A PARTICULATE DNA POLYMERASE ACTIVITY IN ADULT RAT BRAIN

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SUMMARY: A particulate fraction of adult rat brain (sucrose buoyant density 1.24 gm/ml) catalyzed the incorporation of [^{3}H]dTTP into an acid-insoluble product in an endogenously templated reaction sensitive to ribonuclease pretreatment. Upon fractionation, this activity was identified in the cerebellum, pons, frontal lobes and base. The DNA polymerase present in these brain fractions exhibited a strong preference for the synthetic template dT_{12-18}·poly rA rather than dT_{12-18}·poly dA; dT_{10} was completely inactive. Purification and equilibrium Cs_2SO_4 gradient centrifugation of the [^{3}H]DNA product-endogenous template complex suggested that RNA was serving as primer for endogenous DNA synthesis.

The adult rat brain is composed largely of non-dividing cells which do not synthesize DNA (1). An enzyme analogous to DNA polymerase α has been detected only during rat brain growth; no activity was present in the adult brain (2). A second enzyme, resembling DNA polymerase β, has been located tightly bound to the nuclear chromatin in mature rat brain (3). We now report on the presence and distribution of an additional DNA polymerase, located in a particulate and probably cytoplasmic fraction within mature rat brain, that synthesizes DNA in an RNA-primed endogenous reaction.

MATERIALS AND METHODS:

Isolation of particulate fraction. This was a modification of our procedure used to isolate this fraction from human spleen (4). Individual adult Wistar rats (290-420 gm) were asphyxiated with ether and the brain quickly removed. All further manipulations were at 0°C. Following rinsing in 0.01 M N-tris[hydroxymethyl]methyl-2-aminoethane sulfonic acid (TES) pH 7.4, 0.25 M sucrose, 3 mM $MgCl_2$, 12 mM thioglycerol (TMS buffer), the brain, or its various fractions, were minced, 5 vol TMS buffer added and the cells broken with 10 strokes of a Potter-Elvehjem homogenizer. This resulted in rupture of about 80% of the cells while nuclei remained intact, as judged by phase contrast microscopy. The percentage of ruptured nuclei, although small, was not established*. The mixture was centrifuged at 1,500 xg and 16,000 xg to sediment nuclei and mitochondria, respectively. The resultant supernatant was centrifuged at 165,000 xg for 60

*Although it appears likely that we are dealing with a post-nuclear cytoplasmic fraction, we are cognizant of the fact that localization of DNA polymerase activity based on analysis of cell lysates is not foolproof (see 5). Until such time as nuclear rupture or leakage can be quantitatively controlled, the location of this enzyme in brain cells can only be tentatively surmised.

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min to obtain a microsomal pellet fraction. This pellet was resuspended in 1 ml TMS buffer containing 0.8 M KCl and layered on a discontinuous sucrose gradient consisting of 2 ml 50% (w/w) sucrose and 2 ml 25% (w/w) sucrose, both in TMS plus 0.8 M KCl. Following centrifugation in a swinging bucket rotor at 165,000 xg for 90 min, the material at the 25%-50% sucrose interface was carefully removed, diluted to 5 ml with TMS buffer and pelleted by centrifugation at 165,000 xg for 60 min.

Sucrose gradient centrifugation. The cytoplasmic particulate pellet was suspended in 0.5 ml 0.01 M Tris-HCl, pH 7.5, 0.02 M dithiothreitol, 0.002 M EDTA, layered on a 4.8 ml 15-60% (w/w) sucrose gradient in the same buffer, and centrifuged for 16 hr at 165,000 xg in a swinging bucket rotor. Four drop fractions were collected by puncturing the bottom of the tube, density determined from refractive indices, and aliquots assayed for DNA polymerase activity.

DNA polymerase assay. The reaction mixture for endogenous DNA polymerase activity contained in a final vol of 0.125 ml: 40 nmoles each dATP, dCTP and dGTP, 1.875 µmoles $MgCl_2$, 0.008% Triton X-100, 1.625 µmoles Tris-HCl, pH 7.5, 0.5 µmoles dithiothreitol and 4 nmoles dTTP plus 5 µCi [^{3}H]dTTP (42 Ci/mmole) to give a final specific activity of 801 cpm/pmole. Synthetic templated reactions contained in 0.125 ml: 16 nmoles dATP, 1.875 moles $MgCl_2$, 0.4 µg synthetic template, 0.024% Triton X-100, 2.65 µmoles Tri-HCl, pH 7.5, 0.5 µmoles dithiotreitol and 2 nmoles dTTP plus 5 µCi [^{3}H]dTTP (42 Ci/mmole) to give a final specific activity of 1550 cpm/pmole. All reactions were incubated at 37°C for the times specified, the reactions terminated by the addition of 2 ml ice-cold 5% trichloroacetic acid plus 1% sodium pyrophosphate and acid-precipitable radioactivity determined (6). Incubation of the fractions with pancreatic ribonuclease A prior to assaying for endogenous DNA polymerase activity was as described previously (6).

