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Effects of α-Amanitin, Cycloheximide, and Thioacetamide on Low Molecular Weight Nuclear RNA†

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ABSTRACT: Studies were made on the effects of α-amanitin, cycloheximide, and thioacetamide on synthesis and content of low molecular weight nuclear RNA. Cycloheximide, an inhibitor of protein synthesis and the synthesis of 45S pre-rRNA and 5S RNA, also inhibited synthesis of nuclear U1 and U3 RNAs. α-Amanitin, an inhibitor of DNA-dependent RNA polymerase II, inhibited the synthesis of U1 and U2 low molecular weight nuclear RNA. Thioacetamide, which induces nucleolar hypertrophy and increased nucleolar RNA polymerase activity, markedly increased synthesis of 5.8S RNA and U3 RNA. These results show that syntheses of individual low molecular weight nuclear (LMWN) RNAs are controlled by different regulatory mechanisms. In particular, there appears to be a specific relationship between U3 RNA and functional states of the nucleolus.

Of the low molecular weight nuclear RNAs with sedimentation values of 4 to 8 S, the complete nucleotide sequences of 4.5S RNA_I, U1 RNA, and U2 RNA have been defined (Ro-Choi et al., 1972; Reddy et al., 1974; Shibata et al., 1974, 1975). U1 and U2 RNA have the common feature of a highly methylated 5′ terminus which contains the unusual nucleotide $pm_3^{2,2,7}G$ in a pyrophosphate linkage to the 5′ portion of the molecule (Ro-Choi et al., 1974, 1975) and they exist in the nuclei as ribonucleoprotein complexes (Raj et al., 1975). The 4.5S, U1, and U2 RNA are extranucleolar in location; U3 RNA is nucleolus specific (Busch et al., 1971; Ro-Choi and Busch, 1974). These RNA species have been found in tissues of several vertebrate species (Moriyama et al., 1969; Hodnett and Busch, 1968; Weinberg and Penman, 1968; Dingman and Peacock, 1968; Rein and Penman, 1969; Zapisek et al., 1969). U1, U2, and U3 RNA are metabolically very stable and do not appear to be precursors to cytoplasmic products.

The use of inhibitors which have differential effects on different RNA polymerases or interfere with nucleic acid processing should aid in elucidating the functions of these LMWN RNAs. α-Amanitin is a specific inhibitor of nucleoplasmic RNA polymerase II in vitro (Stirpe and Fiume, 1967) but when administered to rats, α-amanitin inhibits both nucleoplasmic and nucleolar RNA synthesis (Jacob et al., 1970;

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Niessing et al., 1970). It has been suggested that the synthesis and maturation of the precursor of rRNA are under the control of nucleoplasmic gene products (Tata et al., 1972; Hadjiolov et al., 1974). Cycloheximide and thioacetamide affect the activity of RNA polymerase I. Following in vivo administration of cycloheximide, RNA polymerase I activity was decreased (Muramatsu et al., 1970); similarly, increased activity of RNA polymerase I was found following thioacetamide treatment (Villalobos et al., 1964).

The present report describes the effect of α-amanitin, cycloheximide, and thioacetamide on ^{32}P-labeled orthophosphate incorporation into LMWN RNA species and their effects on nuclear concentrations of these molecules.

Materials and Methods

Animals. Male Holtzman rats weighing 210 ± 20 g were used. The control and experimental groups each consisted of 10 rats. The α-amanitin, cycloheximide, and thioacetamide were each dissolved in isotonic saline. For maximal effects α-amanitin, 0.25 mg/kg and 0.5 mg/kg (Jacob et al., 1970), or cycloheximide, 20 mg/kg or 40 mg/kg (Muramatsu et al., 1970), were administered intravenously 30 min prior to the administration of $^{32}P_i$.[1] For treatment with thioacetamide, two doses were administered 12 and 24 h prior to injection of $^{32}P_i$. For the labeling of RNA, 10–15 mCi of $^{32}P_i$ in isotonic saline was injected intraperitoneally into each rat 150–180 min prior to sacrifice.

In Vitro Incubations. The Novikoff hepatoma ascites cells were maintained in male Holtzman rats for 6 days at which time the rats were sacrificed and the abdominal fluid was drained into containers. The cells were harvested and washed twice with NKM (0.13 M NaCl, 0.005 M KCl and 0.008 M $MgCl_2$, pH 7.6). Two grams of cells was incubated in 100 ml of medium with carrier-free $^{32}P_i$ (50 mCi) as described by Mauritzen et al. (1971). Cycloheximide was added at a concentration of 40 μg/ml; incubation was carried out for 6 h.

