Physical and Biochemical Properties of Progressive Pneumonia Virus

LAWRENCE B. STONE, KENNETH K. TAKEMOTO, AND MALCOLM A. MARTIN

Laboratory of Viral Diseases and Laboratory of Biology of Viruses, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20014

Received for publication 25 May 1971

The physical and biochemical characteristics of progressive pneumonia virus were found to be remarkably similar to those of the ribonucleic acid (RNA) tumor viruses. Significant findings included the presence of a 60 to 70S RNA genome, RNA-dependent deoxyribonucleic acid polymerase activity, and common morphological properties. This information correlates with previously reported biological observations and supports the provisional inclusion of enveloped RNA-containing "slow viruses" within the RNA tumor virus group.

Progressive pneumonia virus (PPV) and visna virus are serologically related agents capable of inducing slow infections in their natural hosts (11, 18, 20). Both bear striking morphological and physical resemblances to the oncogenic ribonucleic acid (RNA) viruses (20, 22, 23). Our laboratory and others have extended these similarities by finding RNA-dependent deoxyribonucleic acid (DNA) polymerase activity associated with the visna virion (12, 17, 19). Furthermore, we have recently demonstrated that both PPV and visna can cause malignant transformation of murine cells (21) and induce carrier states in cells derived from sheep (L. Stone, *unpublished data*). Such data suggest that RNA tumor viruses and RNA slow viruses may share similar ontogenetic histories and that they be classified within the same taxonomic order. Additional information supporting this hypothesis is presented in the following study of physical and biochemical properties of PPV.

MATERIALS AND METHODS

Viruses and tissue culture. The identity and growth of plaque-purified PPV in a strain of cells derived from sheep testes (ST) has been described (21).

Preparation of labeled virus. Twenty-four hours after PPV infection of ST cell monolayers (MOI = 14), 500 μ Ci of either [3 H]uridine or [3 H]5Me-thymidine (New England Nuclear Corp., Boston, Mass.; > 20 Ci/mmol) was added to the cultures. Supernatant fluids were harvested at 12-hr intervals for the next 48 hr and maintained at 4 C. Conditions for labeling with carrier-free [32 P] α -phosphate (Tracerlab, Waltham, Mass.; 50 mCi/mg) were similar, except that cells were maintained in phosphate-free medium for 12 hr before incorporation and throughout the incorporation period.

Purification of virus and nucleic acid isolation.

Collected virus pools were clarified by low-speed centrifugation (5,000 $\times$ g, 10 min), followed by pelleting in a type 30 Spinco rotor at 30,000 rev/min for 120 min. Pellets were resuspended in buffer containing: tris(hydroxymethyl)aminomethane (Tris), 0.01 M (pH 7.5); ethylenediaminetetraacetic acid (EDTA), 0.001 M; NaCl, 0.1 M. They were then carefully layered over continuous preformed gradients of 20 to 60% (w/v) sucrose (Mann) containing 0.5% bovine serum albumin (BSA; Worthington Biochemicals Corp., Freehold, N.J.). These gradients were then centrifuged in an SW40 rotor at 40,000 rev/min for 18 to 22 hr at 4 C. Banded virus was collected by puncturing the bottom of the centrifuge tube; suspended in a buffer which contained 1% sodium dodecyl sulfate (SDS), polyvinyl sulfate (50 μ g/ml), yeast RNA (1 mg/ml), 0.001 M EDTA, 0.05 M Tris (pH 7.5), and 0.1 M NaCl (1); and then extracted with phenol equilibrated with 0.01 M sodium acetate (pH 5.2). The aqueous phase was collected, and RNA was precipitated with 2 volumes of cold ethanol at -20 C.

DNA polymerase assays. These were performed using unlabeled purified virus as previously described (18).

RESULTS

Properties of purified virus. Isopycnic centrifugation of PPV in sucrose produced a single bluish-white band at densities ranging between 1.16 and 1.175 g/cm³. These values correspond well with those obtained with visna virus in this laboratory. Integrity of the particles was found to be dependent upon the presence of added protein. Therefore, 0.5% BSA was used in all preparative gradients and resuspension buffers. Purification of PPV in cesium chloride or potassium tartrate gradients resulted in poor resolution and re-

duced infectivity. Preliminary concentration with polyethylene glycol 6000 (Union Carbide, New York, N.Y.) (19) was not found to be suitable for nucleic acid studies because of the extended time required to achieve the necessary precipitation.

