EVIDENCE FOR EXTRANUCLEOLAR CONTROL OF RNA SYNTHESIS IN THE NUCLEOLUS

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SUMMARY

Previous studies demonstrate that RNA synthesis by nucleoli isolated from rat liver cells or by solubilized nucleolar polymerase is not inhibited by α-amanitin, whereas this toxin is an effective inhibitor of the extranucleolar polymerase. In contrast, administration of amanitin to intact rats severely reduces synthesis of RNA by liver nucleoli, as shown by the following results obtained 1 hr after injection of the toxin: (a) all species of RNA, including nucleolar RNA, show reduced isotope uptake from an *in vivo* pulse dose of orotic acid; (b) *in vivo* methylation of ribosomal RNA is inhibited; (c) the 45S peak of ribosomal RNA precursor disappears from gradients of nuclear RNA; and (d) RNA synthesis *in vitro* by nucleoli isolated from amanitin-treated rats is inhibited. The contrast between the lack of action of amanitin on RNA synthesis by isolated nucleoli and the effectiveness of the toxin *in vivo* suggests that ribosomal RNA formation in the intact cell is under an extranucleolar control mechanism sensitive to amanitin.

INTRODUCTION

Soluble RNA polymerase extracted from rat liver nuclei has exhibited two separate activities, one being associated with the nucleolus, the other extranucleolar (1-3). When ribonucleoside triphosphates are incubated *in vitro*, polymerization by the extranucleolar enzyme is inhibited by α-amanitin, a toxin extracted from *Amanita phalloides*, whereas this antibiotic does not inhibit the nucleolar enzyme under similar conditions (1,3). This suggests that administration of amanitin to whole animals should selectively inhibit nonribosomal RNA formation while the synthesis of ribosomal RNA continues unimpeded. Such a tool would provide a means for investigating protein synthesis after specific

inhibition of mRNA synthesis. We have therefore examined the effect of administration of amanitin to intact animals on the synthesis of different species of RNA in rat liver and have found unexpectedly that formation of ribosomal RNA as well as other species of RNA is inhibited by the antibiotic under these conditions. This difference between the action of the toxin *in vivo* and its effect *in vitro* suggests that the synthesis of ribosomal RNA in the nucleolus may be under the control of an extranucleolar mechanism sensitive to amanitin.

METHODS AND MATERIALS

Orotic acid-6-^{14}C (spec. act. 35 mC/mmole) and L-methionine-methyl-^{3}H (spec. act. 3 C/mmole) were purchased from Schwarz BioResearch. UTP-^{14}C (spec. act. 200 mC/mmole) was obtained from New England Nuclear, and cycloheximide from Upjohn. Unlabeled nucleoside triphosphates and dithiothreitol were purchased from Calbiochem.

Male albino rats (Charles River), weighing about 150 g, were given α-amanitin (30 µg/g body weight) by jugular vein. Orotic acid-6-^{14}C (0.2 µC/100 g body weight) was then given intravenously 10 min before death. To concentrate the ^{3}H label in the liver, methionine-methyl-^{3}H (50 µC/100 g body weight) was injected into the portal vein 5 min prior to death. Control animals received 0.9% NaCl.

Nuclei and nucleoli were isolated from the livers as described previously (4,5). The RNA was extracted from whole nuclei, isolated nucleoli, and the extranucleolar portion of the nucleus (6,7). Radioactivity was determined in a Nuclear Chicago gas flow counter, and the specific activity was expressed as counts/min/mg RNA. The whole nuclear RNA was also run on sucrose gradients, and the UV profile and distribution of radioactivity in gradient

fractions were measured (7). In the case of rats injected with methionine-methyl-^{3}H, samples of mixed free and membrane-bound polysomes were isolated from the liver (8) and the uptake of radioactivity measured in the total sample obtained from 0.85 g liver. Radioactivity of samples was measured in a Nuclear Chicago liquid scintillation counter.

In similar experiments on whole animals, amanitin was injected and polymerase activity was measured in whole nuclei, isolated nucleoli, and the extranucleolar fraction using methods already described (1,9).

