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PARTIAL PURIFICATION OF DNA-DEPENDENT RNA POLYMERASES FROM SMALL DENSE NUCLEI OF MOUSE BRAIN

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It has been suggested that RNA polymerase plays an important role at the transcriptional level, and that it is also the site of action for many therapeutic and toxic agents (1). The enzymes from many eukaryotic organisms have been studied (2). RNA polymerases have been purified from several mammalian tissues, e.g. calf thymus (3), liver (4) and mouse myeloma (5). Multiple forms of the RNA polymerases have been detected in many of the systems studied. Very little work has been done on this enzyme from brain tissue. However, the brain RNA polymerases have been shown to be affected by the administration of both opiates and ethanol (6). This study describes the isolation and purification of the three RNA polymerases from mouse brain small dense nuclei.

ICR mice (male, 20-25 g) from Simonsen Laboratories, Gilroy, CA, were sacrificed by decapitation. Small dense nuclei were purified from whole brain according to the method described by Oguri et al. (7). Seventy-five mice were usually used in each preparation, being a total of approximately 30 g of brain, wet weight.

The RNA polymerase assay was the same as previously described (8). The assay medium contained: Tris-HCl, 100 mM (pH 7.9, 37°); $(NH_4)_2SO_4$, 35 mM; dithiothreitol (DTT), 1 mM; EDTA, 1 mM; $MgCl_2$, 4 mM; $MnCl_2$, 2 mM; bentonite, 20 μg/ml; ATP, GTP and CTP, 1 mM each; UTP, 0.01 mM; [^{3}H]-UTP, 15 μCi (sp. act. 27.3 mCi/m-mole, New England Nuclear Co., Boston, MA); DNA, 50 μg (calf thymus, Sigma Co., St. Louis, MO); enzyme fraction and water to make up the total volume of 0.5 ml. The reaction mixture was incubated at 37° for 30 min and stopped by addition of 4 ml of 10% TCA-PPi (trichloroacetic acid + 3% sodium pyrophosphate). Bovine serum albumin, 0.15 mg, was added to the mixture and allowed to stand in ice for 15 min. The precipitate was washed on a GFC filter (Whatman Inc., Clifton, NJ) with 8 ml of 5% TCA-PPi five times and rinsed with cold 95% ethanol. The samples were counted in 9 ml Scintiverse (Fisher Scientific, Fairlawn, NJ) LSC mixture in a Beckman LS-100 scintillation counter. One unit of enzyme activity equalled 1 pmole UTP incoporated into TCA-insoluble material in 30 min.

RNA polymerase was solubilized by modification of procedures described by Scwartz et al. (5). The pellet of small dense nuclei was resuspended in 0.1 ml TEMDG (25) (Tris-HCl, 50 mM, pH 7.9, at 4°; EDTA, 1 mM; $MgCl_2$, 4 mM; DTT, 1 mM; glycerol 25%, v/v) buffer per equivalent mouse brain. The suspension was adjusted to 0.32 M ammonium sulfate, pH 7.9. This suspension was then sonicated by using a Branson model W140D Sonifier (microtip, setting 5) for 15-sec intervals until the suspension was no longer clouded and nearly free-flowing (F2). The suspension was then centrifuged in a Beckman SW 65 rotor at 220,000 g for 50 min. The supernatant fraction (F3) was concentrated to 3.0 ml in a Schleicher and Schuell model 100 collodion bag (molecular weight exclusion 25,000) with aspiration. This concentrated solution (F4) was dialyzed against 100 vol. TEMDG (25) in 30 mM ammonium sulfate. The dialysate was changed four times, every 30 min.

