RNA-INSTRUCTED DNA POLYMERASE ACTIVITY IN A CYTOPLASMIC PARTICULATE FRACTION IN BRAINS FROM GUAMANIAN PATIENTS*

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Viruses with characteristics of the RNA tumor virus group (oncornaviruses) cause spontaneous chronic neurological disease in two species. Stansly (1) originally observed a paralytic disorder in mice inoculated with a lymphoma-inducing "C"-type virus. Recently, a neurotropic C-type virus has been isolated from wild mice with a high incidence of lower motor neuron disease (2). This isolate induces a neurological disorder in inoculated mice identical to the spontaneously occurring disease (3). Visna virus, the etiological agent of a slowly progressive meningoencephalitis in sheep, has been the most extensively studied neurotropic C-type virus. The physical and biochemical properties that visna virus shares with oncornaviruses include C-type ultrastructure and mode of replication (4, 5), 70S RNA genome-containing polyadenylic acid tracts (6, 7), and RNA-instructed DNA polymerase (RIDP)[1] (8, 9).

Because of the neurotropic potential of certain oncornaviruses in animals, we have examined central nervous system tissues from patients with idiopathic neurological diseases for evidence of oncornavirus infection. Specifically, RIDP activity was sought in a cytoplasmic particulate fraction having the physical properties of RNA tumor viruses. The methodology used was a modification of that employed in this laboratory to detect biochemical evidence of oncornavirus infection from relatively crude preparations from animal and human tumors (10, 11).

Of particular interest in this study was the examination of brains from patients with amyotrophic lateral sclerosis (ALS) and Parkinsonism-dementia (PD),

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[1] *Abbreviations used in this paper:* ALS, amyotrophic lateral sclerosis, GP, Guamanian patient; NES buffer, 0.05 M Na acetate (pH 5.0) made to 10 mM EDTA and 0.5% SDS; NP-40, Nonidet P-40, PD, Parkinsonism-dementia; RIDP, RNA-instructed DNA polymerase; SDS, sodium dodecyl sulfate; TNE, 0.01 M Tris-HCl (pH 8.3), 0.15 M NaCl, and 0.01 M EDTA.

which are endemic on the island of Guam. These diseases have existed on Guam for centuries and have been under intensive study by the Epidemiology Branch of the National Institute of Neurological Diseases and Stroke (NINDS) since 1956. The epidemiological pattern of this disease complex has been reviewed recently (12, 13). Approximately 30% of adult male and 15% of adult female native Guamanians (Chamorros) are afflicted with one of these fatal neurological disorders. Pertinent to the present study, almost half of the adult Chamorro population who die without symptoms of ALS or PD have neurofibrillary tangles and Hirano bodies that characterize PD and are present in most ALS patients (14).

Materials and Methods

Procurement of Specimens. Tissues were obtained at autopsy by members of the NINDS Research Center in Agana, Guam. All specimens were obtained within 8 h of death, immediately stored at −70°C, and shipped periodically to the U. S. stored in dry ice. Specimens used in this study were stored from 3–12 mo.

Preparation of DNA Polymerase-Containing Cytoplasmic Fraction from Human Brain. 5–10 g of brain or spinal cord were homogenized in 15 ml of 5% sucrose in 0.01 M Tris-HCl (pH 8.3), 0.15 M NaCl, and 0.01 M EDTA (TNE). The suspension was centrifuged at 5,000 *g* for 10 min to remove nuclei, followed by a 12,000 *g* centrifugation for 10 min at 4°C to remove mitochondria and large membrane fragments. The supernate was treated with 1 mg/ml trypsin for 30 min at 37°C to decrease nuclease activity and then inhibited with an equivalent amount of lima bean trypsin inhibitor. The sample was then layered on a 30% glycerol (in TNE) column (8 ml) on a 100% glycerol cushion (6 ml) and centrifuged at 25,000 rpm for 2 h at 4°C in an SW-27 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The particulate material on the glycerol cushion was removed, resuspended in TNE, layered on a 25-60% sucrose (in TNE) linear gradient, and centrifuged at 25,000 rpm at 4°C in an SW-27 rotor for 16 h. Fractions of the sucrose gradient having endogenous RNase-sensitive DNA polymerase activity were pooled, diluted with TNE, and centrifuged at 25,000 rpm at 4°C for 45 min in an SW-27 rotor. The resultant pellet was used as a source of DNA polymerase activity.

