NEW EVIDENCE THAT RELEASE OF RNA POLYMERASE IN ISOLATED NUCLEI REQUIRES TRANSCRIPTION

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Summary: RNA polymerases solubilized by transcription in isolated nuclei at various $(NH_4)_2SO_4$ concentrations were analyzed to determine the proportion of amanitin-sensitive and amanitin-resistant activity. Ionic conditions that stimulated amanitin-sensitive transcription in isolated nuclei increased the amount of RNA polymerase II released. Approximately equal amounts of RNA polymerase I were released at all ionic strengths tested.

Nuclei isolated from many cell types are capable of synthesizing RNA in vitro, but the details of the transcription process are not well characterized. Whereas the fidelity of transcription by purified eukaryotic RNA polymerases is open to question (1), apparently accurate synthesis of ribosomal and pre-ribosomal RNA's in isolated amphibian nuclei (2, 3) and rat liver nucleoli (4) has been reported. Studies of RNA synthesis in isolated nuclei may contribute significantly to a better understanding of transcription, and the regulation thereof, in eukaryotic cells.

A previous report (5) showed that RNA polymerase was solubilized when nuclei isolated from mouse tissues synthesized RNA in vitro. Conditions which reduced transcription of the endogenous DNA template also diminished the amount of RNA polymerase appearing in soluble form. These results were interpreted to mean that transcription was required for release of the RNA polymerase from its endogenous template.

A more direct test of this hypothesis is made possible by the observation that the toxin α-amanitin selectively inhibits soluble RNA polymerase II (6,7) and the Mn^{++}-$(NH_4)_2SO_4$-stimulated RNA synthesis in isolated nuclei (8-11). Thus, the proportion of amanitin-sensitive to amanitin-resistant transcription in isolated nuclei can be altered by adjusting the ionic composition of the medium. This proportion should be reflected in the relative amounts of amanitin-sensitive and amanitin-resistant RNA polymerases solubilized if transcription is required for release of the enzymes from the template. The studies presented here indicate that this is indeed the case.

METHODS

Livers excised from adult male Swiss-Webster mice (40-60 days old) were immersed in ice-cold homogenizing medium (0.02 M Tricine, pH 8.0, 0.25 M sucrose, 2 mM $MgCl_2$, 1 mM dithiothreitol) and nuclei were isolated by the method of Blobel and Potter (12). Isolated nuclei were resuspended in homogenizing medium (1 ml per gram of liver used) and stored at -85°C. RNA synthesis *in vitro* was assayed by the incorporation of [^{3}H]UTP into RNA, measured by the filter paper disc method (13). Composition of the assay mixtures is given in Table 1.

RNA polymerases were solubilized from isolated nuclei by a modification of the method previously described (5). The composition of the release mixtures was the same as the assay mixtures for RNA synthesis, with the following exceptions: the volume was 0.5 ml, [^{3}H]UTP was omitted, and all four nucleoside triphosphates were present at 0.25 mM. After incubation for 30 minutes at 37°, each mixture was homogenized in a glass homo-

genizer with a motor-driven teflon pestle. The homogenates were centrifuged 30 minutes at 30,000 x g, and 25 µl aliquots of the supernatant fractions were assayed for RNA polymerase activity.

RESULTS AND DISCUSSION

Isolated nuclei were assayed at 0-0.5 M $(NH_4)_2SO_4$ in the presence and absence of α-amanitin to determine the effect of these factors on RNA synthesis in vitro. The results presented in Table 1 show that amanitin-sensitive RNA synthesis in the isolated nuclei was greatly stimulated by $(NH_4)_2SO_4$ up to 0.5 M. The amount of amanitin-resistant RNA synthesis remained essent-

Table 1. Effect of α-amanitin and $(NH_4)_2SO_4$ on RNA synthesis in isolated mouse liver nuclei.

$(NH_4)_2SO_4$ (M)	α-amanitin (2 µg/ml)	pmoles UMP incorporated
0	-	8.1
0	+	5.8
0.02	-	9.2
0.02	+	6.1
0.10	-	16.8
0.10	+	6.0
0.50	-	82.4
0.50	+	5.0

Assay mixtures contained in 0.125 ml the following: 0.05 M Tricine (pH 8.0), 2 mM $MnCl_2$, 5 mM $MgCl_2$, $(NH_4)_2SO_4$ as indicated, 5 mM dithiothreitol, 5 mM creatine phosphate, 50 µg/ml creatine phosphokinase, ATP, CTP, GTP, 0.5 mM each, 0.05 mM [^{3}H]UTP (500 mCi/mmole), 25 µl of nuclei in homogenizing medium. Reaction mixtures were incubated 10 minutes at 37°. Each figure represents the average of triplicate assays.

ially constant at all $(NH_4)_2SO_4$ concentrations tested. These results are in good agreement with results of others (8-11).

If RNA polymerases are solubilized as a result of transcription in isolated nuclei, the $(NH_4)_2SO_4$ concentration of the medium should influence the relative amounts of amanitin-sensitive and amanitin-resistant polymerases appearing in soluble form. Specifically, more amanitin-sensitive RNA polymerase should be solubilized at higher $(NH_4)_2SO_4$ concentrations. To test this hypothesis, the following experiment was performed.

