THREE DISTINCT FORMS OF DNA-DEPENDENT RNA POLYMERASE III FROM KIDNEY

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SUMMARY

DNA-dependent RNA polymerases were extracted from nuclei isolated from 1 kg of pig kidney and subjected to DEAE-Sephadex chromatography using a stepwise salt gradient. Fractions corresponding to RNA polymerase III were pooled and rechromatographed on a second DEAE-Sephadex column using a linear salt gradient. At least three distinct peaks, designated as IIIA, IIIB, and IIIC were resolved. These peaks exhibited α-amanitin dose response curves characteristic of RNA polymerase III. Detection of the enzyme was facilitated by assaying with either highly polymerized calf thymus DNA and spermine or with poly [d(A-T)]. The heterogeneity of this enzyme became even more pronounced after further purification. Under the same conditions, both RNA polymerases I and II were resolved at most to two subspecies. The highly heterogeneous nature of RNA polymerase III is consistent with the large number of RNA species believed to be synthesized by this enzyme class.

DNA-dependent RNA polymerase (E.C.2.7.7.6) from eukaryotes exists as multiple species designated as I, II and III (for reviews, see refs. 1 and 2). These enzymes are compartmentalized within the nuclei and show different degrees of sensitivity to the mushroom toxin, α-amanitin. Recently, there is a renewal of interest in the class III enzymes stemming from the following reasons: (a) RNA polymerase III is involved specifically in the synthesis of low molecular weight RNAs including 5S and/or tRNA (3). (b) Class III enzymes can utilize highly polymerized DNA as template in the presence of polyamines (4) and, unlike the other RNA polymerases, can transcribe double stranded DNA very efficiently (5) and (c) Polymerase III levels appear to be dramatically elevated in tumor cells (6).

In lower eukaryotes, RNA polymerase III has been detected as a single form (7,8,9). However, in several mammalian tissues (10,11) and cell types

(12), this enzyme has been shown to exist as multiple species, usually designated as IIIA and IIIB both of which are sensitive to high levels of α-amanitin (3). We now wish to report that unlike RNA polymerases I and II, RNA polymerase III is highly heterogeneous and exhibits several chromatographically separable forms all of which are inhibited only by high levels of α-amanitin.

METHODS

Isolation of nuclei and extraction of RNA polymerases. Nuclei were isolated from 1 kg of pig kidney and DNA-dependent RNA polymerases were extracted from the nuclei as described previously (13). Minor modifications included the addition of 0.25 mM spermine to the homogenization buffer and the dialysis of the final $(NH_4)_2SO_4$ pellet against TGMED-50 buffer (14) before the initial DEAE-Sephadex chromatography.

First DEAE-Sephadex chromatography. The enzyme extract was diluted with TMED buffer to 25% (v/v) glycerol. RNA polymerases were then eluted with a stepwise gradient consisting of one bed volume each of 0.14M$(NH_4)_2SO_4$ and 0.5 M $(NH_4)_2SO_4$ (13). Five ml fractions were collected and alternate fractions were assayed for polymerase activity.

Assay of DNA-dependent RNA polymerase. A standard reaction mixture contained in a total volume of 120 µl: 50 mM Tris-HCl (pH 8.0), 1.25 mM $MnCl_2$, 6.33 mM KCl, 2.5 mM NaF, 0.5 mM DTT, 0.64 mM ATP, GTP and CTP, concentrations of UTP and templates as given in the appropriate legends and 35-40 µl enzyme (14).

RESULTS AND DISCUSSION

RNA polymerases were extracted from the nuclei isolated from 1 kg of pig kidney and subjected to an initial DEAE-Sephadex chromatography using a stepwise gradient. RNA polymerase I was eluted in the 0.14 M salt fraction whereas RNA polymerases II and III were eluted together in the higher salt fractions. RNA polymerase III was identified in the latter fractions by its resistance to low levels (0.5 µg/ml) of α-amanitin which inhibited RNA polymerase II completely (1,2). The pooled α-amanitin-resistant fractions from the 0.5 M salt elution were then subjected to a second DEAE-Sephadex chromatography. Three distinct peaks of RNA polymerase III were detected under these conditions. They were designated as IIIA, IIIB and IIIC (Fig. 1).