Detergent Treatment of Enzyme-Containing Fraction. Previous studies have shown that treatment of RNA tumor viruses with detergent will disrupt the virus and produce virion cores (15, 16). The virion core, containing RIDP, sediments to a denser region of a linear sucrose gradient, and thus can be differentiated from whole virion and membrane-associated DNA polymerase.

A brain specimen was fractionated as described above. The material on the 100% glycerol cushion was removed and divided into two samples. One sample was layered on a 25-60% sucrose gradient, centrifuged, and RIDP assayed from each fraction of the gradient. The other sample was diluted to a protein concentration of 10 mg/ml, 0.01 mg/ml polyvinyl sulfate added, and the sample was made to 0.1 M dithiothreitol. Sterox SL (lot Hf 133; Monsanto Co., St. Louis, Mo.), a polyoxyethelene alcohol nonionic surfactant, was added to a concentration of 2% and the sample was shaken for 15 min at 4°C (16). 1 ml cold ether was added, the sample shaken, and centrifuged for 15 min at 2,000 rpm. The ether was removed and the sample layered on a 25-60% sucrose (in TNE) gradient, centrifuged, and fractions assayed for RIDP activity.

Endogenous RIDP Reaction. A 50-μl sample containing enzyme was treated with 0.1% Nonidet P-40 (NP-40) (Shell Chemical Co., New York) and 25 μmol dithiothreitol for 10 min at 4°C. The detergent-treated sample was added to an endogenous reverse transcriptase reaction mixture (100 μl total vol) containing 6.25 μmol Tris-HCl (pH 8.3), 1 μmol $MgCl_2$, 1.25 μmol NaCl, and 0.2 μmol each dGTP, dATP, dCTP, and [^{3}H]dTTP (50 Ci/mmol). Actinomycin D (100 μg/ml), distamycin (50 μg/ml), and 20 μg oligo $dT_{12\text{-}18}$ were added. The reaction mixture was incubated for a specified time at 37°C and was terminated by adding 0.3 ml water, NaCl, and sodium dodecyl sulfate (SDS) to a final concentration of 0.4 M and 1%, respectively. The sample was made to 10% TCA, 10 μg yeast RNA added, and acid-precipitable radioactivity assayed as described previously (17). In kinetic experiments, 10-μl samples were removed from the reaction mixture and assayed as above.

Cesium Sulfate Gradient Analysis of [^{3}H]DNA Product. The product of a 5 min endogenous RIDP reaction was cleared of protein by phenol-cresol extraction. Unincorporated labeled triphos-

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phates were removed by passing the sample (1.5 ml) through a G-50 Sephadex (coarse) column (0.75 × 74 cm) and the DNA region pooled. Two volumes of ethanol and 20 μg of *Escherichia coli* DNA were added, and the solution was made to 0.4 M lithium chloride. The DNA was precipitated at −20°C for 16 h and centrifuged at 12,000 rpm for 45 min at −20°C. The pelleted [³H]DNA product was assayed by cesium sulfate gradient centrifugation by the method described previously (17). Separate samples of [³H]DNA product were alkali digested in 0.4 NaOH for 3 h at 37°C, heated to 68°C in 50% formamide, and treated with RNase A and RNase T_1 (20 μg/ml each) for 30 min at low salt concentration at 37°C before analysis by cesium sulfate centrifugation.

Purification of [³H]DNA Product. The product of a 10 min endogenous RIDP reaction was purified by phenol-cresol extraction, alkali hydrolysis of RNA, and Sephadex and hydroxyapatite chromatography as described previously (18).