Preparation of Nuclei. Purified liver nuclei were obtained by the citric acid procedure. The minced livers were homogenized in 10 volumes of 1.5 or 5% citric acid and filtered through cheesecloth. Crude nuclei were sedimented at 2500*g* for 15 min and suspended in 0.25 M sucrose containing 0.5% citric acid. This suspension was layered over 2 volumes of 0.88 M sucrose containing 0.5% citric acid and spun at 2500*g* for 20 min.

Nuclei from Novikoff hepatoma cells were prepared by suspending the cells in 10 volumes of 0.01 M Tris-HCl buffer containing 0.01 M NaCl, 1.5 mM $MgCl_2$, and 0.5% Nonidet P-40, pH 7.6, and homogenizing at low speed (600 rpm) with a Teflon glass homogenizer. The suspension was centrifuged at 3000*g* for 15 min and the nuclear pellet was immediately processed.

Isolation and Fractionation of Nuclear RNA. The RNA was extracted by the sodium dodecyl sulfate–hot phenol procedure (Higashi et al., 1966; Hodnett and Busch, 1968; Ro-Choi et al., 1970). The RNA was precipitated with 2.5 volumes of ethanol containing 2% potassium acetate and fractionated by sucrose density gradient centrifugation. The 5–40% linear sucrose gradient was buffered with 0.01 M sodium acetate, pH 5.1, containing 0.14 M sodium chloride and 1 mM EDTA. The centrifugation was done in an SW 27 rotor at 24 000 rpm for 16 h at 4 °C. The gradients were fractionated by an ISCO Model D fractionator and 1-ml fractions were collected and isotope content was determined with a Packard liquid scintillation counter (Clausen, 1968). The low molecular weight RNA fractions were pooled and precipitated with 2.5 volumes of ethanol containing 2% potassium acetate. Gel electrophoresis was carried out as described earlier (Ro-Choi et al., 1973).

[1] Abbreviations used: Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid; P_i, inorganic phosphate; NKM, 0.13 M NaCl, 0.005 M KCl, and 0.008 M $MgCl_2$, pH 7.6; TDC, toluene (400 ml), dioxane (1200 ml), Cellosolve (ethylene glycol monoethyl ether) (1200 ml), naphthalene (224 g), 2,5-diphenyloxazole (30 g), 1,4-bis[2-(5-phenyloxazolyl)]benzene (1.52 g); NHC, Novikoff hepatoma cell; Bis, *N,N'*-methylenebisacrylamide; TA, thioacetamide.

TABLE I: Effects of Various Agents on Nuclear RNA Synthesis in Vivo and in Vitro (Percent Control Specific Activity, cpm/A_{260}).[a]

	Total Nuclear RNA	LMWN RNA	HMWN RNA
Control	100.0	100.0	100.0
α-Amanitin			
0.25 mg/kg	55.0	68.8	44.7
0.50 mg/kg	30.8	45.0	29.5
Cycloheximide			
20 mg/kg	77.3	81.8	
40 mg/kg	56.7	54.5	
40 μg/ml (NHC)	41.4	52.6	
Thioacetamide			
50 mg/kg (1 day)	247.5	222.0	274.0
50 mg/kg (9 days)	130.0	116.0	126.0

[a] Individual values are averages of 2–3 experiments. Abbreviations used are: LMWN RNA, low molecular weight nuclear RNA; HMWN RNA, high molecular weight nuclear RNA (>18S RNA reprecipitated from sucrose density gradient separation); NHC, Novikoff hepatoma cells (for the better labeling of LMWN RNA, Novikoff hepatoma cells were incubated in the flask with $^{32}P_i$).

Results

Effect of Cycloheximide on the Nuclear RNA Synthesis. For the in vitro experiments on Novikoff hepatoma cells, cycloheximide was added at concentrations of 40 μg/ml along of ^{32}P-labeled orthophosphate into nuclear RNA by 59% and that of low molecular weight nuclear RNA by 47% (Table I). The low molecular weight RNA from the control and 40 μg/ml cycloheximide treated sample was analyzed by preparative and slab gel electrophoresis (Figures 1 and 2). Table II shows that labeling of U3 and U1 RNA was most inhibited by cycloheximide treatment (Frederiksen and Hellung-Larsen, 1974). The labeling of 4.5S RNA was least affected by cycloheximide.

