EVIDENCE FOR THE NUCLEAR ORIGIN OF RNA POLYMERASES IDENTIFIED IN THE CYTOSOL: RELEASE OF ENZYMES FROM THE NUCLEI ISOLATED IN ISOTONIC SUCROSE

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SUMMARY

DNA-dependent RNA polymerases were extracted from rat liver nuclei isolated in isotonic or hypertonic sucrose and resolved chromatographically. Activities of RNA polymerases I, II and III from "hypertonic nuclei" were 3.8, 1.5 and 27-fold, respectively, higher than those from "isotonic nuclei". RNA polymerase III was virtually absent in the "isotonic nuclei". Concurrent with the reduced recovery of polymerases from the "isotonic nuclei", the corresponding cytosol fraction contained higher levels of all three enzymes. These studies provide strong evidence for the contention that a large proportion of cytoplasmic RNA polymerase C previously identified in "isotonic cytosol" fraction corresponds to nuclear RNA polymerase III. These data demonstrate the need for the use of hypertonic sucrose in the initial homogenization of tissues in order to prevent leakage of the nuclear enzymes.

All eukaryotic cells examined to date contain three classes of DNA-dependent RNA polymerases, EC 2.7.7.6, (for reviews, see refs. 1 and 2). Class I enzymes are localized in the nucleolus and are responsible for the synthesis of ribosomal RNA. Class II and III polymerases are localized in the nucleoplasm and are involved in the production of mRNA and low molecular weight RNAs, respectively. RNA polymerases II and III are sensitive to low (3-5) and high (6) concentrations of α-amanitin, respectively, whereas RNA polymerase I and its subclasses are insensitive to the toxin. Subsequently, another enzyme has been identified in the cytosol fraction and designated as RNA polymerase C (7). The remarkable similarities between this enzyme and RNA polymerase III have prompted some investigators to suggest that the two enzymes may be identical. In this paper, we wish to present evidence to

indicate that at least the bulk of the cytoplasmic RNA polymerase is indeed nuclear RNA polymerase III which is released into the cytosol.

METHODS

Preparation of nuclear and cytosol fractions by hypertonic and isotonic sucrose methods. Liver from Fischer female rats was homogenized in hypertonic buffer (7 ml/g liver) containing 2.2-2.3 M sucrose, 15 mM $MgCl_2$ and 0.25 mM spermine essentially as described previously (8,9). The major modification was that the volume of the medium was reduced to 7 ml/g wet weight of tissue. This did not cause any detectable change in the recovery of the enzyme in the nuclei or the cytosol, but facilitated handling of the cytosol fraction obtained in this hypertonic medium. The pellet obtained after filtering and centrifugation at 40,000 x g for 70 min, was designated as the "hypertonic nuclei". The supernatant was subsequently used to prepare the "hypertonic cytosol" fraction.

For preparation of isotonic fractions, liver was homogenized in buffer (6 ml/g liver) containing 0.05 M Tris-HCl pH 7.6, 0.01 M $MgCl_2$, 0.025 M KCl and 0.25 M sucrose. Nuclei were obtained by centrifugation at 1000 x g for 7 min. The centrifugation was repeated to completely sediment nuclei. The pellets designated as "isotonic nuclei", were combined and further purified by homogenization in 2.2 M sucrose containing 1 mM $MgCl_2$ (2.5 ml/g liver) followed by centrifugation at 40,000 x g for 90 min. The supernatants from the low-speed and high-speed centrifugations were combined and used to prepare the "isotonic cytosol".

The supernatant fractions from both hypertonic and isotonic preparations were diluted to 0.44 M sucrose by addition of buffer containing 0.05 M Tris-HCl pH 7.5, 0.01 M $MgCl_2$, 0.025 M KCl, 0.5 mM dithiothreitol and 0.1 mM EDTA. Mitochondria and microsomes were removed from the cytosol by successive centrifugation at 40,000 x g for 10 min followed by 100,000 x g for 90 min.

Extraction of RNA polymerases. Extraction of nuclear RNA polymerases and $(NH_4)_2SO_4$ precipitation were similar to that described by us previously (10-12). Cytosol fractions were precipitated directly with $(NH_4)_2SO_4$ (0.42 g/ml).

RESULTS

To investigate the levels of RNA polymerases retained in nuclei isolated in isotonic and hypertonic media, enzymes were extracted from the respective nuclei and resolved chromatographically. Figure 1 shows the DEAE-Sephadex chromatographic profile of RNA polymerases from the "isotonic" and "hypertonic" nuclei. It is evident that all three RNA polymerases were present in significantly higher levels in the "hypertonic nuclei". Fractions containing the three peaks of nuclear enzyme activity were pooled, dialyzed overnight and assayed with optimal salt concentrations. Activities of RNA polymerases I, II and III were 3.8-, 1.5- and 27-fold higher in the "hypertonic nuclei" relative to "isotonic nuclei" (see Table 1).