RNA Extraction. RNA was extracted from the final enzyme-containing pellet by a modification of the method of Lee et al. (19). The pellet was suspended in 10 ml of 0.05 M Na acetate (pH 5.0) made to 10 mM EDTA and 0.5% SDS (NES buffer). An equal vol of phenol-chloroform (1:1) saturated with NES buffer was added, shaken vigorously at room temperature, chilled to 4°C, and the aqueous phase separated by centrifugation at 12,000 rpm for 10 min. The interface was re-extracted five times. Nucleic acid was precipitated by addition of two volumes of ethanol and lithium chloride to 0.4 M at −20°C for 16 h and centrifugation at 12,000 rpm for 45 min at −20°C. The pellet was dissolved in 0.01 M Tris-HCl (pH 7.0), 0.01 M NaCl, and 3 mM $MnCl_2$. 10 μg/ml RNase-free DNase was added and incubated at 25°C for 1 h. The protein was removed by phenol-cresol extraction and the RNA reprecipitated in ethanol.

Viral 70S RNA was extracted from purified virus by the method described previously (17). All RNA preparations had an OD_{260} to OD_{280} ratio of greater than 1.8.

RNA-[³H]DNA hybridization. Hybridization mixtures contained six OD_{260} units of cellular RNA, 500 cpm of [³H]DNA probe, 5 mM EDTA, and 0.4% SDS in 25 μl of 0.96 M phosphate buffer. The annealing reaction was performed in siliconized DNA-coated heat-sealed capillary pipettes and incubated at 68°C to a C_rt value of 5,000 (20). Viral RNA-[³H]DNA hybridization reactions were performed under the same conditions and carried to a C_rt value of 1.0. The melting profile of RNA-[³H]DNA hybrids was performed by hydroxyapatite chromatography as described previously (18). $T_{m(e)}$ represents the temperature at which one half of the RNA-DNA hybrids are eluted from the hydroxyapatite column.

Results

Endogenous RNase-Sensitive DNA Polymerase Activity from Brain Cytoplasmic Fractions. Actinomycin D-and distamycin-resistant endogenous DNA polymerase activity in fractions from an isopycnic sucrose gradient of the temporal lobe from a Guamanian patient (GP) with ALS (GP 168) is depicted in Fig. 1 *a*. There was a peak of RNase-sensitive DNA polymerase activity in fractions from 1.16-1.18. g/ml region of the gradient. This peak was not present in cytoplasmic fractions from two control brains from U. S residents with no evidence of neuropathology (Fig. 1 *b* and *c*). The peak fractions (nos. 17–19) of RNase-sensitive DNA polymerase activity from the ALS brain were pooled and subjected to two further isopycnic sucrose gradient centrifugations. There was a clearing of OD_{260} absorbing material from the gradient; however, the RNase-sensitive DNA polymerase activity persisted in the 1.16–1.18 g/ml region.

Another ALS brain specimen (GP 214) was fractionated as above; however, the material on the 100% glycerol cushion was treated with Sterox before being applied to the linear sucrose gradient, as described in the Materials and Methods. Fig. 2 shows the RNase-sensitive DNA polymerase activity from fractions of a linear sucrose gradient of a nontreated (*a*) and Sterox-treated (*b*) ALS brain specimen. Again there was a peak of RNase-sensitive DNA polymerase activity in the 1.17 g/ml region of the gradient of the untreated sample; the peak of DNA