Cycloheximide treatment in vivo inhibited incorporation of ^{32}P-labeled orthophosphate into nuclear high molecular weight RNA (Figure 3) in agreement with earlier findings of Muramatsu et al. (1970). At doses of 20 and 40 mg of cycloheximide per kg of body weight, respectively (Table I), the percent inhibitions of [^{32}P]orthophosphate incorporation into total nuclear RNA were 23 and 43% and into low molecular weight RNA were 18 and 45%. However, the relative radioactivity in 28S and 45S RNA was unchanged, indicating processing of 45S RNA was parallel to its synthesis.

Preparative gel electrophoresis of LMWN RNA showed the labeling of 5S RNA was most inhibited, i.e., 55%, relative to other RNA components at both the concentrations of cycloheximide used (Table II). As noted with α-amanitin, labeling of 4.5S RNA was relatively unaffected.

Effect of α-Amanitin on Nuclear RNA Synthesis. The effects of α-amanitin (0.25 mg/kg) on the synthesis of nuclear RNA were analyzed by sucrose density gradient centrifugation

TABLE II: Effects of Various Agents on LMWN RNA (Percent Control Specific Activity).[a]

	4S	4.5S	5S	U1	U2	U3
Control	100.0	100.0	100.0	100.0	100.0	100.0
α-Amanitin						
0.25 mg/kg	42.4	95.3	43.5	26.3	44.2	
0.50 mg/kg	47.6	87.3	46.1	27.4	39.5	
Cycloheximide						
20 mg/kg	78.7	107.0	49.2	77.7		
40 mg/kg	67.0	118.4	45.1	67.9		
40 μg/ml (NHC)	51.9	72.6	38.8	28.0	45.7	27.3
Thioacetamide[b]						
50 mg/kg (1 day)	193	198	229	426	354	678
50 mg/kg (9 days)	131	73	80	230	158	128

[a] Specific activities (counts per A_{260} unit) were determined after separation of LMWN RNA by preparative gel electrophoresis (Canalco PD 320) and individual values are averages of 2–3 experiments. [b] Specific activities were determined indirectly from distribution of LMWN RNA by amount from stained gel and distribution of radioactivity in individual species of LMWN RNA. After 9 days of thioacetamide treatment, accumulating 4.5S, 5S, and U3 RNA occurs while content of U1 and U2 RNA remains constant. A slight decrease in the content of 4S RNA was observed.[2]

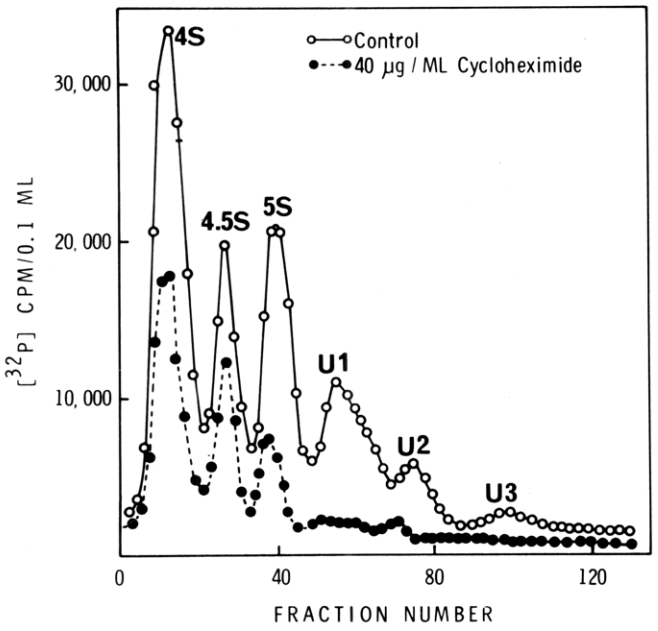

FIGURE 1: Elution profiles of LMWN RNA of Novikoff hepatoma cells from preparative polyacrylamide gel electrophoresis. Two grams of Novikoff hepatoma cells was incubated in 100 ml of minimum Eagle's medium with 50 mCi of $^{32}P_i$ for 6 h in the presence and absence of cycloheximide. (O–O) Control; (●–●) 40 μg/ml cycloheximide treated.