In order to determine whether the loss of enzymes from "isotonic nuclei"

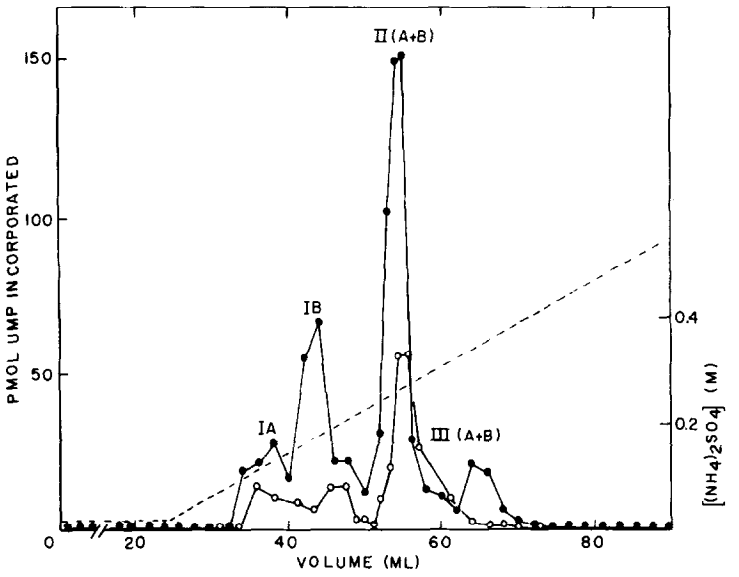

Figure 1. DEAE-Sephadex chromatography of RNA polymerases extracted from rat liver nuclei

"Hypertonic" and "isotonic" nuclei were prepared from 14 g rat liver as described in METHODS. RNA polymerases were extracted, dialyzed against buffer containing 50 mM Tris-HCl (pH 7.9), 25% (v/v) glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA and 0.5 mM dithiothreitol (Buffer I), and centrifuged as described previously (12). Enzyme extracts were applied to DEAE-Sephadex (A25) columns (0.9 x 8 cm) previously equilibrated in Buffer I. The columns were washed with Buffer I containing 0.01 M $(NH_4)_2SO_4$ and were eluted with a linear gradient of 0.01 M - 0.6 M $(NH_4)_2SO_4$. Fractions (1 ml) were collected in the presence of bovine serum albumin (1 mg) and aliquots (35 µl) were taken to analyze for RNA polymerase activity as described previously (12) with the inclusion of 1 mM spermine in the assay (15). (●——●) "Hypertonic nuclei"; (○——○) "isotonic nuclei".

was correlated with an increase in RNA polymerase activity in the "isotonic cytosol", the corresponding cytosol fractions were prepared and subjected to DEAE-Sephadex chromatography. Figure 2 shows the column chromatographic profiles of RNA polymerases obtained from the "hypertonic" and "isotonic" cytosol fractions. RNA polymerases II (A + B) and III (A + B) were sensitive to low and high concentrations of α-amanitin, respectively, whereas RNA polymerases I (A + B) were completely insensitive to the toxin. All RNA polymerases were present in much higher proportions in the "isotonic cytosol" fraction. The better chromatographic resolution of RNA polymerases from

TABLE 1. Comparison of RNA Polymerase Activity from Nuclear and Cytosol Fractions

Cellular Fraction	Isolation Medium (Sucrose)	Protein in Extract (mg)	Recovery of DNA (mg/g)	RNA polymerase activity (pmole of UMP incorporated) I	II	III
Nuclei	Isotonic	8	0.636	510	4,375	50
	Hypertonic	8	0.676	1,921	6,630	1,350
Cytosol	Isotonic	200	---	1,838	5,924	4,190
	Hypertonic	204	---	370	3,743	969

Fractions containing I (IA + IB), II (IIA + IIB) and III (IIIA + IIIB) were individually pooled, dialyzed overnight against buffer containing 50 mM Tris-HCl (pH 8), 50% (v/v) glycerol, 0.1 mM EDTA and 0.5 mM dithiothreitol. Fractions were assayed for RNA polymerase activity (12), using $(NH_4)_2SO_4$ at 30 mM for polymerase I and 100 mM for enzymes II and III in the presence of excess DNA. RNA polymerase activity, calculated from several enzyme concentrations, is expressed as total pmol of UMP incorporated from 14 g liver. Protein in the extracts was determined by the procedure described by Bennett (19) using bovine serum albumin as a standard and refers to the total recovery in the extracts (from 14 g liver) just prior to chromatography. DNA in the nuclear fractions was estimated as described by Burton (20).

the cytosol fractions relative to those from the nuclear extracts was due to the larger columns used to accommodate large quantities of protein in the cytosol. It should also be noted that much larger fractions were collected for the cytosol fractions and the values in the ordinates for Figures 1 and 2 were not identical. Fractions containing I (A + B), II (A + B) and III (A + B) were pooled, dialyzed overnight and assayed with optimal levels of $(NH_4)_2SO_4$. The recovery of RNA polymerases I, II and III was approximately 5-fold, 1.6-fold and 4-fold higher in the "isotonic cytosol" fractions (Table 1) as compared to the corresponding enzyme activities in the "hypertonic cytosol" fraction. The combined activities of polymerase I in the "isotonic" nuclei and cytosol roughly corresponded to the sum of their

activities in the "hypertonic" nuclei and cytosol (Table 1). This was also the case with RNA polymerase II.