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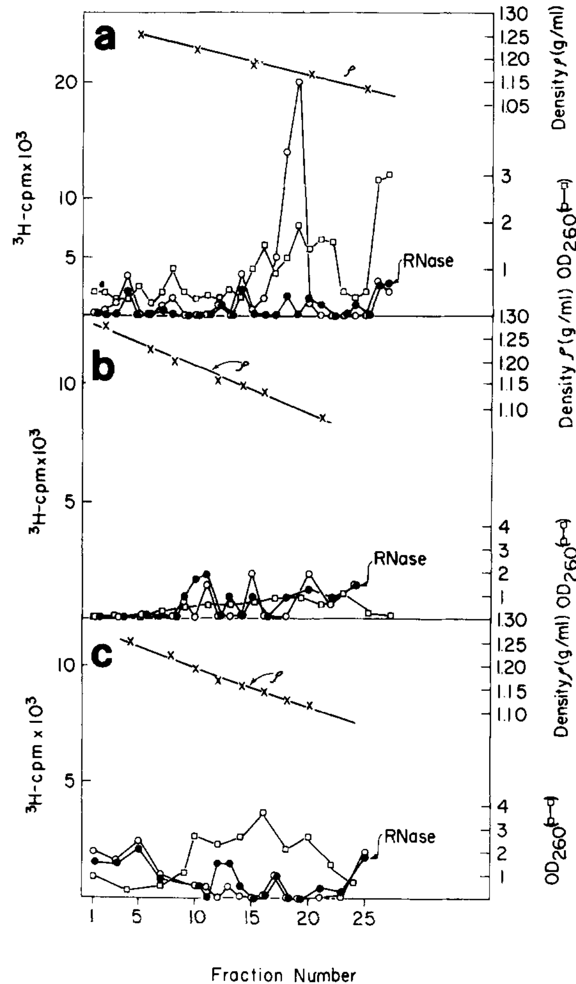

FIG. 1. Isopycnic sucrose gradient of cytoplasmic particulate fractions from (*a*) Guamanian ALS brain (GP 168) and (*b* and *c*) normal brains from two U. S. individuals. Endogenous DNA polymerase activity in 50-μl samples was assayed with (●—●) and without (O—O) prior incubation of gradient fraction with RNase. Reaction mixtures were as described in the Materials and Methods.

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polymerase activity in the Sterox-treated sample sediments to a denser (1.22-1.24 g/ml) region of the gradient. We were unable to alter the density of the particulate fraction containing the polymerase activity using phospholipase "C" (21) or the nonionic detergent NP-40 (16), which have been employed in other studies to produce cores from whole virions. Caution should be used in interpreting the results of Sterox treatment as unequivocal evidence for the presence of polymerase activity in a virion core structure. We have occasionally observed an increase in the buoyant density of membrane-bound DNA-dependent DNA polymerase activity after detergent treatment.

Requirements of Endogenous RIDP Reaction. The 1.16-1.18 g/ml region of a linear sucrose gradient from an ALS brain (GP 168) was pooled and centrifuged

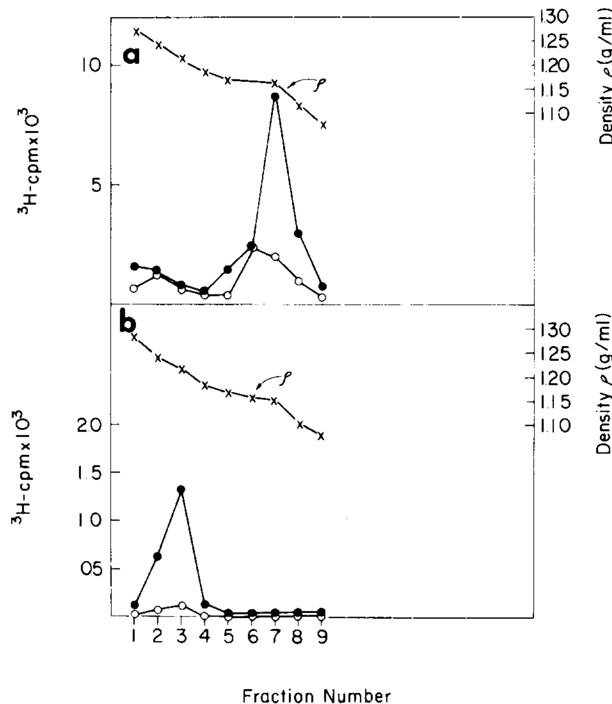

FIG. 2. Effect of Sterox treatment on endogenous DNA polymerase activity of cytoplasmic particulate fraction from Guamanian ALS brain (GP 214). Control (*a*) and Sterox-treated (*b*) samples were subjected to isopycnic sucrose gradient centrifugation. Each fraction was diluted on TNE and centrifuged for 1 h at 25,000 rpm at 4°C in an SW-27 rotor. The pellet was divided and one sample treated with RNase (O—O) and the other with water (●—●) and then assayed for DNA polymerase activity as described in the Materials and Methods.

