DIFFERENT RESPONSES OF SOLUBLE WHOLE NUCLEAR RNA POLYMERASE AND SOLUBLE NUCLEOLAR RNA POLYMERASE TO DIVALENT CATIONS AND TO INHIBITION BY α-AMANITIN

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SUMMARY

RNA polymerase activities of isolated nuclei and nucleoli and of the solubilized enzyme extracted from these organelles were examined in the presence of divalent cations and α-amanitin. The ratio of enzyme activity in presence of Mg^{2+} to activity in presence of Mn^{2+} and ammonium sulfate was higher for the enzyme preparations made from nucleoli than for the corresponding preparations made from whole nuclei. When incubated in presence of ammonium sulfate and Mn^{2+}, the polymerase activities of the whole nuclei and of the soluble enzyme preparation made from whole nuclei were extensively inhibited by amanitin, whereas the polymerase activity of the nucleolar enzyme was only slightly affected by the toxin under the same conditions. From the differing degrees of response to amanitin and to Mg^{2+} and Mn^{2+}, it has been concluded that the RNA polymerase of nucleoli differs structurally from the chromatin-associated polymerase extractable from whole nuclei.

INTRODUCTION

Isolated nuclei show two types of RNA polymerase activity. When incubated in the presence of Mg^{2+} ion, they appear to synthesize mostly a ribosomal type of RNA; in the presence of Mn^{2+} and ammonium sulfate they make RNA with a base ratio resembling that of DNA (1). By means of autoradiography, it has been shown (2,3) that the RNA made by nuclei incubated with Mg^{2+} accumulates in the nucleoli, the accepted site of ribosomal RNA synthesis, whereas RNA formed during incubation in Mn^{2+} and a low concentration of ammonium sulfate is mainly extranucleolar. This distinction between the divalent cation requirements for nucleolar and extranucleolar polymerase activity is not absolute; we have demonstrated that the capacity of isolated nucleoli to synthesize RNA can in fact be increased above the level achieved with Mg^{2+} if Mn^{2+} and

a sufficiently high concentration of ammonium sulfate is present in the incubation medium (4). Recently, Roeder and Rutter (5) have chromatographically separated two polymerase activities from rat liver nuclei; one of these showed greater activity in presence of Mg^{2+}, and was thought to be associated with the nucleoli.

Stirpe and his colleagues (6,7) have found that α-amanitin, a toxic peptide obtained from *Amanita phalloides*, causes extensive inhibition of the RNA polymerase activity of crude nuclear enzyme preparations incubated in the presence of Mn^{2+} and ammonium sulfate, but little inhibition of activity assayed in presence of Mg^{2+}. We have recently demonstrated that amanitin inhibits soluble RNA polymerase extracted from whole liver nuclei by a direct action on the enzyme protein and not on the DNA template (8). Since the soluble enzyme obtained from whole liver nuclei shows little activity except in the presence of Mn^{2+} and ammonium sulfate and seems mainly representative of extranucleolar polymerase, we have now prepared a soluble polymerase from isolated nucleoli and have examined its activity in the presence of Mg^{2+} and of Mn^{2+}, and its response to addition of amanitin.

ISOLATION OF SOLUBLE POLYMERASES

The soluble enzyme was obtained from whole nuclei by a procedure described earlier (9); nuclei isolated from the liver of male albino rats (Charles River Breeding Laboratories, Wilmington, Massachusetts) were lyzed by gentle homogenization with 0.05 M Tris-HCl buffer, pH 8.9, containing 0.025 M KCl, 0.5 mM dithiothreitol and 2 mM Mg acetate, then incubated at 37° for 30 min. Glycerol was added to a final concentration of 30%, and the mixture was shaken vigorously at room temperature for 2 min. The enzyme-free residue was removed by centrifugation at 105,000 × *g* for 30 min. This procedure resulted in almost complete release of RNA polymerase from whole nuclei. To prepare nucleolar soluble enzyme, nucleoli were isolated from rat liver nuclei (4) and the procedure described above for obtaining soluble enzyme from whole nuclei was applied except that the nucleoli were sonicated for 1 min in the buffer, and that incubation at 37° and shaking were eliminated. The DNA content of the preparations was measured as described previously (9). Protein was estimated by a modification

of Lowry's procedure (10). Both the whole nuclear and the nucleolar soluble enzymes depended on exogenous DNA for activity. Unlike the soluble enzyme extracted from whole nuclei, the nucleolar preparation was quite unstable and assays had to be carried out immediately after preparation.