Negative staining of purified unlabeled virus by the method of de-Thé (4) resolved 80 to 100-nm enveloped particles. None appeared to have "spikes" as was reported by Thormar and Cruickshank for visna virus (23). Occasionally, phosphotungstate penetrated the viral envelope to reveal a densely staining spherical internal component (4) of 64-nm diameter (Fig. 1). This particle appears very similar to the avian leukosis (3), murine leukemia (4, 24), mouse mammary tumor (13), and feline leukemia (15) viruses.

The viral nucleic acid. Figure 2 indicates the pattern of nucleoside incorporation into PPV. Preferential utilization of uridine was noted as infectivity coincided with the pattern of [³H]-uridine labeling. [³²P]-*o*-phosphate was similarly incorporated into infectious PPV. There was no evidence of thymidine incorporation, suggesting that the viral nucleic acid is RNA.

The sedimentation constant of phenol-SDS-extracted PPV RNA was determined by centrifugation through 15 to 30% (w/v) sucrose gradients using simian virus 40 (SV40) DNA 1 as a marker. [³H]-uridine-labeled nucleic acid was generally represented as a homogeneous, rapidly migrating species having a sedimentation coefficient of about 64 to 65S as calculated by the method of Martin and Ames (14). This material was always accompanied by a distinct, slowly sedimenting component of 4 to 7S. The immunologically related visna virus recently was found to have 60 to 70S RNA by Lin and Thormar (12) and Harter et al. (9).

In later experiments, a third species of RNA sedimenting at 36S was also detected (Fig. 3). Incubation of these three components (64 to 65S, 36S, 4 to 7S) with 10 μ g of pancreatic ribonuclease A (Worthington Biochemical Corp. Freehold, N.J.) for 60 min at 37 C resulted in nearly complete digestion (Table 1). Duesberg (5) was able to convert the 70S Rous sarcoma virus RNA to components sedimenting at 36S by melting or dimethyl sulfoxide treatment and proposed that the large RNA species was an aggregate molecule. Exposure of the rapidly sedimenting PPV RNA to 100 C for 3 min did not effect a conversion to the 36S state. Only a heterogeneous pattern of 4 to 7S components was observed. Melting the total extracted PPV RNA for the same period of time also yielded only the slowly sedimenting RNA.

Depending upon how the virus was stored and

manipulated, the 4S component comprised between 15 and 100% of the total radioactivity. When the virus was frozen and thawed once before RNA extraction, virtually all of the nucleic acid was recovered in the slowly sedimenting region (4 to 7S) of the gradient. Virus stored at 4 C for 12 hr and then extracted contained only the 36S and 4S RNA components. Harvesting virus at the time of maximal cytopathic effect resulted in the isolation of only 4S RNA. The highest proportion of 60 to 70S RNA was prepared from freshly harvested and phenol-SDS-treated virus which was obtained after pelleting clarified supernatant fluids in a type 50.1 rotor at 50,000 rev/min for 60 min. These findings suggest that the 60 to 70S RNA molecule is readily degraded to more slowly sedimenting components after prolonged storage or exposure to heat. Others have observed a similar phenomenon during extraction of RNA from Rous sarcoma virus (5, 16), avian myeloblastosis virus (7) murine leukemia virus (1, 2), feline leukemia virus (10), and the C-type viper agent (8).

The sedimentation constant of intact PPV. The results of velocity sedimentation of PPV are shown in Fig. 4. A mixture of purified [³²P]-labeled virus and [³H]thymidine SV40 was layered over gradients of 15 to 30% (w/v) sucrose and centrifuged for 50 min at 30,000 rev/min in an SW56 rotor. As can be seen, PPV migrated more rapidly than the SV40 marker. A sedimentation constant of approximately 600 can be calculated from this experiment.

Viral RNA-dependent DNA polymerase. Table 2 shows the requirements of PPV DNA polymerase. A 15-fold increase in the incorporation of [³H]thymidine monophosphate into DNA over the zero-time control can be seen. Maximal synthesis required all four deoxynucleoside triphosphates, a divalent cation, a nonionic detergent (Triton X-100), and a sulfhydryl agent. DNA synthesis was inhibited by ribonuclease A treatment of disrupted virus, suggesting that the template was RNA. Use of the synthetic polyribonucleotide polyriboadenylic acid polyribo-uridylic acid greatly enhanced utilization of labeled precursor and did not require the presence of either deoxyadenosine triphosphate, deoxyguanosine triphosphate, or deoxycytidine triphosphate. Optimum DNA synthesis was observed at a concentration of 0.1% Triton X-100, which is greater than that reported for the RNA tumor viruses, and identical to that for the visna enzyme (19). As seen in Table 3, the polymerase product was insensitive to both alkaline hydrolysis and ribonuclease A treatment, whereas more than 94% was degraded by pancreatic deoxyribonu-

FIG. 1. *Negatively stained progressive pneumonia virus from 1.16 g/cm³ region of sucrose gradient. The lower particle demonstrates internal component. Magnification 240,000.*

clease. This material therefore has the properties of DNA.