at 100,000 *g* for 1 h in an SW-27 rotor. 1.5 μg of the resultant pellet was used in an endogenous polymerase reaction as described in the Materials and Methods. The kinetic curve of DNA polymerase activity shows a peak of DNA synthesis at 15 min, which was 73% RNase sensitive (Fig. 3).

The requirements of this endogenous DNA polymerase reaction are summarized in Table I. The marked decrease in DNA synthesis with the omission of dATP or dGTP indicates a heteropolymeric polydeoxynucleotide was synthesized, and it is unlikely that we were observing terminal deoxynucleotidyl transferase activity (22). The omission of the oligo $dT_{12\text{-}18}$ primer caused a markedly limited reaction. DNA synthesis occurred at 70% of control levels when Mg^{++} was omitted. Further inhibition of DNA synthesis probably was not observed because of the presence of cation in the enzyme-containing cytoplasmic pellet. The NP-40 concentration producing maximum synthesis was 0.1%. It should be noted that significant DNA synthesis occurred in the absence of NP-40 pretreatment of the enzyme-containing sample.

Cesium Sulfate Gradient Analysis of DNA Product. In an attempt to differentiate an RNA-primed from an RNA-templated DNA polymerase reaction, the endogenous product of a DNA polymerase reaction was analyzed by cesium sulfate centrifugation. Brain sample (temporal lobe) of a patient with

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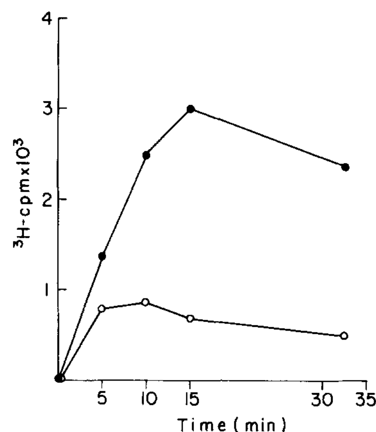

FIG. 3. Kinetics of incorporation of [^{3}H]dTTP by cytoplasmic particulate fraction from Guamanian ALS brain (GP 168). 1.5 μg protein from pellet of 1.16-1.18 g/ml region of final sucrose gradient was used (see Materials and Methods). The reaction was performed with (O—O) and without (●—●) prior incubation of sample with RNase).

TABLE I
Requirements of Endogenous DNA Polymerase Reaction from ALS Brain (GP 168) Cytoplasmic Particulate Fraction

Condition	[^{3}H]TTP incorporated
	pmol
Complete	1.40
Minus dATP	0.16
Minus dGTP	0.10
Minus Mg^+	1.06
Minus oligo $dT_{12\text{-}18}$	0.50
Minus trypsin pretreatment	0.52
Plus RNase A (50 μg/ml)	0.40
NP-40 titration	
Without NP-40	0.40
0.001% NP-40	0.48
0.01% NP-40	0.52
0.1% NP-40	1.40
0.25% NP-40	0.64

Conditions for complete reaction are described in the Materials and Methods; the reaction mixture was incubated at 37°C for 15 min.