DEAE-Sephadex (A-25) was suspended in TEMDG (25) buffer in 1 M ammonium sulfate and washed with 10 vol. TEMDG (25) in 30 mM ammonium sulfate. The dialyzed F5 solution was applied to the DEAE-Sephadex column at about 10 μg protein/ml bed volume. The usual column volume for a preparation of seventy-five mice was 9 ml, the column being 0.9 cm in diameter with a bed height of 14 cm. The column was then washed with two column volumes of TEMDG (25) in 30 mM $(NH_4)_2 SO_4$. The RNA polymerase activities were eluted by ammonium sulfate concentrations increased in steps: two column volumes each of TEMDG (25) in 225 mM $(NH_4)_2SO_4$, TEMDG (25) in 275 mM $(NH_4)_2SO_4$, and TEMDG (25) in 1 M $(NH_4)_2SO_4$ were applied. Fractions equal to one-third the column volume were collected. These fractions (as well as F1 to F5) were dialyzed four times against 10 volumes of TEMDG (25), and the dialysate was changed every 30 min. These dialyzed fractions were then assayed for RNA polymerase activity. All the purification procedures were performed at 0-4°.

Protein concentrations were determined according to the method of Lowry et al. (9) after the samples were dialyzed against distilled water. DNA concentrations were determined by the diphenylamine assay of Leyva and Kelley (10).

As reported by Oguri et al. (7), the nuclear population obtained by this procedure showed a 97 per cent homogeneity by morphology. The nuclei were small, dense, and darkly staining. These nuclei were identified by the criteria of Austoker et al. (11) as being primarily oligodendroglial in their origin. The RNA polymerases were solubilized and partially purified from this nuclear population. The results of the solubilization and purification are summarized in Table 1. In each assay, a limited amount of the fraction was used in order to insure linearity of enzyme activity.

The percentage yield of nuclei from the total brain homogenate is low, a recovery of 10 per cent of total brain homogenate DNA (8). Thus, the initial fractionation and sonication (F2) resulted in an activity equal to 15 per cent of that from total brain homogenate (F1).

Table 1. Purification of RNA polymerase from small dense nuclei of mouse brain*

Fraction	Total activity (units)	Total protein (mg)	Specific activity (units/mg protein)	Purification factor	Activity recovery (%)
F1	8298	2630	3.16	1	100
F2	1286	25.8	49.8	16	15
F3	859	18.3	46.9	15	10
F4	320	4.2	76.2	24	4
DEAE-Sephadex	234	3.5			3
Peak 1	67	0.77	147+	47	< 1
Peak 2	80	0.47	373+	118	1
Peak 3	56	1.73	85+	27	< 1

*Experimental details of fraction preparation and designation are described in the text.
+Specific activities of peaks 1, 2, and 3 represent fractions of maximum specific activity within each peak.

The ammonium sulfate elution profile on a DEAE-Sephadex column is shown in Fig. 1. Three enzymatic activities, referred to as peak 1, peak 2 and peak 3, were detected with different ammonium sulfate concentrations, 0.225, 0.275 and 1 M respectively. Among the

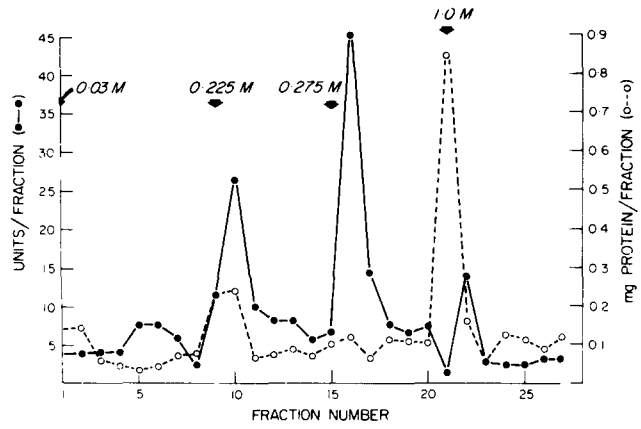

Fig. 1. DEAE-Sephadex A-25 elution profile of small dense nuclear RNA polymerases. Elution was by $(NH_4)_2SO_4$, pH 7.9. Enzyme was applied at 0.03 M $(NH_4)_2SO_4$, and elution was by increasing steps of $(NH_4)_2SO_4$ concentration. Arrows indicate first fractions containing $(NH_4)_2SO_4$ at the concentrations indicated. Fraction volumes of one-third column volume were collected and assayed for enzyme activity (●—●) and protein concentration (o · o) as described.