RESULTS AND DISCUSSION

Table 1 shows the effect of Mg^{2+} and of Mn^{2+} with ammonium sulfate on RNA polymerase activity assayed in whole nuclei and in isolated nucleoli, and on the activity of soluble polymerase prepared from each of these organelles. Our results agree with those of other workers using soluble polymerase (5,12); maximal enzyme activity with Mn^{2+} required addition of 0.06 M $(NH_4)_2SO_4$ to the soluble enzymes, whereas 0.4 M salt is needed to activate maximally the crude polymerase in whole nuclei (1) or nucleoli (4). The ratio of Mg^{2+}-dependent activity to Mn^{2+}-dependent activity is higher in the crude nucleolar preparation than in whole nuclei (Table 1). The different response to divalent cations is accentuated when the soluble enzymes prepared from whole nuclei and from nucleoli were compared, using rat liver DNA as the template. This is due to a much greater Mg^{2+} activity per mg protein in the case of the nucleolar soluble enzyme, a finding which corresponds to the demonstration by Roeder and Rutter (5) of two soluble enzyme activities in liver nuclei that differ in their relative stimulation by Mg^{2+} and Mn^{2+}.

Since the nucleolar enzyme is much less stable (a phenomenon observed by Akamatsu *et al.* (11) in their preparation of soluble nucleolar polymerase), the whole nuclear soluble preparation probably contains little of the nucleolar soluble enzyme because of its destruction during preparation and storage before use.

The action of α-amanitin on the crude and soluble polymerase preparations is shown in Table 2. In agreement with Stirpe (7), amanitin had little inhibitory effect on the Mg^{2+}-dependent activity of the crude soluble preparations in the absence of $(NH_4)_2SO_4$. In the presence of $(NH_4)_2SO_4$ with Mn^{2+}, the activities of the crude whole nuclear enzyme and of the whole nuclear soluble preparation were depressed by 70–90%. However, the same

Table 1. Comparison of the RNA polymerase activities of whole nuclei, isolated nucleoli, soluble whole nuclear enzyme, and soluble nucleolar enzyme assayed with Mg^{2+} or with Mn^{2+} plus ammonium sulfate.

Type of enzyme preparation	No. of expts.	Enzyme activity		Mg/Mn ratio
		Mg^{2+}	Mn^{2+} + $(NH_4)_2SO_4$	
Whole nuclei	3	1180	2865	0.41
Isolated nucleoli	4	11840	18780	0.63
Nuclear soluble	7	152	1302	0.12
Nucleolar soluble	4	620	1280	0.48

In the case of the whole nuclei, the assay conditions were based on those of Widnell and Tata (1). The assay mixture for the Mg^{2+}-dependent reaction contained in 0.5 ml (μmoles): Tris-HCl (pH 8.5), 50; $MgCl_2$, 2.5; dithiothreitol, 5; NaF, 3; ATP, GTP and CTP, 0.3 each; UTP, 0.1; UTP-^{14}C (366 mc/m mole from Schwartz BioResearch, Orangeburg, N.Y.), 0.0002, along with nuclei containing 250 μg DNA. The Mn^{2+}-$(NH_4)_2SO_4$-activated reaction contained the same ingredients except that Mg^{2+} was replaced by $MnCl_2$ (2 μmoles); the mixture also contained $(NH_4)_2SO_4$ (0.2 mmoles) and fluoride was omitted. The Mg^{2+}-dependent reaction was carried out for 20 min and the Mn^{2+} reaction for 40 min at 37° in order to reach plateau values (1). The reaction was terminated at the end of incubation and uptake of ^{14}C was measured as described previously (9). The activities are expressed as μμmoles nucleotide incorporated/mg DNA. The data for the polymerase activity of isolated nucleoli are taken from the paper of Jacob *et al.* (4).

In the case of the two soluble enzyme preparations, the reaction mixtures were based on those used by Jacob *et al.* (9) to study soluble nuclear enzymes. The Mg^{2+}-dependent reaction mixture for the soluble enzyme contained in 0.75 ml (μmoles): Tris-HCl (pH 8.0), 40; $MgCl_2$, 2.0; NaF, 2.0; spermine, 2.0; ATP, 0.35; CTP and GTP, 0.3 each; UTP, 0.07; UTP-^{14}C, 0.0002, and in addition 60 μg purified rat liver DNA and 0.3 mg enzyme protein for the whole nuclear extract and 0.06 mg enzyme protein for the nucleolar extract. The Mn^{2+}-dependent reaction mixture for the soluble enzyme contained all the cofactors required for the Mg^{2+}-dependent reaction, except that Mg^{2+} was replaced by $MnCl_2$ (1.5 μmoles) and $(NH_4)_2SO_4$ was included at a final concentration of 0.06 M. Concentrations of $(NH_4)_2SO_4$ higher than 0.06 M inhibit the activity of soluble enzyme. This salt was added after 4 min of preincubation of the reaction mixture, since higher activities were obtained by this delay, probably because of the known inhibitory action of salts on binding of enzyme to DNA if added at the start of incubation. The soluble enzymes were incubated for 30 min at 37°, which gave plateau values for both Mg^{2+} and Mn^{2+} activities. The reaction was terminated and the uptake of ^{14}C was measured as described previously (9). The activities are expressed as μμmoles nucleotide incorporated/mg protein in the enzyme preparation.

