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EFFECT OF HUMAN PLACENTAL RIBONUCLEASE INHIBITOR IN CELL-FREE RIBOSOMAL RNA SYNTHESIS

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SUMMARY

Exposure to purified placental ribonuclease inhibitor caused a marked reduction in ribonuclease activity associated with intact nucleoli. Addition of the placental RNase inhibitor to a nucleolar RNA synthesizing system, prior to the addition of ribonucleoside triphosphates, resulted in a significant increase in the size but did not alter the yield of the RNA products. The effect of the RNase inhibitor on the size of distribution of in vitro transcriptional products could be reversed by the addition of N-ethylmaleimide after transcription was terminated. The properties of the ribonuclease(s) affected by the inhibitor were indicated by its resistance to N-ethylmaleimide and EDTA inhibition and by its limited endonucleolytic cleavage of the in vitro transcript.

The ultimate expression of a newly synthesized transcript in mammalian cells is dependent on a series of reactions which alter the molecular form of that primary transcript to a functionally competent form (1,16,20). Although the processing reactions for a particular type of RNA may vary (i.e., preribosomal RNA versus premessenger RNA), each pathway has in common the feature that a significant proportion of the precursor RNA molecule is degraded during the maturation process (1,16,20). Such findings have then made it reasonable to propose that enzymes (ribonucleases) which degrade RNA must play an important role in the regulation of nuclear RNA metabolism (1,8,9). For this reason, characterization of ribonucleases and the mechanisms which control their action are needed in order to gain further understanding of RNA metabolism and of the mechanism of gene expression in mammalian cells.

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To establish the role that ribonucleases play in nuclear RNA metabolism, the complexity of the nuclear transcriptional system must be reduced. The localization of ribosomal gene expression and the enzymes responsible for ribosomal RNA synthesis and processing in nucleoli (2,7,11,15,18) offers such a system for study, and provides a relatively pure source of ribosomal RNA precursors and processed products, as well as starting material for identifying enzymatic components (2,7,12,15).

In this report we present data to show that purified human placental ribonuclease inhibitor can be used to inhibit endonucleolytic activity associated with Ehrlich cell nucleoli. Our findings also suggest that the inhibitor may provide a useful probe to facilitate the separation and identification of specific ribonuclease activities associated with purified nucleoli.

MATERIALS AND METHODS

Materials

Ultrapure sucrose was purchased from Schwarz/Mann. Unlabeled ribonucleoside triphosphates and pancreatic RNase were obtained from Sigma Chemical Co. [^{3}H]UTP (40 Ci/mmol) was obtained from ICN Pharmaceuticals and [α^{32}P]UTP (660 Ci/mmol) and Omnifluor from New England Nuclear. Ehrlich nucleolar endoribonuclease (Fraction IV) was purified as described by Eichler and Tatar (10). Bacteriophage f_2[^{32}P]RNA (1 to 6 x 10^4 cpm/nmol of nucleotide) was prepared according to the procedure of Glitz et al. (14) for MS2 RNA. [^{3}H]18S and 28S ribosomal RNA markers were prepared from Ehrlich cells according to the procedure of Winicov and Perry (22). Purified human placental ribonuclease inhibitor was kindly provided by Dr. Peter Blackburn (Rockefeller University) or prepared from human placenta by the method of Blackburn (4).

RNase assay

The assay measures conversion of f_2[^{32}P]RNA to acid-soluble nucleotides as described previously (10). One unit of ribonuclease activity is that amount which produces 1 nmol of acid-soluble nucleotide in 30 min.

Purification of nucleoli

Nucleoli were prepared from Ehrlich ascites tumor cells essentially according to the method of Muramatsu et al. (19). The nucleolar pellets were taken up in 25% glycerol, 50 mM Tris-HCl, pH 8.0, 5 mM $Mg(Ac)_2$, 5 mM dithiothreitol, 0.1 mM EDTA. This material was immediately frozen and stored in 100 µl aliquots in liquid nitrogen.

Transcriptional assay

The reaction mixture (0.1 ml) contained 10 mM Tris-HCl, pH 8.0, 7.5% glycerol, 5 mM $MgCl_2$, 2.5 mM dithiothreitol, 100 mM KCl, 0.05 mM EDTA, 0.3 mM GTP, 0.3 mM CTP, 1 mM ATP, 0.05 mM either [α^{32}P]UTP (100 to 500 cpm/pmol) or [^{3}H]UTP (350 cpm/pmol). After incubation for 15 min at 30°, 0.4 ml of a

solution consisting of 0.01 M $Na_4P_2O_7$, 1 mg/ml transfer RNA, and 0.010 M EDTA were added, followed by 1 ml of chilled 15% trichloroacetic acid. The precipitated sample was collected on glass fiber filters (Whatman GF/C) and washed with 25 ml of 5% trichloroacetic acid. The filter was dried and radioactivity determined using a toluene-based scintillation fluid in a Beckman LS-7000 counter.