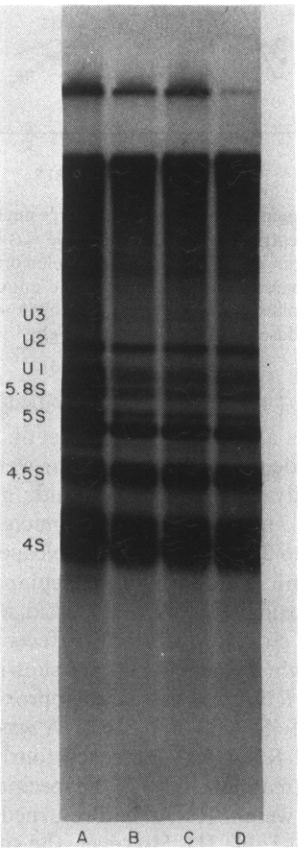

FIGURE 2: Autoradiogram of a slab gel electrophoresis separation of low molecular weight RNA components from Novikoff hepatoma nuclear RNA. Marked inhibition of U1 and U3 RNA labeling was observed. (A) Control; (B) cycloheximide at a concentration of 10 μg/ml; (C) cycloheximide at a concentration of 20 μg/ml; (D) cycloheximide at a concentration of 40 μg/ml.

(Figure 4). α-Amanitin inhibited the incorporation of [^{32}P]-orthophosphate into RNA species sedimenting at 18S, 28S, and 45S RNA (HMWN RNA) by approximately 55 and 70% at doses of 0.25 and 0.50 mg/kg of body weight, respectively (Table I). The labeling of nuclear 4-8S RNA was decreased by 31 and 55% at 0.25 and 0.50 mg of α-amanitin/kg of body weight (Table I). The radioactivity in 45S RNA was higher relative to 18S and 28S RNA in α-amanitin-treated samples than in the controls. This result suggests α-amanitin interferes with both the processing and synthesis of 45S RNA (Figure 4).

The effect of α-amanitin on labeling of 4-8S RNA species was analyzed in more detail by polyacrylamide gel electrophoresis. Autoradiography of the LMWN RNA (Figure 5) shows that labeling of U1 RNA was inhibited at both the concentrations of α-amanitin employed. α-Amanitin (0.5 mg/kg of body weight) inhibited labeling of 4S and 5S RNA by 52 and 54%, respectively, while that of 4.5S RNA was not significantly decreased. The labeling of U1 and U2 RNAs was most inhibited, i.e., 73 and 60%, respectively, as shown in Table II.

Effects of Thioacetamide on the Nuclear RNA Synthesis. In vivo, thioacetamide produces a remarkable nucleolar hy-

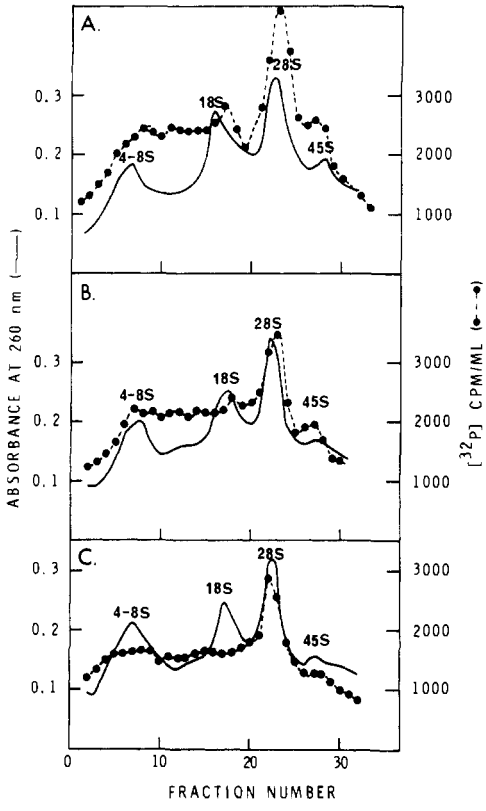

FIGURE 3: Sedimentation profiles of rat liver nuclear RNA on sucrose density gradient centrifugation. Cycloheximide was injected intravenously into rats and 30 min later 15 mCi of ^{32}P-labeled orthophosphate was injected intraperitoneally. After 150 min, the animals were killed. (A) Control; (B) cycloheximide at a dose of 20 mg/kg body weight; (C) cycloheximide at a dose of 40 mg/kg body weight.