DISCUSSION

Currently available evidence supports the inclusion of enveloped RNA-containing slow vi-

ruses within the RNA tumor virus group described by Nowinski et al. (15). The similarities between these agents are summarized in Table 4. Sedimentation properties of PPV suggest that it contains a significant lipid constituent, as it reaches equilibrium between 1.16 and 1.175

FIG. 2. Equilibrium density gradient centrifugation of concentrated $[^3\text{H}]$ -uridine labeled progressive pneumonia virus. Virus was layered over gradients of 20 to 60% (w/v) sucrose containing 0.5% (w/v) bovine serum albumin, 0.01 M tris(hydroxymethyl)aminomethane (pH 7.5), 0.001 M ethylenediaminetetraacetic acid, and 0.1 M NaCl and centrifuged in the SW40 rotor of a Beckman L2-65B ultracentrifuge for 22 hr at 40,000 rev/min at 4 C. Fractions were collected from the bottom of the tube.

g/cm³ in sucrose. These values are comparable to the buoyant densities observed for visna and the RNA tumor viruses (12). The calculated sedimentation constant for PPV was approximately the same as that noted by Robinson et al. (16) for the Bryan strain of Rous sarcoma virus. Remarkable similarity between negatively stained PPV preparations and published electronmicrographs of the oncornaviruses (15) provided compelling data relating dimension morphology and internal organization. Both groups resemble each other in size, surface structure, and compact spherical internal component. Furthermore, previous morphogenic observations (20) suggested parallel developmental histories involving laminar intracytoplasmic (type A-like) and mature budding particles. Comparative electron microscopy of these viruses is being planned to test further their observed resemblance.

Significant among the common physical and biochemical properties of the enveloped slow viruses and the RNA tumor viruses are the RNA-dependent DNA polymerase and the 60 to 70S viral RNA genome. The possible role that a reverse transcriptase might play in either virus

FIG. 3. Velocity centrifugation of $[^{32}\text{P}]$ -labeled PPV RNA with $[^3\text{H}]$ -simian virus 40 DNA I marker. A mixture of PPV RNA and simian virus 40 DNA I in 250 μ liters was layered over a 15 to 30% (w/v) sucrose gradient containing 0.01 M tris(hydroxymethyl)aminomethane (pH 7.5), 0.001 M ethylenediaminetetraacetic acid, 0.1 M NaCl, and 0.015% (v/v) diethylpyrocyanate and centrifuged in an SW56 rotor for 60 min at 56,000 rev/min at 4 C. Fractions were collected from the bottom of the tube and precipitated onto nitrocellulose filters with 5% (w/v) trichloroacetic acid.

group has already been speculated upon greatly and will not be discussed in this report. Previously, 60 to 70S RNA species were found only among the oncogenic RNA viruses (1, 2, 5, 10, 16). Isolation of this large RNA from visna and PPV further establishes the close relationship among these agents. Molecular hybridization of the RNA from PPV and visna to the RNA or the DNA products of oncornaviruses might yield additional information concerning their evolutionary relatedness or diversity. Whether the 36S RNA represents a subunit of the 70S molecule (5) or the product of untwisting as Bader and Steck suggested for murine leukemia virus is not clear at this time.

The most interesting criterion for inclusion of enveloped slow viruses within the RNA tumor virus group would be biologic evidence of oncogenicity. The ability of visna and PPV to induce malignant transformation in vitro has been

FIG. 4. Velocity sedimentation of intact progressive pneumonia virus. A mixture of $[^{32}\text{P}]$ -labeled PPV and $[^3\text{H}]$ -labeled simian virus 40 DNA I (small plaque variant) was layered over a 15 to 30% (w/v) sucrose gradient and centrifuged in a SW56 rotor for 50 min at 30,000 rev/min at 4 C. Fractions were collected from the bottom of the tube, and radioactivity was determined after precipitation with 5% (w/v) trichloroacetic acid.