Characterization of endogenous product-template complex. The peak fractions of endogenous DNA polymerase activity from two successive sucrose bandings were pelleted by centrifugation at 165,000 xg for 60 min, resuspended in 0.2 ml 0.05 M Tris-HCl, pH 7.5, 0.01 M dithiothreitol and added to 0.3 ml of the following reaction mixture: 0.8 µmoles each dATP, dCTP, dGTP and dTTP, 7.5 µmoles $MgCl_2$, 0.013% Triton X-100, 50 µg actinomycin D, 8.8 µmoles Tris-HCl, pH 7.5, plus 100 µCi each [^{3}H]dTTP (42 Ci/mmole) and [^{3}H]dGTP (12 Ci/mmole). Following incubation at 37°C for the specified times, the reaction was terminated by the sequential addition of 1% sodium dodecyl sulfate, 0.4 M NaCl and 100 µg calf thymus DNA. An equal vol of a 1:1 mixture of Tris-HCl (pH 7.5) equilibrated phenol: $CHCl_3$ plus 1% isoamylalcohol was then added, the mixture shaken gently, and nucleic acid precipitated from the resulting aqueous phase with 3 vol cold 95% ethanol. The nucleic acids were collected by centrifugation, residual ethanol removed with a gentle stream of N_2, the pellet resuspended in 0.01 M Tris-HCl, pH 7.5, 0.01 M NaCl and centrifuged to equilibrium in Cs_2SO_4 gradients, as described previously (7).

Materials. [Methyl-^{3}H]dTTP and [8-^{3}H]dGTP were purchased from New England Nuclear, Boston, Mass. The synthetic templates dT_{10}, dT_{12-18}·poly rA and dT_{12-18}·poly dA were from P-L Biochemicals, Milwaukee, Wisconsin. Unlabeled deoxyribonucleoside triphosphates were produced by Calbiochem, San Diego, California. Pancreatic ribonuclease A, from Miles Laboratory, Elkhart, Indiana was heated (2 mg/ml) at 100°C for 10 min prior to use.

RESULTS: Endogenous DNA polymerase activity in whole brain. The post-nuclear particulate fraction, obtained from the brain of a single rat as described in Materials and Methods, was centrifuged to equilibrium, fractionated and assayed for endogenous DNA polymerase activity in a 15 min reaction (Fig. 1, top). The

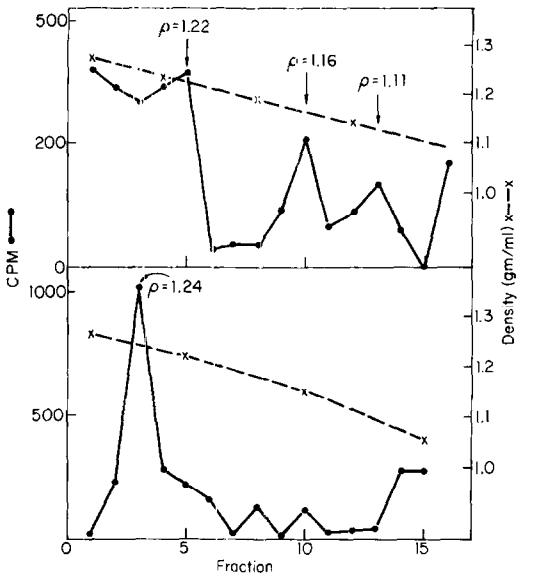

Fig. 1 - Sucrose density gradient centrifugation of brain cytoplasmic particulate fraction. Top: The cytoplasmic particulate fraction was purified from a single rat brain (see Materials and Methods), centrifuged to equilibrium in a 15-60% sucrose gradient, fractionated, and assayed for endogenous DNA polymerase activity. Bottom: Fractions 3-5 of the above gradient were pooled, centrifuged at 165,000 xg for 60 min, the pellet resuspended and subjected to a second equilibrium sucrose centrifugation and assay.

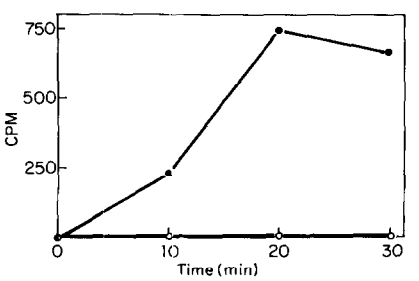

Fig. 2 - Kinetics plus ribonuclease sensitivity of the endogenous reaction. The active fractions from two consecutive sucrose gradients, as in Fig. 1, were pooled, pelleted and resuspended in 0.05 M Tris-HCl, pH 7.5 containing 0.02 M dithiothreitol and 0.03% Triton X-100. The sample was divided into two equal parts; one part received 100 µg/ml ribonuclease A (0-0) while an equivalent volume of Tris-HCl was added to the remaining sample (0-0). Following 30 min at 22°C, components of the endogenous DNA polymerase assay mixture were added, the samples placed at 37°C and aliquots removed at timed intervals for determination of acid-insoluble radioactivity.