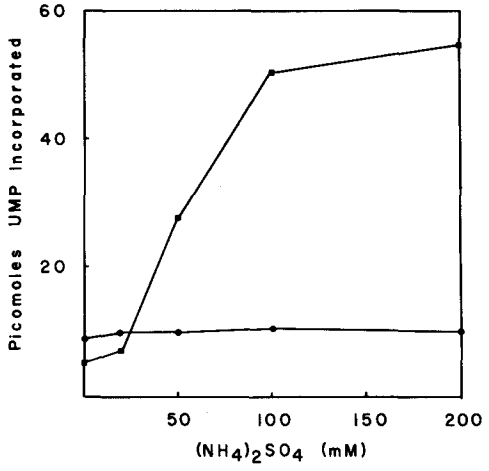

Figure 1. Sensitivity to α-amanitin of RNA polymerases solubilized at various $(NH_4)_2SO_4$ concentrations. Release mixtures containing the indicated concentrations of $(NH_4)_2SO_4$ were prepared and fractionated as described under Methods. Triplicate 25 µl aliquots of the resulting soluble RNA polymerases were assayed with and without α-amanitin (2 µg/ml). All assay mixtures contained 0.04 M $(NH_4)_2SO_4$ and 25 µg heat-denatured calf thymus DNA. Results are plotted as RNA polymerase activity (pmoles UMP incorporated) vs. the $(NH_4)_2SO_4$ concentration at which the polymerases were solubilized.

Symbols: ● amanitin-resistant polymerase; ■, amanitin-sensitive polymerase.

Solubilization mixtures containing 0-0.2 M $(NH_4)_2SO_4$ were incubated and fractionated as described under Methods. The soluble RNA polymerases obtained were assayed in the presence

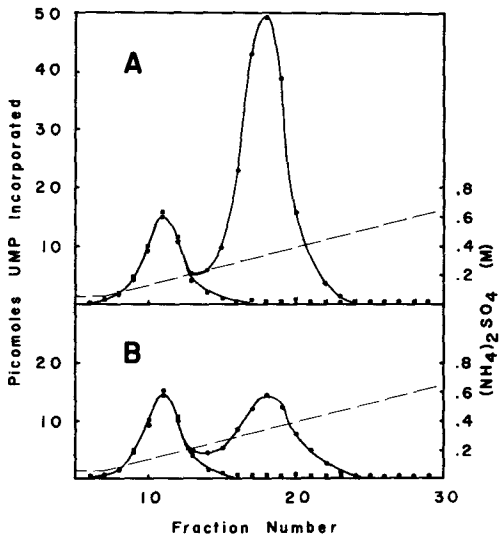

Figure 2. Chromatographic fractionation of RNA polymerase. Polymerases solubilized at (A) 0.10 M and (B) 0.02 M $(NH_4)_2SO_4$ were applied to identical DEAE-cellulose columns equilbrated with 0.05 M $(NH_4)_2SO_4$ in TGMED buffer (0.02 M Tricine (pH 8.0), 5 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM dithiothreitol, 20% glycerol by volume). Each column was washed with one bed volume of starting buffer, and both columns were developed simultaneously with two lines from a single gradient generator (0.05-0.70 M $(NH_4)_2SO_4$ gradient). Aliquots (25 µl) of each column fraction were assayed with (■) and without (●)α-amanitin.

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and absence of α-amanitin with denatured calf thymus DNA as template. The results are shown in Figure 1. The majority of RNA polymerase activity solubilized at 0 or 0.02 M $(NH_4)_2SO_4$ was amanitin-resistant. At $(NH_4)_2SO_4$ concentrations of 0.05 M or higher, the amount of amanitin-sensitive RNA polymerase was greatly increased, but the amount of amanitin-resistant RNA polymerase remained constant. Concentrations of $(NH_4)_2SO_4$ above 0.2 M were not used because the nuclei formed a gel that could not be separated into soluble and insoluble fractions by the centrifugation conditions used.

To confirm the distributions of RNA polymerases, the soluble polymerases obtained at 0.10 and 0.02 M $(NH_4)_2SO_4$ were fraction-

ated on columns of DEAE-cellulose. The results are shown in Figure 2. The amount of RNA polymerase I (amanitin-resistant) was the same in both cases, but far more RNA polymerase II (amanitin-sensitive) was solubilized at 0.10 M $(NH_4)_2SO_4$. No RNA polymerase III was found in either case.

RNA polymerases in isolated nuclei are generally found in an insoluble or "aggregate" form (14), presumably bound to the chromatin template. During RNA synthesis *in vitro*, RNA polymerases become soluble (5). The results presented here support the hypothesis that transcription of the endogenous template is required for release of RNA polymerases from the enzyme-template complex. When ionic conditions were adjusted to alter the proportion of amanitin-sensitive to amanitin-resistant transcription of the endogenous template in isolated nuclei, parallel alterations in the amounts of RNA polymerases I and II occurred. It may be that a molecule of RNA polymerase must reach a particular site, perhaps a termination signal, before being released from the chromatin complex.

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