distinguishing properties of the three RNA polymerases typical of eukaryotes are their elution profiles from DEAE-Sephadex and their α-amanitin sensitivities (12). The activity in peak 2 was most sensitive to α-amanitin (Table 2). In the presence of a low concentration of α-amanitin, 10 ng/ml, the activity was reduced to 29.5 per cent of the control. Higher concentration of the toxin, 5 μg/ml, inhibited virtually all the enzyme activity. However,

Table 2. Sensitivities of RNA polymerases of DEAE-Sephadex peak fractions to α-amanitin

Peak	% of control of remaining activities	
	+ α-Amanitin (10 ng/ml)	+ α-Amanitin (5 μg/ml)
1	73.5	36.0
2	29.5	4.5
3	76.5	45.5

the α-amanitin inhibitions for peak 1 and peak 3 were not so clear. α-Amanitin, at 10 ng/ml, inhibited both peaks to about the same degree. When the α-amanitin concentration was increased to 5 μg/ml, the activities for peak 1 and peak 3 were inhibited to 36 and 45.5 per cent of control respectively. It was, therefore, difficult to identify peak 1 and peak 3 according to α-amanitin sensitivity. Peak 2 was most sensitive to α-amanitin and, thus, it appears that peak 2 represents the RNA polymerase responsible for HnRNA.

The RNA polymerase activities in peaks 2 and 3 depended on the addition of exogenous DNA (Table 3). Without exogenous DNA added, there was essentially no UTP incorporation. However, peak 1 showed only partial dependence on additional DNA. This is likely due to the contamination of this fraction by endogenous DNA. The peak 1 fraction contained DNA at 69 μg/ml, while peaks 2 and 3 contained 14 and 21 μg/ml respectively.

As previously reported (8), ribonuclease contamination of the nuclear preparation was insignificant. When similarly examined, ribonuclease contaminations of each of the three peaks were also found to be insignificant.

We have found that RNA polymerase in the nuclear fraction can be assayed in the presence of 0.35 M ammonium sulfate. As previously reported (8), addition of calf thymus DNA to the nuclear fraction did not affect the RNA polymerase activity in 0.35 M ammonium sulfate. However, in the presence of 0.035 M ammonium sulfate, addition of exogenous DNA resulted in a four-fold increase in specific activity. Hyman and Davidson (13), using *Escherichia coli* RNA polymerase, reported that the initiation process did not occur in the high salt condition.

We have found that there is no measurable activity in soluble fractions assayed in high salt. For this reason, our enzymes were routinely assayed in the presence of low ammonium sulfate.

Table 3. DNA dependencies of RNA polymerase fractions from DEAE-Sephadex chromatography

Peak	Specific activity (units/mg protein)	
	Without exogenous DNA	With 100 μg/ml calf thymus DNA
1	21.3	50.9
2	3.4	239
3	0	64.8

The fractionation of RNA polymerases by the methods reported in this study yielded three RNA polymerase fractions. The degrees of purification relative to whole brain homogenate were 47-, 118- and 27-fold respectively. We believe that this is the first study reporting the solubilization and partial purification of three RNA polymerases from brain small dense nuclei. Further studies are in progress to characterize these three enzymes. Recent work in this laboratory (14) has shown differences in the activities of two enzyme systems, protein kinase and histone methyl transferase, studied in oligodendroglial-rich and neuronal mixed nuclear preparations from mouse brain. In both nuclear preparations, part of these activities are tightly bound to the chromatin. Austoker et al. (11) have reported that RNA synthesis in oligodendroglial nuclei is different from that in neuronal and astroglial nuclei. We will attempt to isolate and purify the RNA polymerases from other brain cell types and to investigate further the differences in RNA synthesis.

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