TABLE 1. Ribonuclease hydrolysis of progressive pneumonia virus ribonucleic acid^a

RNA species	Trichloroacetic acid-insoluble radioactivity (dpm)	Amt digested ^b (%)
70S		
Untreated ^c	740	0
Ribonuclease (10 μg)	68	90.8
Ribonuclease (50 μg)	55	92.5
36S		
Untreated	2,687	0
Ribonuclease (10 μg)	60	97.7
Ribonuclease (50 μg)	56	97.9
4S		
Untreated	5,550	0
Ribonuclease (10 μg)	70	98.7
Ribonuclease (50 μg)	56	99.0

^a Samples of 70, 36, and 4S $[^3\text{H}]$ -PPV RNA isolated from velocity sedimentation runs were exposed to either 10 or 50 μg of ribonuclease A in 0.2 M NaCl at 37 C for 60 min and then precipitated with trichloroacetic acid onto nitrocellulose filters.

^b Indicates fraction of RNA converted to acid-soluble nucleotides.

^c Untreated control value is derived from RNA incubated with phosphate-buffered saline (pH 7.2) for 60 min at 37 C.

TABLE 2. Requirements of the progressive pneumonia virus DNA polymerase^a

Reaction system	$[^3\text{H}]$ dTMP incorporated (pmoles)
Complete	1.40
Without dATP	0.02
Without dATP, dGTP, dCTP	0.02
Without virus	<0.01
Without Triton	<0.01
Without dithiothreitol	0.02
Without Mg^{2+}	0.02
With poly rA·rU	4.65
With poly rA·rU—dATP, dGTP, dCTP	4.50
With poly rA·rU—virus	0.02
With ribonuclease A (50 $\mu\text{g}/\text{ml}$)	0.30

^a Each complete reaction mixture was incubated for 90 min and contained in 50 μl of: 0.04 M tris-(hydroxymethyl)aminomethane-hydrochloride (pH 7.8); 0.06 M potassium chloride; 0.01 M magnesium acetate; 5×10^{-4} M deoxyadenosine triphosphate (dATP), deoxycytidine triphosphate (dCTP), deoxyguanosine triphosphate (dGTP); 2×10^{-5} M $[^3\text{H}]$ -methyl thymidine triphosphate (9000 counts/min per pmoles); 8×10^{-3} M dithiothreitol; polyriboadenylic acid:polyribouridylic acid (poly rA·rU; 5 $\mu\text{g}/\text{ml}$) where indicated; 0.1% (v/v) Triton X-100; and 10^{-7} tissue culture infective dose₅₀ of virus. Ribonuclease A was heated for 15 min at 90 C to inactivate any possible contaminating deoxyribonuclease. The zero time value in the complete system of 0.09 pmoles has been subtracted from all values.

TABLE 3. Characterization of product of progressive pneumonia virus DNA polymerase

Treatment ^a	Trichloroacetic acid-insoluble radioactivity	Percentage undigested
Untreated	6,450	100
Deoxyribonuclease (20 $\mu\text{g}/\text{ml}$)	350	5.5
Ribonuclease (20 $\mu\text{g}/\text{ml}$)	6,200	96
Alkaline hydrolysis (0.3 N KOH $\times$ 18 hr)	6,000	93

^a The product of an extensive reaction mixture (three times the quantities described in Table 2) was divided into portions and subjected to the various treatments. Incubation with 20 μg of ribonuclease or deoxyribonuclease was for 60 min at 37 C. Samples were then chilled, precipitated onto cellulose nitrate filters with 5% (w/v) trichloroacetic acid, and counted. The untreated control was incubated with phosphate-buffered saline (pH 7.2) for 18 hr at 37 C.

clearly demonstrated (22). However, studies have not been made documenting the occurrence of neoplasms in sheep, the natural host for these diseases. Furthermore, it is not known whether animals dying of visna or progressive pneumonia

TABLE 4. *Observed common properties of PPV-maedi-visna and the RNA tumor viruses*

Property	Virus group	
	PPV-visna-maedi	RNA tumor viruses
Particle diameter	80 to 100 nm	80 to 100 nm
Morphology	Enveloped spherical viruses with compact internal component	Enveloped spherical viruses with compact internal component
Buoyant density	1.16 to 1.175 (sucrose)	1.16 to 1.18 (sucrose)
RNA size	60 to 70S	60 to 70S
Reverse transcriptase	Present	Present
Oncogenicity	Transformation in vitro, undefined in vivo	Oncogenic in vivo and in vitro
Site of maturation	Budding from cell membrane	Budding from cell membrane

virus have had concurrent tumors. The possibility that neither virus causes neoplasia in vivo provides a basis for speculation upon the potential role that host interaction may play in mediating slow degenerative infections, thus differentiating them from those slow infections which we now recognize as being oncogenic.