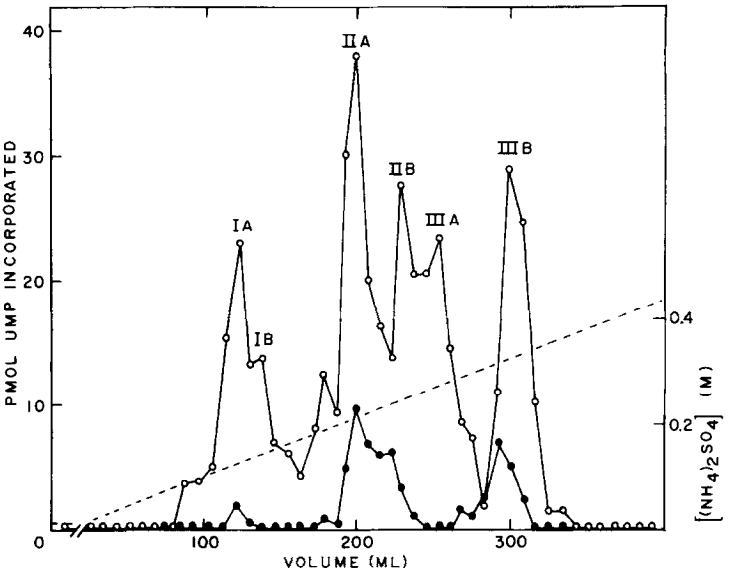

Figure 2. DEAE-Sephadex chromatography of RNA polymerases from rat liver cytosol.

"Isotonic" and "hypertonic" cytosol fractions were isolated from 14 g rat liver as described in METHODS. After dialysis against Buffer I, the preparations were clarified by centrifugation (80,000 x g for 40 min), applied to DEAE-Sephadex columns (2.5 x 16 cm), eluted with a linear gradient and analyzed as described in the legend to Figure 1. Note that the values on the ordinates are different from those in Figure 1. (●——●) "Hypertonic cytosol"; (o——o) "isotonic cytosol".

DISCUSSION

The present studies clearly demonstrate that DNA-dependent RNA polymerases I, II and III are maximally retained in nuclei isolated by initial homogenization of tissue in hypertonic sucrose, a method commonly used for the preparation of pure nuclei. These enzymes can be released into the cytoplasm during homogenization in isotonic sucrose. Similar observations were made with RNA polymerases from other tissues (data not shown). Since the nuclear and cytosol fractions obtained in isotonic or hypertonic buffers were further processed under similar conditions, it is very unlikely that

alterations in the recovery of RNA polymerases were due to differential processing of these fractions. Although free RNA polymerases have been shown to be easily extractable by homogenizing the "hypertonic nuclei" in isotonic sucrose (13), no investigation to date has attempted to compare the levels of RNA polymerases from the "isotonic cytosol" directly with those from the "hypertonic cytosol" and hence failed to recognize the overwhelming loss of all RNA polymerases, particularly polymerase III, from the "isotonic nuclei". The almost complete release of polymerase III into the "isotonic cytosol" may explain the ease with which the latter enzyme can be detected in this fraction (7,14,15). To ensure maximal retention of all RNA polymerases in the cell nucleus, the nuclei should hence be prepared with hypertonic sucrose (2.2-2.3 M). The nuclear pellet should not be further processed in isotonic buffers, as commonly practiced, except perhaps in the case of certain solid tumors where removal of contaminating lipids and membranes by brief washing with Triton X-100 does not result in a significant loss of enzyme activity (12). This could be a special situation which might be typical of neoplastic tissues.

The release of RNA polymerases from the nuclei during tissue homogenization is of paramount importance for the determination of the levels of cellular RNA polymerases, especially those of II and III. In particular, this presents a problem in the case of cells in culture, which are not easily broken by homogenization in dense media. Clearly the procedures that are commonly employed for the isolation of nuclei from such systems have grossly underestimated the actual levels of RNA polymerases present in that organelle. Such errors could be further magnified when the alterations in the levels of enzymes are investigated under various physiological and pathological conditions. This problem can be resolved by extracting the enzyme directly from whole cells. Indeed, such an approach has been adopted in some laboratories for quantitative extraction of RNA polymerases from cell cultures (16,17).

Finally, the recovery of protein in the respective fractions deserves some comment. There was no significant difference in the amount of protein recovered from the cytosol under the isotonic and hypertonic conditions. Similarly, the recovery of DNA and protein in the nuclei isolated in isotonic and hypertonic media was identical. Yet, much higher levels of all RNA polymerases were seen in the "isotonic cytosol" as opposed to the "hypertonic cytosol". This might imply that relatively few soluble proteins in the nuclei are easily released into the cytosol under isotonic conditions. They include poly(A) polymerase (18) and RNA polymerase.

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