Table 1 - Endogenous DNA polymerase activity of 1.20-1.25 g/ml sucrose buoyant density fractions obtained from various rat brain compartments

Brain compartment[a]	Activity[b]	
	pmols/fraction	pmols/gm tissue
Whole brain	4.1	2.1
Cerebellum	0.8	2.9
Pons	4.0	13.8
Frontal lobes	1.1	1.1
Base	1.1	1.9

[a]Single rat brains were processed as described in Methods
[b]Activity was measured by the incorporation of [^{3}H]dTTP into an acid-insoluble product formed in a 15-min reaction

majority of the activity was exhibited as a broad peak in the bottom one-third of the gradient. When these fractions were pooled, concentrated by centrifugation at 165,000 xg for 60 min, and the pellet resuspended and rebanded in a second sucrose gradient, a sharp peak of activity was observed at a density of 1.24 gm/ml (Fig. 1, bottom). The kinetics of this reaction is shown in Fig. 2. Incorporation proceeded for 20 min before reaching a plateau value. Preincubation of the 1.24 gm/ml density fraction with 100 µg/ml ribonuclease completely obliterated this activity (Fig. 2), suggesting the involvement of RNA in this DNA polymerase reaction.

Distribution of DNA polymerase activity in various brain compartments. Individual rat brains were dissected into cerebellum, pons, frontal lobes and base. The 1.20-1.25 gm/ml sucrose buoyant density region of particulate fractions from each compartment were assayed for endogenous DNA polymerase activity (Table 1). Each brain compartment appeared to possess this activity, with the pons exhibiting the highest specific activity. The polymerase activities in the 1.22 gm/ml gradient density peak fractions from pons, base and cerebellum were further characterized by the use of synthetic templates. Each of the enzyme fractions preferred dT_{12-18}·poly rA over dT_{12-18}·poly dA as template and

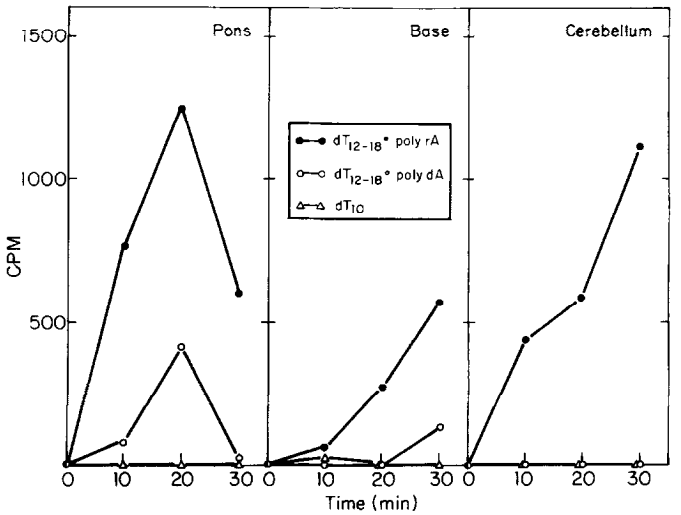

Fig. 3 - Synthetic template utilization by the cytoplasmic particulate DNA polymerase of different areas of the brain. A single rat brain was dissected into pons, base and cerebellum fractions, the cytoplasmic particulate fraction of each obtained and centrifuged to equilibrium in 15-60% sucrose density gradients. The ability of the fractions with a density of 1.22 gm/ml to utilize dT_{12-18}·poly rA (●-●), dT_{12-18}·poly dA (O-O) or dT_{10} (Δ-Δ) as templates for the polymerization of [^{3}H]dTTP was assayed, as described in Materials and Methods.

was unable to function with only dT_{10} (Fig. 3). The inability of the fractions to catalyze polymerization when two deoxyribonucleoside triphosphates (dATP and dTTP) were present in the reaction mixtures rules out the possibility that what we were measuring was a terminal transferase-like activity and instead strongly suggests a DNA polymerase-type reaction. Among the cellular polymerases, a preference for the oligodeoxyribonucleotide:polyribonucleotide template is characteristic of DNA polymerase γ (8-10).