Table 2. Comparison of the inhibitory action of α-amanitin on RNA polymerase in whole liver nuclei, liver nucleoli, soluble whole nuclear polymerase, and soluble nucleolar polymerase incubated under various ionic conditions.

Type of enzyme preparation	Enzyme activity			
	Mg^{2+}		Mn^{2+} + $(NH_4)_2SO_4$	
	Control	Amanitin	Control	Amanitin
Whole nuclei	1000	820 (-18%)	2215	620 (-72%)
Isolated nucleoli	3930	2980 (-25%)	8050	7370 (-8%)
Nuclear soluble	365	325 (-11%)	3185	220 (-93%)
Nucleolar soluble	1590	1650 (+4%)	3360	2775 (-18%)

The enzymes were prepared and incubated as described in Table 1. The final concentration of Mg^{2+} was 5 mM for the whole nuclei and 2.5 mM for the soluble enzymes; for Mn^{2+} it was 4 mM and 2 mM, respectively; and for ammonium sulfate it was 0.4 M and 0.06 M respectively. α-Amanitin was added at the start of incubation at a concentration of 0.03 µg/ml. Activities are expressed as µµmoles incorporated/mg DNA in the case of the whole nuclei and µµmoles incorporated/mg protein in the case of the soluble enzyme preparations. The experiment was replicated 2 or 3 times.

concentration of amanitin (0.03 µg/ml) inhibited the polymerase activity of the nucleolus and of the nucleolar extract only slightly and not in every experiment. The action of higher concentrations of amanitin was examined in studies on isolated nucleoli. At levels of 0.3, 3, and 30 µg/ml, no further depression of nucleolar RNA synthesis was achieved than is recorded in Table 2 for 0.03 µg/ml. The slight action of amanitin on polymerase activity in the isolated nucleoli may be due to traces of extranucleolar polymerase contaminating the nucleoli. High doses of α-amanitin did not depress the whole nuclear polymerase activity by more than 80%, thus indicating the presence of at least 20% of resistant enzyme, presumably of nucleolar origin.

These results substantiate the difference between polymerase activity in nucleoli and in the extranucleolar region of the liver cell nucleus. It was demonstrated earlier with soluble polymerase made from whole nuclei (8) that amanitin acts on polymerase protein and not on the DNA template. The present data suggest that it is selective for the protein of the extranucleolar enzyme.

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REFERENCES

1. Widnell, C.C. and Tata, J.R. *Biochim. Biophys. Acta*, 123: 478 (1966).
2. Pogo, A.O., Littau, V.C., Allfrey, V.G., and Mirsky, A.E. *Proc. Nat. Acad. Sci. U.S.*, 57: 743 (1967).
3. Maul, G.G. and Hamilton, T.H. *Proc. Nat. Acad. Sci. U.S.*, 57: 1371 (1967).
4. Jacob, S.T., Sajdel, E.M., and Munro, H.N. *Biochim. Biophys. Acta*, 157: 421 (1968).
5. Roeder, R.G. and Rutter, W.J. *Nature*, 224: 234 (1969).
6. Stirpe, F. and Fiume, L. *Biochem. J.*, 105: 779 (1967).
7. Novello, F. and Stirpe, F. *Biochem. J.*, 112: 721 (1969).
8. Jacob, S.T., Sajdel, E.M., and Munro, H.N. *Nature* (In press).
9. Jacob, S.T., Sajdel, E.M., and Munro, H.N. *Biochem. Biophys. Res. Commun.*, 32: 831 (1968).
10. Bennett, T.P. *Nature*, 213: 1131 (1967).
11. Akamatsu, N., Maeda, H., Kamiya, T., and Miura, Y. *J. Biochem. (Tokyo)*, 66: 101 (1969).
12. Cunningham, D.D., Cho, S., and Steiner, D.F. *Biochim. Biophys. Acta*, 171: 67 (1969).