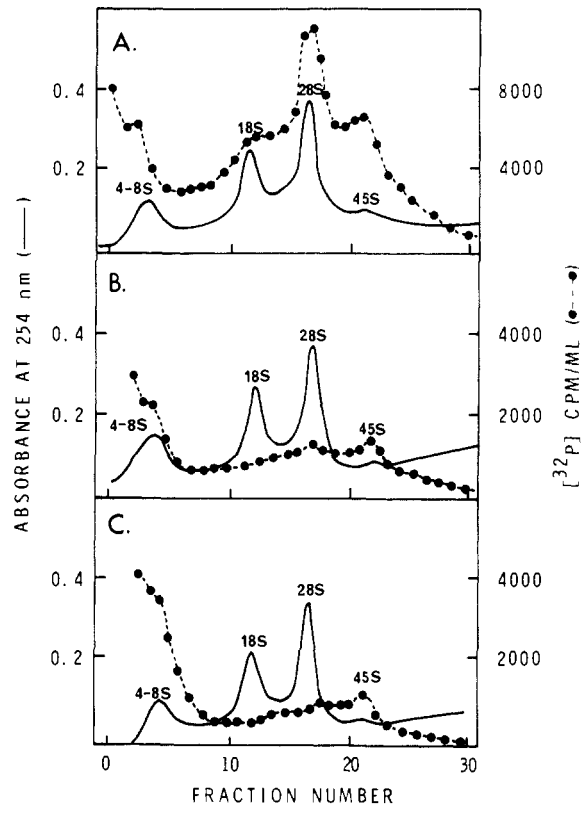

FIGURE 4: Action of α-amanitin on the sedimentation profiles of rat liver nuclear RNA. α-Amanitin was injected intravenously into rats and 30 min later 15 mCi of ^{32}P-labeled orthophosphate was injected intraperitoneally. After 150 min, the animals were killed. (A) Control; (B) α-amanitin at a dose of 25 μg/100 g body weight; (C) α-amanitin at a dose of 50 μg/100 g body weight.

pertrophy and a large increase in nucleolar pre-rRNA synthesis (Steele et al., 1965; Villalobos et al., 1964). The effect of thioacetamide on the low molecular weight RNA components was analyzed by polyacrylamide gel electrophoresis (Figures 6 and 7). Labeling of whole nuclear RNA (Table I) was increased to 247% after 24 h of thioacetamide treatment. Labeling of both low and high molecular weight RNA was increased approximately two- to threefold, indicating that both fractions were similarly effected. Effects on individual low molecular weight nuclear RNA are shown in Table II. The labeling of U3 RNA was increased approximately sevenfold. The labeling of 4S, 4.5S, and 5S RNA was doubled and that of U1 and U2 RNA was increased fourfold. At 9 days of thioacetamide treatment, most of the specific activity of various low molecular weight RNAs had returned to approximately control values (Table II). However, the specific activities of U1 and U2 RNA were still elevated over the control values. Although some changes occurred in the relative amounts of these RNA species per gram of tissue, the most marked change was in the U3 RNA which increased 3.5 times over the control amount.[2]

Discussion

Most of the available clues as to the function of low molecular weight RNAs are indirect. Some drugs which induce changes in nuclear structure and function produce metabolic variations in nuclear low molecular weight RNAs (Weinberg and Penman, 1969; Howard and Stubblefield, 1972; Frederiksen and Hellung-Larsen, 1974; Simard et al., 1974).

These data provide a good correlation between synthesis and content of U3 RNA and nucleolar biosynthetic activity. The inhibition of protein synthesis by cycloheximide rapidly suppresses nucleolar synthesis of 45S pre-rRNA (Willems et al., 1969; Warner et al., 1966). In vitro studies revealed the inhibition of RNA polymerase I activity in cycloheximide-treated rats (Muramatsu et al., 1970). On the other hand, treatment of rats with thioacetamide enhances the RNA polymerase I activity (Villalobos et al., 1964) and also the synthesis of 45S pre-rRNA (Steele et al., 1965). The observations that U3 RNA synthesis was markedly reduced by cycloheximide at 3 h and markedly enhanced by thioacetamide at 27 h after administration correlate with the changes in RNA polymerase I activity and the specific localization of U3 RNA in the nucleolus.[3] These results suggest that U3 RNA may have a role in the production of preribosomal RNA. Interestingly, Goldstein and Ko (1974) reported that this is a "shuttling" RNA.