Fractions corresponding to IIIA and IIIB were pooled, dialyzed against TGMED buffer as described previously and rechromatographed on a third DEAE-Sephadex column (Fig. 2). Peaks IIIA and IIIB were resolved completely as two major

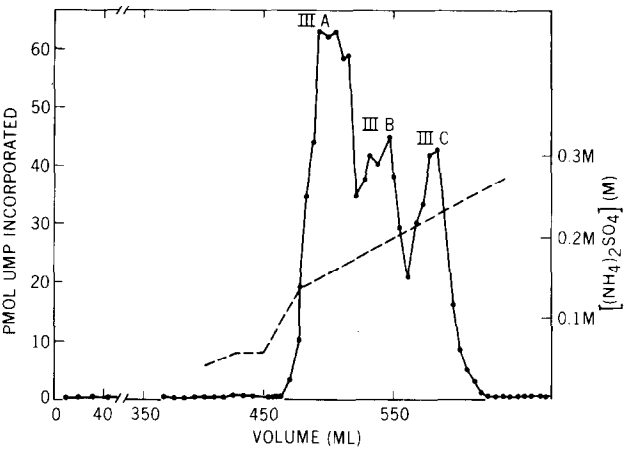

Figure 1. Rechromatography of RNA polymerase III on DEAE-Sephadex column.

Fractions containing RNA polymerase III activity from the 0.5 M $(NH_4)_2SO_4$ step of an initial DEAE-Sephadex (A-25) column chromatography were dialyzed and subjected to chromatography on a second DEAE-Sephadex column (2.5 x 12 cm) which had previously been equilibrated with 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 dithioreitol (TGMED buffer) and 10 mM $(NH_4)_2SO_4$. The column was washed with 1.5 column volumes of the same buffer and fractionated using a linear gradient of 30 to 630 mM ammonium sulfate in TGMED buffer. Alqiuots of 35 µl from alternate fractions (3 ml) were taken for the enzyme assay. The assay included calf thymus DNA (133 µg/ml) as template in the presence of 5 mM spermine, 0.5 µg/ml of α-amanitin and 64 µM [^{3}H]UTP (specific activity 28 x 10^3 cpm/nmole). Fractions corresponding to enzyme activity representing IIIA and IIIB were pooled together, IIIC was pooled separately. Bovine serum albumin was added to a final concentration of 1 mg/ml and the fractions were dialyzed against 20 vol of TGMED buffer containing 50% (v/v) glycerol.

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peaks. A minor peak of IIIC that had been previously associated with IIIB was also resolved.

Figs. 1 and 2 show that in addition to distinct peaks IIIA, IIIB, and IIIC, the chromatographic profile contained minor peaks which were not completely separated from the major species. This was particularly evident in Fig. 2 where minor peaks began to emerge between IIIA and IIIB. Further experiments are required to prove unequivocally whether these peaks between IIIA and IIIB represent authentic species of RNA polymerase III. Nevertheless, the heterogeneity of this enzyme species was evident for several other reasons. For example, in another set of experiments, the sequence of chromatography was