Sucrose gradient sedimentation

RNA samples (0.2 ml) in 70% formamide, 3 mM Tris-HCl, pH 7.5, 3 mM EDTA, 0.5% sarcosyl were incubated 15 min. at 37° before being layered onto a 40% sucrose gradient (3.8 ml) made up in the same 70% formamide buffer. After centrifugation at 35,000 rpm for 16 h in an SW60 rotor at 25°, fractions (0.13 ml) were collected from the bottom of the tube. Fractions were precipitated by the addition of 0.4 ml 1 mg/ml transfer RNA, 0.01 M $Na_4P_2O_7$, 0.010 M EDTA followed by 1 ml of 15% trichloroacetic acid. After standing 10 min at 0°, fractions were collected on Whatman GF/C filters and processed for counting as previously described in this paper.

Other Methods

Protein was determined by the method of Lowry *et al.* (17). DNA was determined by the method of Schneider (21).

RESULTS AND DISCUSSION

It is well known that isolated nucleoli are able to incorporate labeled nucleotides into acid-precipitable material (2,11,15,18). However, the RNA synthesized by isolated nucleoli typically has a lower sedimentation coefficient than that of nucleolar RNA produced *in vivo* (18). In order to define the types of activities responsible for this *in vitro* effect, nucleoli were incubated initially in the presence of an exogenous labeled RNA. Ribonuclease activities associated with intact nucleoli capable of degrading this RNA to acid-soluble products were distinguished by their sensitivity to the chelating agent EDTA, the sulfhydryl reagent N-ethylmaleimide, and human placental RNase inhibitor. As shown in Table I, the bulk of the ribonuclease activity associated with intact nucleoli was not sensitive to N-ethylmaleimide or EDTA. However, a significant proportion of the ribonuclease activity was sensitive to human placental RNase inhibitor.

To more specifically address the nucleolar involvement of the ribonuclease activity which was sensitive to the inhibitor, the effect of these compounds on *in vitro* transcription was tested. The *in vitro* synthesis of RNA by isolated nucleoli was shown to be dependent on the amount of nucleoli

Table I. Properties of Ribonuclease Activity of Purified Nucleoli

Addition	Relative Activity
None	1.00
10 mM N-ethylmaleimide	0.88
10 mM EDTA	0.73
67 µg/ml human placental ribonuclease inhibitor	0.27
133 µg/ml human placental ribonuclease inhibitor	0.03
0.5% sodium dodecyl sulfate	0.00

The reaction mixture (0.15 ml) contained 50 mM Tris-HCl, pH 7.5, 75 mM KCl, 0.1 mg per ml bovine serum albumin, 4.5 nmol of $f_2[^{32}P]$RNA (sp. act. 18.4 cpm/pmol) and 6.4 µg nucleoli (based on DNA content). After 30 min. at 30°, the reaction mixture was stopped and processed as described in METHODS.

added, and as can be seen in Figure 1, the rate and extent of synthesis was not affected by α-amanitin. These results are consistent with the interpretation that the RNA synthesized *in vitro* is catalyzed by RNA polymerase I (2,6,11,15,18,19) and that the observed transcription may be restricted to the elongation of nascent RNA chains (15). Although the extent of RNA synthesis was not affected by the inclusion of the placental RNase inhibitor in the reaction mixture, the size of the *in vitro* transcriptional product was significantly affected (Figure 2). The RNA product synthesized in the presence of RNase inhibitor included material in the 45S size range consistent with the reported size of preribosomal RNA synthesized *in vivo* (1,20). When transcription was terminated by the addition of EDTA, the protection of the transcriptional products by the placental RNase inhibitor could be reversed by inactivating the inhibitor with N-ethylmaleimide (Figure 3). These results suggest that the placental RNase inhibitor protects the larger transcriptional products from a limited endonucleolytic