Product-template characterization. The twice-banded activities from whole brains of each of two rats were utilized in large scale endogenous reactions of 7 and 15 min, respectively; the [^{3}H]DNA product-template complexes purified and both centrifuged to equilibrium in Cs_2SO_4 gradients. Results are summarized in Fig. 4. Almost all of the radioactivity from the 7-min reaction was located at the Cs_2SO_4 density of RNA (1.62 gm/ml), while the 15-min product was identifiable

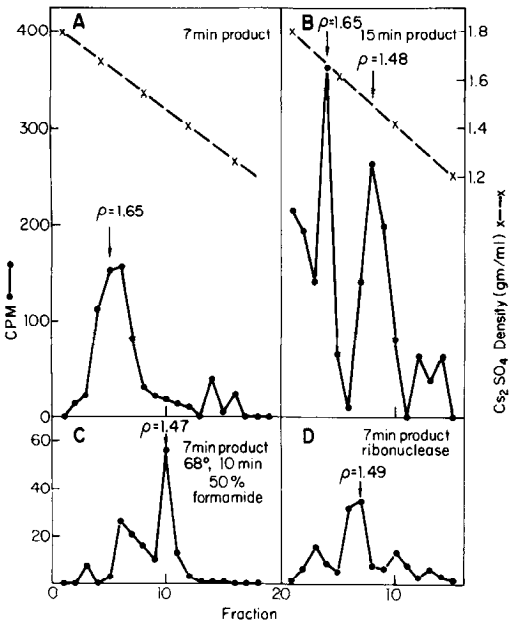

Fig. 4 - Cs_2SO_4 density gradient analysis of the DNA product from brain cytoplasmic particulate endogenous reaction. [^{3}H]DNA products were prepared and purified from the cytoplasmic particulate 7-min and 15-min endogenous reactions using enzyme preparations from two adult rat brains, as described in Materials and methods. A) The 7-min DNA product was centrifuged to equilibrium in Cs_2SO_4 gradients (SW 56 rotor, 36,000 rev/min, 65 hr, 15°C), fractionated, and acid-insoluble radioactivity determined. B) The 15-min DNA product of an endogenous reaction mixture identical in size to the 7-min reaction was treated as in A. C) The purified 7-min DNA product was diluted with an equal vol of formamide, heated at 68°C for 10 min, quickly cooled and processed as in A. D) The purified 7-min DNA product was incubated at 37°C for 30 min in the presence of 100 µg/ml ribonuclease A prior to banding as in A.

in two very distinct almost equal-size peaks at the densities characteristic of RNA and DNA (1.48 gm/ml) respectively. Subjecting the 7-min product-template complex to heating or ribonuclease prior to banding resulted in the solubilization of 70-80% of the radioactivity. The remaining [^{3}H]DNA product yielded a wide density profile with the appearance of a prominent peak at the density of DNA. No activity was identifiable when the [^{3}H]DNA product was treated with 5 µg deoxyribonuclease plus 5 mM Mg^{++} for 30-min at 37°C (data not shown). We interpret these results as indicating that by 7-min endogenous DNA synthesis is initiated by the polymerization of very short DNA chains covalently attached

to an RNA primer. The appearance of a very prominent DNA peak by 15 min suggests that longer stretches of DNA are synthesized by this fraction. Further experimentation is required before a rigorous conclusion can be reached regarding the identity of the endogenous template.

DISCUSSION: An enzyme with the synthetic template capabilities of DNA polymerase γ, located in a particulate fraction of rat brain, has been shown to utilize an RNA primer for the endogenous synthesis of DNA. RNA has been shown to be the requisite primer for bacteriophage (11), bacterial (12), and polyoma virus (13) DNA synthesis; a similar role in mammalian DNA replication is probable (14, 15) but not definitively established. Chargaff and co-workers have suggested that since DNA polymerase is primer-dependent while RNA polymerase is primer-independent and produces primers hydrogen-bonded to DNA that the involvement of RNA in DNA synthesis may be a general phenomenon (16).

In a survey of adult rat tissues (brain, spleen, kidney, bladder, prostate, seminal vesicles, testicle, lymph nodes, pancreas), only the brain and prostate gland were found to contain post-nuclear particulate ribonuclease-sensitive endogenous DNA polymerase activity (S.S. Witkin, W. Whitmore, M. Lorenc, unpublished). Like the brain, DNA synthesis in normal rat prostate is extremely low but the activity of DNA polymerase γ is strikingly high (17). The presence of this enzyme only in fractions of specific tissues would argue against its role as a generalized DNA repair enzyme and would instead suggest some organ-specific function, such as selective gene amplification. An enzyme similar in properties to that reported here, but regarded as an RNA-instructed DNA polymerase by virtue of its ability to transcribe RNA, has been loated in the brains of asymptomatic and neurologically impaired persons from the island of Guam (18). Although DNA polymerase γ has not been shown to transcribe natural RNA into DNA *in vitro* (19), this does not negate that it may well do so *in vivo* under as yet undefined reaction conditions. The biochemical distinction between DNA polymerase γ and RNA-instructed DNA polymerase (reverse transcriptase) may thus be more artifactual than real.

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