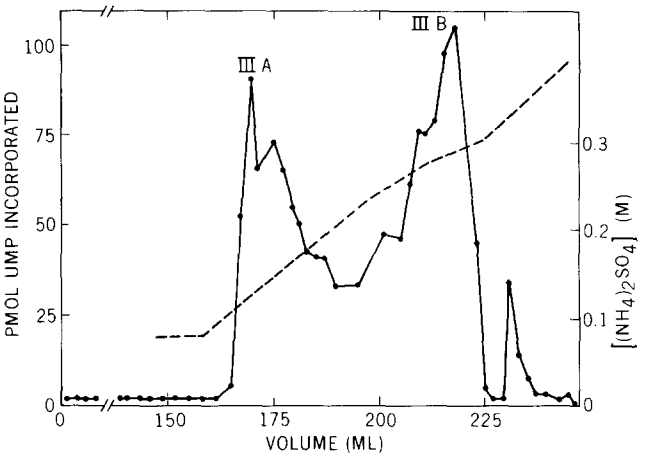

Figure 2. Rechromatography of pooled peaks IIIA and IIIB on DEAE-Sephadex column.

The dialysate containing IIIA and IIIB enzyme activity obtained as described in the legend to Fig. 1 was diluted to 25% glycerol with TMED buffer and applied to a 1.8 x 12.5 cm DEAE-Sephadex column equilibrated as in Fig. 1. The column was then washed with 1.5 column volumes of 100 mM $(NH_4)_2SO_4$ in TGMED and the enzymes were eluted with a linear gradient (3 column volumes) of 120 to 550 mM $(NH_4)_2SO_4$ in TMED buffer. One ml fractions were collected and 35 µl aliquots were analyzed for polymerase activity in the presence of 0.5 µg/ml α-amanitin, 30 µg/ml poly [d(A-T)] as template and 10 µM [^{3}H]UTP (specific activity 70 x 10^3 cpm/nmole).

altered to see whether similar heterogeneity was obtained. Thus the fractions corresponding to RNA polymerases II and III from the first DEAE-Sephadex chromatography were pooled and subjected to CM-Sephadex chromatography as described previously (10). RNA polymerase II was eluted in the wash fractions of this column whereas RNA polymerase III was retained and eluted at 0.07 M $(NH_4)_2SO_4$. The CM-Sephadex column fractions were then subjected to DEAE-Sephadex chromatography. Again, the heterogeneity of RNA polymerase III was observed (Fig. 3).

It may be argued that the heterogeneity of RNA polymerase III is an artifact of the assay or is induced by proteolytic conversion of the enzyme into different forms during several chromatographic fractionations. Such a possibility seems unlikely for the following reasons: (a) phenylmethylsulfonylfluoride,

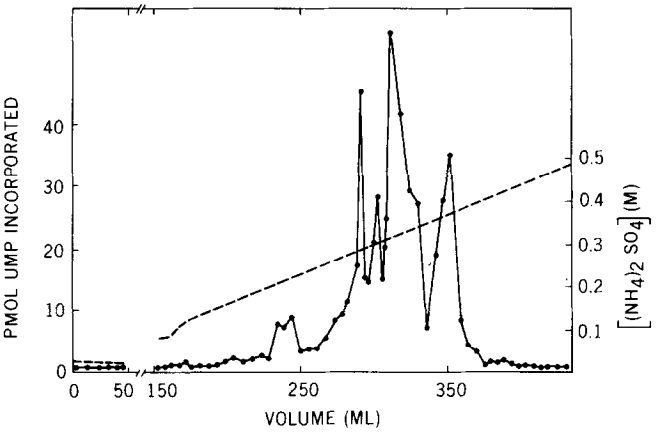

Figure 3. Rechromatography of RNA polymerase III obtained by chromatography on CM-Sephadex.

RNA polymerases II and III were obtained from the initial DEAE-Sephadex column by eluting with 0.5 M $(NH_4)_2SO_4$ as described in Methods. The fractions containing enzyme activity were dialyzed and subjected to CM-Sephadex (A-25) chromatography essentially as described previously (10). RNA polymerase III was retained on CM-Sephadex and eluted as a single peak at 0.07 M $(NH_4)_2SO_4$. Fractions containing enzyme III activity were pooled, dialyzed and subjected to rechromatography on a DEAE-Sephadex column (1.5 x 12.5 cm) equilibrated in TGMED buffer with 30 mM $(NH_4)_2SO_4$ and using a linear gradient of 50 mM to 600 mM $(NH_4)_2SO_4$. Aliquots were taken to assay as described in the legend to Figure 2.