RESULTS

Amanitin was administered to intact rats, and at various times thereafter a pulse of orotic acid-6-^{14}C was given and the labeling of whole nuclear RNA and of nucleolar RNA were examined 10 min later. Fig. 1 shows that, within 30-60 min of administra-

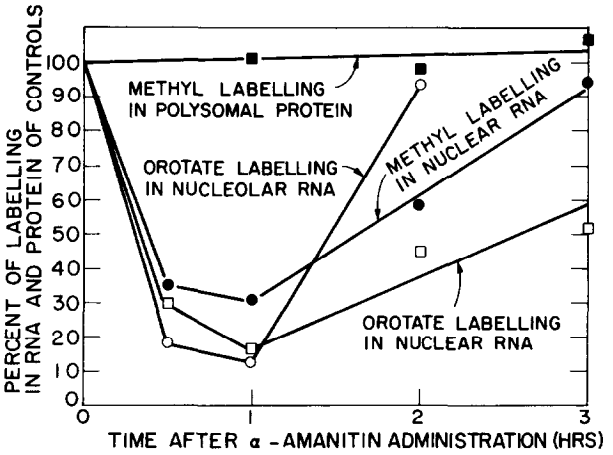

Fig. 1. Effect of α-amanitin administration *in vivo* on the labeling of nuclear and nucleolar RNA. To obtain results at each time point, rats were used in groups of two for nuclei and groups of four for nucleoli. The animals were given α-amanitin intravenously (30 μg/100 g body weight) while the control rats received 0.9% NaCl. After specified periods (0.5, 1, 2, and 3 hr), the animals were injected via the jugular vein with orotic acid-6-^{14}C or via the portal vein with methionine-methyl-^{3}H, and were killed 10 and 5 min after injection, respectively. The radioactivity in the RNA or polysome fractions is expressed as counts/min/mg of extracted RNA; the control (untreated) animals are given a value of 100.

tion of amanitin, uptake of orotic acid into whole nuclear RNA and nucleolar RNA was inhibited by more than 80%. The effect of the inhibitor on nucleolar RNA was transient, so that uptake from the pulse dose had returned to control levels by 2 hr after amanitin treatment. Whole nuclear RNA uptake showed a much slower recovery and was still 50% inhibited 3 hr after amanitin administration. Since whole nuclear RNA includes the nucleolar fraction, which shows rapid recovery, it can be inferred that the extranucleolar RNA must undergo little recovery. This proved technically difficult to measure due to the breakage of nucleoli after α-amanitin treatment, but in one experiment (not included in Fig. 1) in which the extranucleolar fraction was recovered free of broken nucleoli, uptake of orotate into extranucleolar RNA was still 78% inhibited 4 hr after injection of the toxin, whereas whole nuclear RNA uptake was only 30% reduced. Consequently, rats treated 2 hr or more beforehand with amanitin are fully capable of ribosomal RNA synthesis, without reduced capacity for synthesis of other RNA species.

The effect of the inhibitor on RNA made in the nucleolus was confirmed by using a short pulse of methionine-methyl-^{3}H, which acts mainly as the precursor for methylation of ribosomal RNA. Fig. 1 shows that amanitin inhibited incorporation of methyl label into whole nuclear RNA, with some recovery of incorporating capacity 3 hr after the inhibitor was given. In the same experiment nascent peptides labeled with methionine-methyl-^{3}H were recovered from the liver polysomes. Fig. 1 shows that the pulse dose of methionine labeled these to an equal extent at all times after amanitin administration. This not only demonstrates that amanitin has no direct effect on general protein synthesis, but also excludes the possibility that the inhibitor affects the

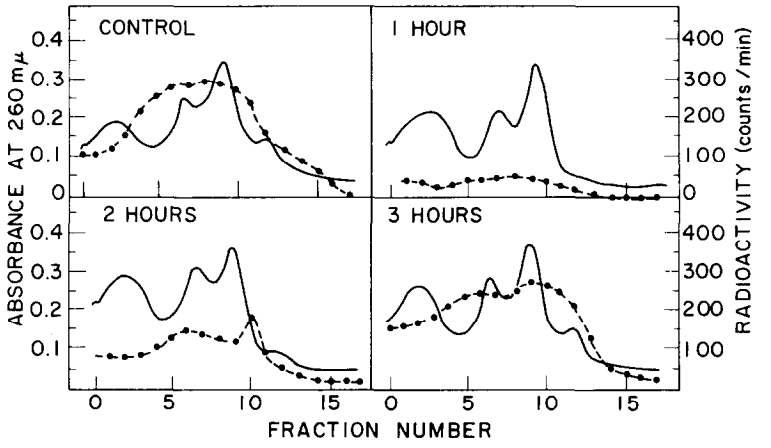

Fig. 2. Effect of α-amanitin administration *in vivo* on the sucrose density gradient profile of nuclear RNA after a pulse dose of orotic acid-6-^{14}C. The experimental details are given in the legend to Fig. 1. ——, absorbance of nuclear RNA; ●-●-●, radioactivity, expressed as counts/min.

uptake of labeled precursor into the free methionine pool of the liver, since this pool is used for both protein synthesis and ribosomal RNA methylation.