Kanehisa et al. (1972) have reported that some low molecular weight RNA stimulated the transcription in isolated chick chromatin and *E. coli* RNA polymerase systems. α-Amanitin acts as a highly selective inhibitor of RNA polymerase II both in vivo and in vitro (Tata et al., 1972; Smuckler and Hadjiolov,

[2] At 9 days the relative amounts of 4S, 4.5S, 5S, U1, U2, and U3 RNA were 0.83, 1.45, 1.61, 1.27, 1.13, and 3.57, respectively, by comparison with control values.

[3] These experiments were carried out at 27 h, a time when rRNA synthesis is rapidly increasing (Steele et al., 1965). At earlier times, there is an inhibition of RNA synthesis (Steele et al., 1965).

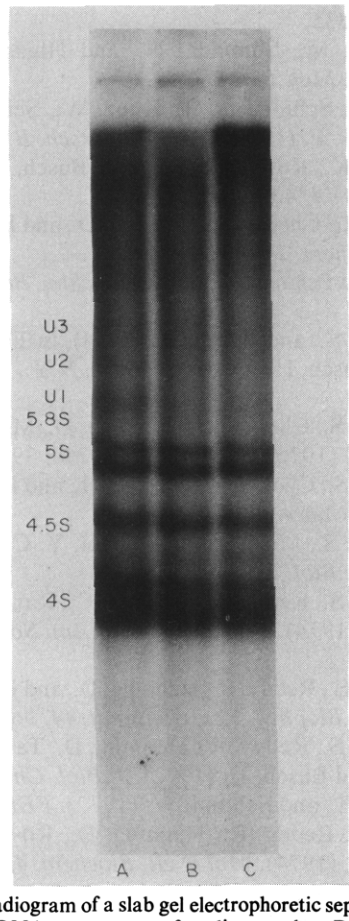

FIGURE 5: Autoradiogram of a slab gel electrophoretic separation of low molecular weight RNA components of rat liver nuclear RNA. Low molecular weight RNA fraction from Figure 4 was pooled and precipitated with ethanol. RNA was separated by 10% polyacrylamide gel electrophoresis. (A) Control; (B) α-amanitin at a dose of 25 μg/100 g body weight; (C) α-amanitin at a dose of 50 μg/100 g body weight.

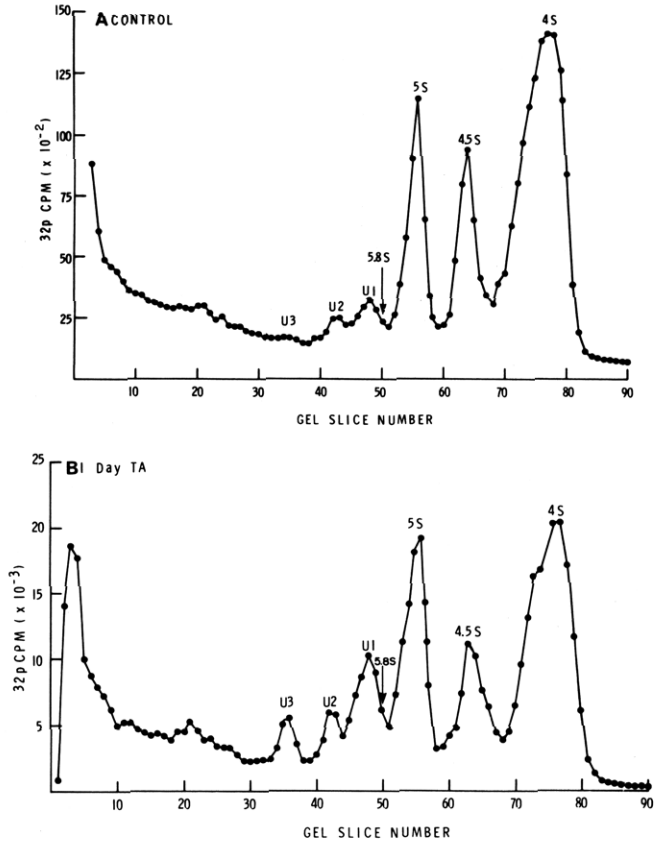

FIGURE 6: Radioactivity profile of LMWN RNA after slab gel electrophoresis. The gel consisted of 10% polyacrylamide with an acrylamide–Bis ratio of 39:1. Electrophoresis was performed in 0.04 M Tris acetate, 0.02 M sodium acetate, 1 mM EDTA (pH 7.2) and was continued until brom phenol blue dye had migrated 30 cm. Each gel track (2 cm) was sliced into 3-mm slices; slices were dissolved in 1 ml of 30% H_2O_2 per slice by heating at 60 °C for 16 h, neutralized with 0.1 ml 1 M Tris-Cl, pH 8, and counted in 10 ml of TDC. (A) LMWN RNA from control animals; (B) LMWN RNA from animals treated with 50 mg/kg thioacetamide for 1 day.