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a potent protease inhibitor, was included during enzyme extraction. (b) RNA polymerase II which eluted together with polymerase III in the initial DEAE-Sephadex chromatography was not resolved to more than two distinct subspecies, IIA and IIB, after several chromatographic fractionations (data not shown). Analysis of RNA polymerase I under similar conditions also produced only two peaks corresponding to IA and IB either in the presence of DNA and spermine or in the presence of poly[d(A-T)]. (c) Results similar to those given in Fig. 3 were obtained after a single DEAE-Sephadex chromatography of the enzyme extracted from smaller amounts (20 gm) of rat liver or kidney and assayed with low levels of α-amanitin.

That IIIA, IIIB, and IIIC indeed represent RNA polymerase III was confirmed by their sensitivity to high levels of α-amanitin. A typical dose response curve reported for RNA polymerase III (3) was observed for all three

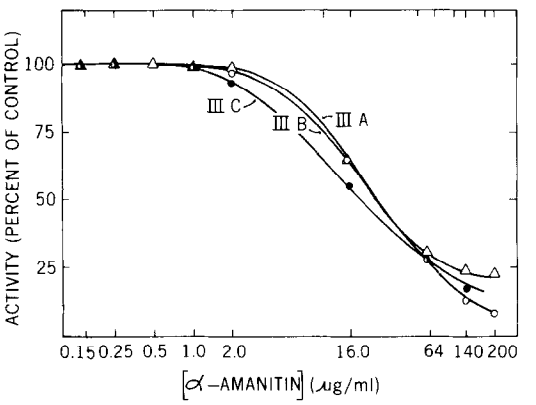

Figure 4. Sensitivity of RNA polymerases IIIA, IIIB and IIIC to α-amanitin.

Purified RNA polymerases IIIA, IIIB and IIIC were obtained by chromatography on DEAE-Sephadex as described in the legends to Figs. 1 and 2. Thus, enzyme IIIC was resolved after the second DEAE-Sephadex chromatography while enzymes IIIA and IIIB were only separated after an additional chromatographic step. RNA polymerases IIIA, IIIB, and IIIC were dialyzed separately as described in the legend to Fig. 1 and each form of the enzyme was then subjected to subsequent CM-Sephadex chromatography using a linear gradient of 30 to 250 mM $(NH_4)_2SO_4$ (6). Aliquots (35 µl) were taken to assay as described in the legend to Fig. 2. Fractions containing enzyme III activity were pooled and dialyzed. The sensitivity of the purified enzyme preparations to varying concentrations of α-amanitin was determined in the presence of calf thymus DNA (133 µg/ml), 80 mM $(NH_4)_2SO_4$ and 30 µM [^{3}H]UTP (specific acitvity 90 x 10^3 cpm/nmole).

enzymes, 50% inhibition being achieved at approximately 20 µg/ml of the toxin (Fig. 4). It should also be pointed out that the specific activities of the RNA polymerases at this stage of purification were comparable to those reported for class III enzymes.

The detection of the heterogeneity of RNA polymerase III was made possible by the use of either poly [d(A-T)] or highly polymerized DNA in the presence of spermine as templates (4). The enzyme activity was stimulated several fold under these conditions. In addition, the collection of small volume fractions facilitated detection of the subspecies of the enzyme.

The heterogeneous nature of RNA polymerase III is certainly consistent with the unusually large number of RNA species synthesized by this class of enzyme. In addition to 5S and transfer RNA, this enzyme also appears to be involved in the synthesis of other low molecular weight RNAs in the nuclei (15).

Since the nucleus contains as many as 11 discrete species of such RNAs (16-18) and if each of the molecules is synthesized by a distinct RNA polymerase, it is conceivable that class III RNA polymerase can in fact exist as multiple species.

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