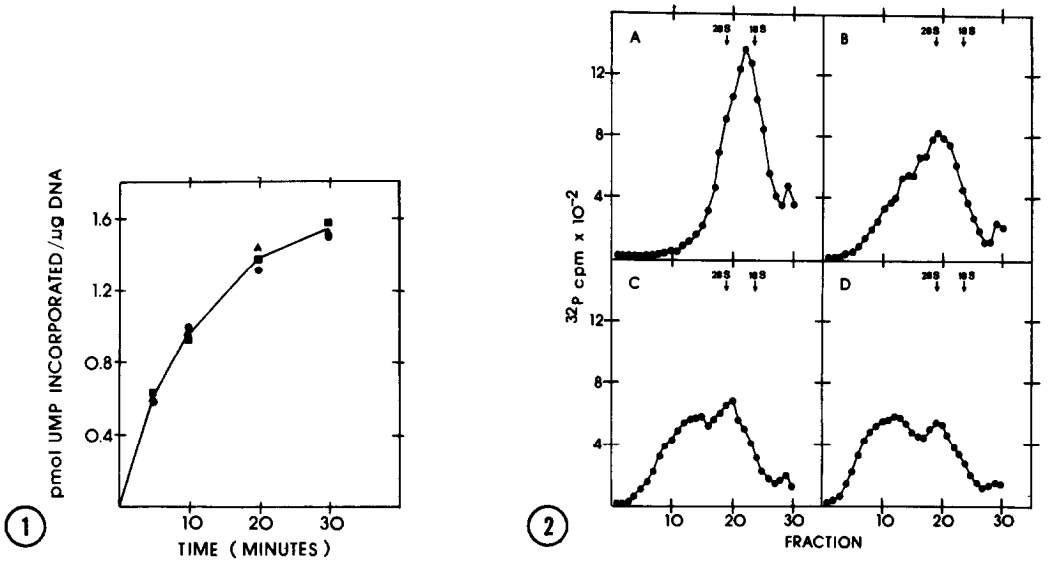

Figure 1. Time course of transcription by isolated nucleoli. Reaction mixtures (0.6 ml) contained 0.01 M Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 0.1 M KCl, 7.5% glycerol, 5 mM dithiothreitol, 0.05 mM EDTA, 0.3 mM GTP, 0.3 mM CTP, 1 mM ATP, 0.05 mM [^{3}H] UTP (350 cpm/pmol) and 115 µg nucleolar DNA. Reactions were run at 30° and started by the addition of substrate. Aliquots (0.1 ml) were taken at the indicated times into 0.4 ml 1 mg/ml tRNA, 0.01 M $Na_4P_2O_7$, 0.01 M EDTA at 0° and precipitated by the addition of 1 ml 15% trichloroacetic acid. After 10 min at 0°, samples were collected on glass fiber filters and counted. (▲—▲) control; (●—●) 0.5 µg/ml α-amanitin; (■—■) 66.7 µg/ml human placental RNase inhibitor.

Figure 2. Effect of human placental ribonuclease inhibitor on the size of RNA synthesized by isolated nucleoli. Reaction mixtures (0.6 ml) contained 0.01 M Tris-HCl, pH 8.0, 5 mM $MgCl_2$, 0.1 M KCl, 7.5% glycerol, 5 mM dithiothreitol, 0.05 mM EDTA, 0.3 mM GTP, 0.3 mM CTP, 1 mM ATP, 0.05 mM [α^{32}P]UTP (164 cpm/pmol) and 57.6 µg nucleolar DNA. Reactions were started by the addition of substrate. The reaction was stopped after 15 min. at 30° by adjusting the reaction mixture to 0.5% sodium dodecyl sulfate, 10 mM EDTA. The reaction mixture was extracted with phenol and the ethanol-precipitated nucleic acid from the aqueous phase was run on a formamide-sucrose gradient as described in METHODS. (A) control (no inhibitor); (B), (C), and (D) 1, 2, and 4 µg of human placental RNase inhibitor added respectively.

attack of an RNase (or RNases) which is insensitive to 10 mM EDTA and 10 mM N-ethylmaleimide.