ALS (GP 174) was fractionated as described in the Materials and Methods and an endogenous RIDP reaction performed with 1.0 μg protein of the enzyme-containing pellet in the presence of actinomycin D and distamycin. The early DNA product (5-min reaction) was subjected to analysis by Cs_2SO_4 gradient centrifugation and is shown in Fig. 4. There was a peak of acid-precipitable radioactivity in the RNA-DNA hybrid region of the gradient, which shifted to the DNA region of the gradient (1.45 g/ml) by conditions that hydrolyze RNA but not DNA (alkali and RNase) or that disrupt hydrogen but not covalent bonds (heat and alkali). These results are in contrast to studies with mammalian RNA-primed

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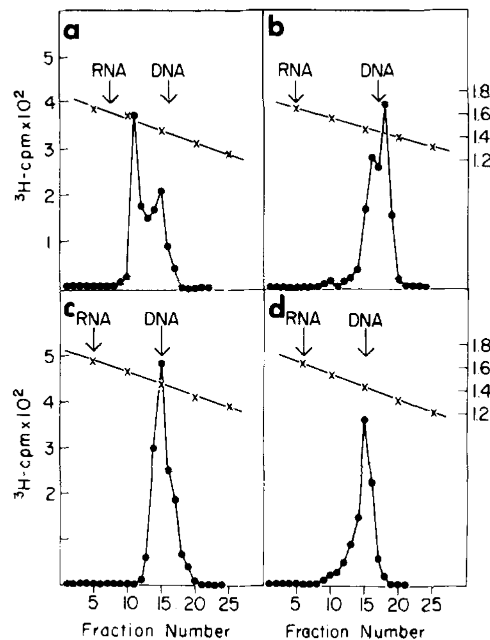

FIG. 4. Cs_2SO_4 equilibrium density gradient centrifugation of ALS brain (GP 174) product (*a*). Equivalent amounts of [^{3}H]DNA product were treated with heat (*b*), ribonuclease (*c*), and alkali (*d*) as described in the Materials and Methods.

DNA-instructed DNA polymerase, which catalyzes synthesis of nascent DNA strands covalently linked to RNA primer molecules, which are unaffected by heat treatment (23).

Fig. 5 shows the Cs_2SO_4 gradient analysis of the endogenous [^{3}H]DNA product from temporal lobes from brains of a normal Chamorro (GP 219) and from U. S. patients with multiple sclerosis, subacute sclerosing panencephalitis, and schizophrenia. Only the reaction product from the normal Guamanian brain shows acid-precipitable radioactivity in the hybrid region of the gradient; this peak was completely heat sensitive (i.e., it shifted to the DNA region).

Table II summarizes the Cs_2so_4 analysis of reaction products from a number of brain specimens including those depicted in Figs. 3 and 4. Only the brain specimens from two of two ALS patients, one of two PD patients, and two Guamanian controls exhibited a positive reaction that consisted of acid-precipitable radioactivity in the RNA or RNA-DNA hybrid region of the gradient, which sedimented to the DNA region of the gradient after heat or RNase treatment.

Sizing of the DNA Product. The purified DNA product of the endogenous RIDP reaction had a sedimentation coefficient of 4-5S as determined by velocity alkaline sucrose density gradient centrifugation (24). The peak of acid-precipitable radioactivity was DNase sensitive but RNase and pronase resistant.

RNA-[^{3}H]DNA Hybridization. Definitive proof of the presence of reverse transcriptase rests on the demonstration that the DNA product of a polymerase reaction is complementary to heteropolymeric regions of an RNA template. In this relatively crude system, the RNA template has not been purified. Thus, hybridization experiments were attempted with the endogenous [^{3}H]DNA product and whole RNA extracted from the final enzyme-containing pellet.

