cent of control incubation. a)RNA synthesis on endogenous template. Control incubation (5 minutes reaction with control nuclei) represents 4424±631 ^{14}C-GTP cpm per mg DNA per 10^5 input cpm. Data are the average of three experiments. b)RNA synthesis on poly d(A-T) template. Control incubation (10 minute reaction with control nuclei) represents 1282±411 ^{14}C-UTP cpm per mg DNA per 10^5 input cpm. All reactions contain poly d(A-T), 0.17 to 0.66 mg/ml. Each point is the average of 3 to 5 determinations.

heximide administration *in vivo* (8,18). More recently it has been reported that, following similar *in vivo* administration of cycloheximide, the total amount of RNA polymerase I activity which could be extracted from livers of such cycloheximide treated animals was undiminished as compared to control animals that did not receive the antibiotic (3). The latter assays were, of course, conducted upon solubilized extracts of such tissues employing exogenous DNA templates. The present findings support the view that cycloheximide treatment resulted in the inhibited synthesis of a polypeptide which is not required for the functioning of RNA polymerase I with exogenous templates, but which determines the proportion of the polymerase I molecules which are actively transcribing the endogenous DNA template while the total number of polymerase I molecules remains unchanged. Thus, a rapidly turning over polypeptide, acting either on RNA polymerase I or, upon the nucleolar genome, may regulate the functional intranuclear RNA polymerase I distribution and the intracellular rate of ribosomal RNA synthesis.

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