Previously, we described the characterization of a highly purified nucleolar endoribonuclease (10). Since this nucleolar RNase represents the major nuclease activity associated with nucleoli which was not sensitive to EDTA or N-ethylmaleimide inhibition (10), it was of interest to determine whether the enzyme would then be sensitive to the RNase inhibitor purified from human placental tissue (4). When using native f_2RNA as a substrate, the

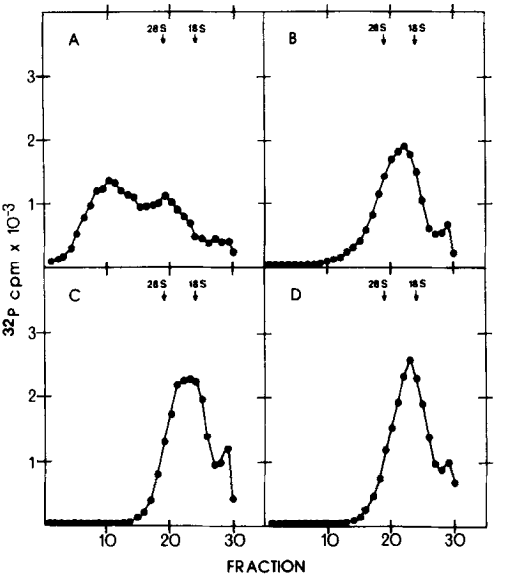

Figure 3. Effect of N-ethylmaleimide on the size of RNA synthesized in the presence of human placental RNase inhibitor. Reaction mixtures (0.6 ml) contained 0.01 M Tris HCl, pH 8.0, 5 mM $MgCl_2$, 0.1 M KCl, 7.5% glycerol, 5 mM dithiothreitol, 0.05 mM EDTA, 0.3 mM GTP, 0.3 mM CTP, 1 mM ATP, 0.05 mM [α^{32}P] UTP (500 cpm/pmol) and 57.6 µg nucleolar DNA. Reactions were started by the addition of substrate. After 15 min. at 30° the reaction mixtures were brought to 10 mM in EDTA and the indicated concentration of N-ethylmaleimide. The reactions were allowed to continue for 15 min. at 30° and were then stopped by adjusting the reaction mixture to 0.5% sodium dodecyl sulfate. The RNA from the reaction mixtures was isolated and run on formamide-sucrose gradients as described in METHODS. (A) contained 20 µg human placental RNase inhibitor during transcription; (B) same as A except that the reaction was adjusted to 10 mM N-ethylmaleimide after 15 min. of transcription; (C) control (no additions during transcription); (D) same as C except that 10 mM N-ethylmaleimide was added after 15 min. of transcription.

Ehrlich nucleolar RNase was found to be inhibited by the placental RNase inhibitor (Figure 4), although to a lesser extent than that observed with a comparable level of pancreatic RNase activity. Nevertheless, the inhibition of the Ehrlich nucleolar enzyme by the placental RNase inhibitor could be reversed by the addition of N-ethylmaleimide in a similar fashion as that described by Blackburn *et al.* (5) for the pancreatic RNase-inhibitor complex.

Since the properties of the purified nucleolar endoribonuclease (10) are consistent with those affected by the placental RNase inhibitor in the nucleolar transcriptional system, this suggests that this enzyme may, in part, be responsible for the smaller size distribution of *in vitro* tran-

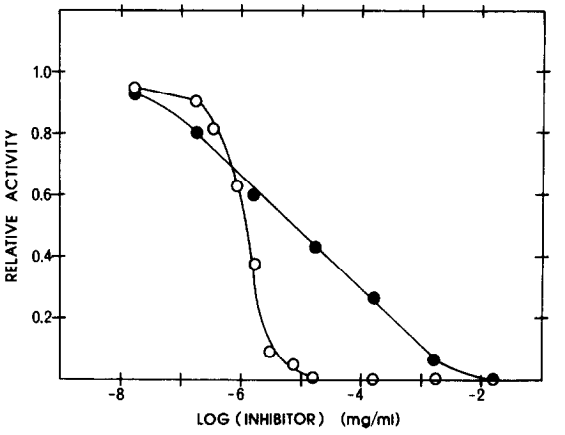

Figure 4. Effect of human placental RNase inhibitor on the degradation of RNA by pancreatic RNase and Ehrlich nucleolar RNase. The reaction mixtures (0.15 ml) contained 0.05 M Tris-HCl, pH 7.5, 0.075 M NaCl, 0.1 mg/ml bovine serum albumin, 5 mM EDTA and either 3 units of Ehrlich nucleolar RNase (●—●) or 13 picograms pancreatic RNase (○—○). The amount of inhibitor was varied as indicated. Reaction mixtures were preincubated 5 min. at 37° and the reaction was started by the addition of 4.5 nmoles $f_2[^{32}p]$ RNA (sp. act. 56 cpm/pmol). Reaction mixtures were stopped after 30 min. and processed as described in METHODS.

scriptional products. These data may also have important implications in permiting further differentiation of nucleolar ribonuclease activities in order to facilitate the determination of their involvement in ribosomal RNA metabolism.

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