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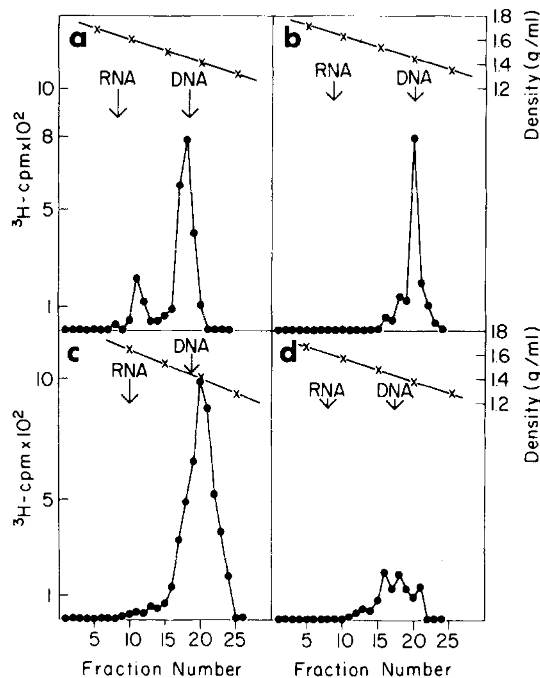

FIG. 5. Cs_2SO_4 equilibrium density gradient centrifugation of product from normal Chamorro brain (*a*) (GP 219) and from brains of patients with multiple sclerosis (*b*), subacute sclerosing panencephalitis (*c*), and schizophrenia (*d*).

Similar experiments have been performed using cytoplasmic pellet RNA from human leukemic cells (25).

Table III summarizes the hybridization results. There was more extensive hybridization of the [^{3}H]DNA product from the brain of a PD patient (GP 185) with RNA from PD brain cytoplasmic pellet as compared with RNA from a U. S. control brain. The homology of the [^{3}H]DNA probe with normal brain RNA indicates that there are normal nucleotide sequences (RNA or DNA) in the relatively crude enzyme pellet that were also copied in the polymerase reaction. A small but significant portion of the [^{3}H]DNA probe annealed to Rauscher leukemia virus RNA, visna virus RNA, and synthetic poly A. Thus it appears that a portion of the [^{3}H]DNA probe contains poly T regions that annealed with the poly A tracts known to be present in oncornavirus RNA and may be present in brain RNA. We do not know the poly A content of the various RNA preparations used in these experiments. However, the poly A content of the synthetic poly A was probably in vast excess over the poly A content of the other RNA preparations; hybridization mixtures contained 6 OD_{260} units of cellular RNA, 0.5 μg of viral RNA, and 6.0 μg of poly A. Thus, the more extensive hybridization and higher $T_{m(e)}$ of the PD RNA-[^{3}H]DNA hybrid indicates that it is likely that the probe annealed with heteropolymeric regions of the PD RNA.

Discussion

These studies were designed to determine if RIDP were present in a particulate fraction of brains from patients with ALS and PD. Evidence that we have de-

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TABLE II

Summary of Results of Cs_2SO_4 Equilibrium Density Gradient Centrifugation of Product from Normal and Diseased Brains

Brain origin	RNA and hybrid region	Reaction
	cpm	
Normal-U. S. A.	0	–
Normal-Guam (GP 11)	447	+*
Normal-Guam (GP 219)	325	+
Schizophrenia	0	–
Jacob-Creuzfeldt	0	–
Multiple sclerosis	0	–
Multiple sclerosis	0	–
Subacute sclerosing panencephalitis	30	–
ALS (GP 168)	325	+
ALS (GP 172)	723	+
PD (GP 185)	263	+
PD (GP 70)	19	–

* Positive result indicates that acid-precipitable radioactivity band in the RNA or RNA-DNA hybrid region of the gradient could be displaced to the DNA region of the gradient after heat and RNase treatment.

TABLE III

Hybridization of Endogenous Reverse Transcriptase [³H]DNA Product from PD (GP 185) Brain with Various RNA Preparations

	Hybridized		$T_{m(e)}$
	>60°	>85°	
	%	%	°C
PD-RNA	53	16.3	83
Normal brain RNA	35	10.9	80
Rauscher leukemia virus RNA	15.6	2.0	72.5
Visna virus RNA	17	5.0	68
Poly rA	25	2.2	71

Annealing conditions were as described in Materials and Methods.

tected RIDP is: (*a*) DNA polymerase activity persists in the presence of concentrations of actinomycin D and distamycin that inhibit most DNA-directed DNA synthesis (26); (*b*) the majority of endogenous DNA polymerase activity is sensitive to prior treatment with RNase; (*c*) the early reaction product is a 4-5S DNA heteropolymer joined by hydrogen bonds to an RNA molecule; and (*d*) the purified [^{3}H]DNA product anneals to RNA extracted from the enzyme-containing pellet more extensively than to normal brain RNA or poly rA.