The effect of amanitin on ribosomal RNA formation was confirmed by gradient analysis of whole nuclear RNA profiles and uptake of pulse doses of orotate-6-^{14}C (Fig. 2). Whereas a distinct O.D. peak of 45S RNA is seen in the profile of animals not given amanitin, it disappears 1 hr after giving the antibiotic and returns to its normal abundance by 3 hr after injection. The pulse label of orotic acid shows the typical labeling pattern of nuclear RNA in the control sample. Nucleoli isolated from such nuclei show a sharp peak of activity at 45S (4,7) but this is obscured in the whole nuclear RNA profiles such as that of Fig. 1. At 1 hr after amanitin, the labeling pattern is uniformly low. At 2 hr there is a partial return of activity, particularly in the high molecular weight RNA, while at 3 hr the labeling pattern is similar to that in the control sample.

Table 1. The effect of *in vivo* administration of α-amanitin on nuclear, nucleolar, and extranucleolar RNA polymerase activities.

Type of enzyme preparation	Control	Enzyme activity (% of control) 1 hr	2 hr	3 hr	4 hr	5 hr
Whole nuclei	100 (5160)	42	-	41	-	-
	100 (7350)	32	26	-	26	-
Whole nucleoli	100 (7445)	35	-	42	-	-
	100 (2860)	34	-	-	-	47
	100 (8070)	21	-	-	31	-
	100 (2635)	54	-	-	-	-
Extranucleolar fraction	100 (440)	56	-	-	-	60

The animals were given α-amanitin as described in the legend to Fig. 1 except that 4-hr and 5-hr time points were also included in some experiments. The nuclear, nucleolar, and extranucleolar fractions were isolated as described in the text. The RNA polymerase activities were determined as described previously (1). The enzyme activity is expressed as μμmoles UMP incorporated/ mg DNA; the control, untreated animals were given a value of 100. The enzyme assay was performed in triplicate.

Whole nuclei, isolated nucleoli and extranucleolar fraction from animals treated with amanitin were also examined for RNA polymerase activity by incubating these organelles with triphosphates. Table 1 shows that polymerase activity in both preparations under optimal conditions (in the presence of Mn^{2+} and ammonium sulfate) was depressed 1 hr after injection of the inhibitor. Recovery of the polymerase activity of the nucleoli was somewhat slower than suggested by the labeling studies (Fig. 1).

DISCUSSION

The action of amanitin on RNA synthesis *in vivo* differs from its action *in vitro*. Whole nucleoli and soluble nucleolar polymerase are insensitive to large doses of amanitin added to the enzyme under *in vitro* conditions (1). However, nucleoli recovered

from rats treated *in vivo* with amanitin have much reduced nucleolar polymerase activity (Table 1), accompanied by a lack of incorporation of precursors into nucleolar RNA (Fig. 1) and a loss of the 45S ribosomal RNA precursor peak seen in gradients of nuclear RNA (Fig. 2). This suggests that in the intact cell the function of nucleolar polymerase is regulated by some extranucleolar factor sensitive to amanitin. Since the *in vivo* action of amanitin on nucleolar RNA synthesis is transient (Fig. 1), it is tempting to conclude that amanitin binds to a rapidly turning-over extranucleolar factor that regulates nucleolar ribosomal RNA synthesis.

There are several ways in which extranucleolar control of nucleolar RNA synthesis could be exerted. First, it is possible that the synthesis of a species of RNA in the extranucleolar compartment exerts a direct regulatory function over ribosomal RNA formation. Second, RNA formed in the extranucleolar compartment may code for synthesis of a regulatory protein made in the cytoplasm. In either of these cases, amanitin would have an inhibitory effect through its action on extranucleolar polymerase. Third, amanitin may be converted metabolically into a toxin that inactivates nucleolar enzyme; this would explain the lack of direct *in vitro* action of amanitin on nucleolar polymerase (1,3). Since *in vivo* nucleolar RNA synthesis recovers rapidly after amanitin inhibition, the action of such a metabolite may not be directly on the enzyme but on a rapidly renewed cofactor made extranucleolarly. Finally, amanitin may interact directly with a cytoplasmic regulatory factor. In relation to this possibility, it has been shown in HeLa cells that cycloheximide inhibits RNA synthesis, apparently by suppressing cytoplasmic formation of protein (10), and we have observed that treatment of rats with cycloheximide inhibits liver nucleolar polymerase activity

(unpublished data). It is unlikely, however, that the amanitin suppresses cytoplasmic synthesis of nucleolar polymerase; amanitin does not have a direct action on general protein synthesis (Fig. 1), and the continued activity of the enzyme for 50 min or more during *in vitro* incubation of nucleoli (9) does not suggest a rapid rate of renewal.

Our earlier studies (1) showed that α-amanitin added *in vitro* inhibits only the extranucleolar enzyme. The present data suggest that this may be because, for optimal activity, the nucleolar polymerase requires an additional factor that might be analogous to the sigma initiating factor of bacterial polymerase (11).

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