1972; Sekeris and Schmid, 1972; Hadjiolov et al., 1974; Jacob et al., 1970). However, α-amanitin in vivo inhibits the labeling of rRNA (Jacob et al., 1970; Niessing et al., 1970; Tata et al., 1972) presumably by inhibiting the maturation and the synthesis of 45S pre-rRNA (Hadjiolov et al., 1974).

The present results reveal U1 RNA labeling is inhibited markedly by α-amanitin under the conditions when HnRNA synthesis is blocked and the activity of RNA polymerase II is impaired (Zylber and Penman, 1971). The inhibition of synthesis of U1 RNA by α-amanitin may be due to the inhibition of RNA polymerase II activity.[4]

Cycloheximide in vitro also inhibits labeling of U1 RNA appreciably. This effect could be due to the suppression of extranucleolar RNA polymerase II activity since this drug also inhibits the extranucleolar RNA synthesis during the late stages of the drug treatment (Muramatsu et al., 1970).

The 5S RNA is transcribed by an extranucleolar RNA polymerase III which is sensitive to α-amanitin only at higher

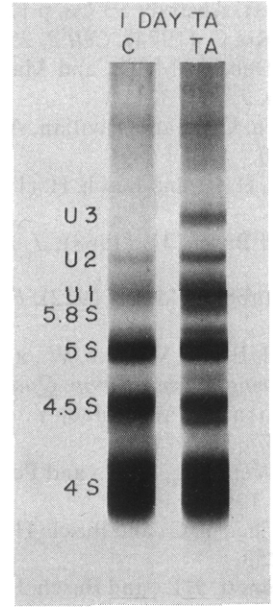

FIGURE 7: Autoradiogram of rat liver LMWN RNA after slab gel electrophoresis. Electrophoresis was performed as described in Figure 6. The gel was wrapped in Saran Wrap and exposed to x-ray film for several hours. (C) LMWN RNA from control animals; (TA) LMWN RNA from animals treated with 50 mg/kg thioacetamide for 1 day.

[4] U1 RNA is largely localized to the chromatin (Ro-Choi and Busch, 1974). Approximately 2000–3000 sequences in DNA code for U1 RNA (Engberg et al., 1973). If these sequences correspond to the regulatory regions in the genome, it is possible that U1 RNA serves in gene control. In addition, a "shuttling" behavior of U1 RNA between the cytoplasmic and nucleus was reported by Goldstein and Ko (1974). On the other hand, the particulate nature of the U1 RNA–protein complex in the nucleus and its low turnover rate indicate that U1 RNA may have structural functions.

concentrations (Weinman and Roeder, 1974; Aloni et al., 1971). In addition to extranucleolar nuclear 5S RNA_{III}, there are two types of ribosomal 5S RNA and a class of 5S RNA that shuttles between cytoplasm and nucleus (Leibowitz et al., 1973; Zehavi-Willner and Danon, 1972; Goldstein and Ko, 1974).

Interestingly, the labeling of 4.5S RNA was least affected by any of the agents employed. The role of these unique RNA species (Ro-Choi et al., 1972) is not yet defined. However, it is interesting that labeling of 4.5S RNA was stimulated at 24 h of thioacetamide treatment while at 9 days of thioacetamide treatment the rate of labeling of 4.5S RNA returned to control values. Thioacetamide also produces transient stimulation of DNA synthesis at an early period of treatment (Steele et al., 1965).

Synthesis of 4S RNA was inhibited by α-amanitin to the same extent as 5S RNA synthesis although less by cycloheximide. Stimulation of 5S RNA labeling by thioacetamide was also different from that of 4S RNA labeling at 24 h and 9 days of thioacetamide treatment.

The different results for stimulation and inhibition of synthesis of the various low molecular weight nuclear RNA species indicate complex regulatory mechanisms exist for the synthesis of these different types of RNAs.

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