The enzyme activity is in a cytoplasmic particle that can be sedimented at high speed and has the buoyant density of RNA tumor viruses (1.16-1.18 g/ml). This particulate fraction is not disrupted by physical manipulation and maintains its characteristic density with repeated centrifugations. Treatment with the nonionic surfactant, Sterox, changes the buoyant density of the enzyme-containing particle to 1.24 g/ml, the density of the oncornavirus virion core.

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Synthesis of RNA-DNA hybrids by an endogenous reverse transcriptase reaction was found only in normal and diseased Chamorro brains. Examination of a limited number of normal and diseased brains from individuals who lived in the U. S. produced negative results. Our inability to distinguish afflicted from asymptomatic Chamorros was not surprising since neuropathological lesions (neurofibrillary tangles and Hirano bodies) are present in most Chamorros regardless of clinical status (14).

Definitive characterization of this polymerase activity and identification as a true viral polymerase will depend on purification of biochemically active quantities of this polymerase to determine its template specificities, cation preference, fidelity of its transcription product, as well as its antigenic relationship to animal virus and human leukemic RIDP. Of critical importance in these studies will be the examination of the DNA polymerases from fresh normal brains. There is evidence (27, 28) that some properties of rat brain DNA polymerases differ from those extracted from other organs and we have observed differences in the synthetic template preferences of brain as compared to liver DNA-dependent DNA polymerases (unpublished results).

There have been a number of theories proposed for the etiology of ALS and PD that invoke a genetic basis (29) or widespread exposure to a neurotoxin (30). In view of the studies reported here, a viral etiology should be reconsidered. Attempts to transmit this disease to nonhuman primates by cell preparations of diseased brain (in the manner that was successful for the transmission of kuru) have thus far been unsuccessful (31).

Chamorros who migrate to the U. S. appear not to lose their susceptibility to ALS and PD (32), a finding consistent with ubiquitous infection or with chromosomal transmission of the RNA tumor virus information, a phenomenon that has been observed in certain inbred mouse strains (33). The introduction of viral information into the Chamorro germ line could have occurred when the population of Guam was small (latter half of 17th century) and its persistence could be the result of a relatively restricted gene pool (12).

Summary

Nervous system tissues from a number of patients with idiopathic neurological disorders were examined for biochemical evidence of RNA tumor virus infection. RNase-sensitive DNA polymerase activity was found in a cytoplasmic particulate fraction from two patients with Guamanian amyotrophic lateral sclerosis (ALS) but not in brains from two normal U. S. individuals. The buoyant density of the enzyme-containing fraction was 1.16-1.18 g/ml and could be converted to a denser region of the gradient (1.24 g/ml) by treatment with the nonionic surfactant, Sterox. The cation and detergent requirements for the endogenous RNase-sensitive DNA polymerase reaction were determined.

The early (5 min) endogenous reverse transcriptase product was analyzed by cesium sulfate gradient centrifugation. RNase- and heat-sensitive RNA-DNA hybrids were detected in the product analysis of two ALS, one Parkinsonism-dementia (PD) brain, and two brains from asymptomatic Chamorros but not in brains from normal U. S. individuals and a number of patients with neuro-psychiatric disorders. The DNA product was a 4.5S heteropolymer that hybridized

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more extensively to RNA extracted from the enzyme-containing pellet from PD brain as compared to a similar fraction from normal U. S. brain. The DNA product appeared to be unrelated to Rauscher or visna virus 70S RNA as determined by RNA-[^{3}H]DNA hybridization.

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