LITERATURE CITED

- Bader, J. P., and T. L. Steck. 1969. Analysis of the ribonucleic acid of murine leukemia virus. *J. Virol.* 4:454-459.
- Blair, C. D., and P. H. Duesberg. 1968. Structure of Rauscher mouse leukemia virus RNA. *Nature (London)* 220:396-399.
- Bonar, R. A., V. Heine, D. Beard, and J. W. Beard. 1963. Virus of avian myeloblastosis (BAI strain A). XXIII. Morphology of virus and comparison with strain R (erythroblastosis). *J. Nat. Cancer Inst.* 30:949-998.
- de-Thé, G., and T. E. O'Connor. 1966. Structure of a murine leukemia virus after disruption with Tween-ether and comparison with two myxoviruses. *Virology* 28:713-728.
- Duesberg, P. H. 1968. Physical properties of Rous sarcoma virus RNA. *Proc. Nat. Acad. Sci. U.S.A.* 60:1511-1518.
- Duesberg, P. H., and R. D. Cardiff. 1968. Structural relationships between the RNA of mammary tumor virus and those of other RNA tumor viruses. *Virology* 36:696-700.
- Erickson, R. 1969. Studies on the RNA from avian myeloblastosis virus. *Virology* 37:124-131.
- Gilden, R. V., Y. K. Lee, S. Oroszlan, J. L. Walker, and R. J. Huebner. 1970. Reptilian C-type virus: biophysical, biological and immunological properties. *Virology* 41:187-190.
- Harter, D. H., J. Schlom, and S. Spiegelman. 1971. Characterization of visna virus nucleic acid. *Biochim. Biophys. Acta* 240:435-441.
- Jarrett, O., J. D. Pitts, J. M. Whalley, A. E. Clason, and J. Hay. 1971. Isolation of the nucleic acid of feline leukemia virus. *Virology* 43:317-320.
- Kennedy, R. C., E. M. Eklund, C. Lopez, and W. J. Hadlow. 1968. Isolation of a virus from the lungs of Montana sheep affected with progressive pneumonia. *Virology* 35:483-484.
- Lin, F. H., and H. Thormar. 1970. Ribonucleic acid-dependent deoxyribonucleic acid polymerase in visna virus. *J. Virol.* 6:702-704.
- Lyons, M. J., and D. H. Moore. 1965. Isolation of the mouse mammary tumor virus: chemical and morphological studies. *J. Nat. Cancer Inst.* 35:549-566.
- Martin, R. G., and B. N. Ames. 1961. A method for determining the sedimentation behavior of enzymes: application to protein mixtures. *J. Biol. Chem.* 236:1372-1379.
- Nowinski, R. C., L. J. Old, N. H. Sarkar, and D. H. Moore. 1970. Common properties of oncogenic RNA viruses (oncornaviruses). *Virology* 42:1152-1157.
- Robinson, W. S., A. Pitkanen, and H. Rubin. 1965. The nucleic acid of the Bryan strain of Rous sarcoma virus: purification of the virus and isolation of the nucleic acid. *Proc. Nat. Acad. Sci. U.S.A.* 54:137-144.
- Schlom, J., D. H. Harter, A. Burny, and S. Spiegelman. 1971. DNA polymerase activities in virions of visna virus, a causative agent of "slow" neurological disease. *Proc. Nat. Acad. Sci. U.S.A.* 68:182-186.
- Sigurdsson, B., P. A. Palsson, and H. Grimsson. 1957. Visna of sheep. A slow, demyelinating infection. *Brit. J. Exp. Neurol.* 39:519-528.
- Stone, L. B., E. A. Scolnick, K. K. Takemoto, and S. A. Aaronson. 1970. Visna virus: a slow virus with an RNA dependent DNA polymerase. *Nature (London)* 229:257-258.
- Takemoto, K. K., C. F. T. Mattern, L. B. Stone, J. E. Coe, and G. Lavelle. 1971. Antigenic and morphologic similarities of progressive pneumonia virus, a recently isolated "slow virus" of sheep, to visna and maedi viruses. *J. Virol.* 7:301-308.
- Takemoto, K. K., and L. B. Stone. 1971. Transformation of murine cells by two "slow viruses," visna virus and progressive pneumonia virus. *J. Virol.* 7:770-775.
- Thormar, H. 1965. Physical, chemical and biological properties of visna virus and its relationship to other animal viruses, p. 335-339. *In* NINDB Monograph No. 2. National Institutes of Health, Bethesda, Md.
- Thormar, H., and J. G. Cruickshank. 1965. The structure of visna virus studied by the negative staining technique. *Virology* 25:145-148.
- Zeigal, R. F., and F. J. Rauscher. 1964. Electron microscopic and bioassay studies on a murine leukemia virus (Rauscher) I. Effect of physico-chemical treatments on the morphology and biological activity of the virus. *J. Nat. Cancer Inst.* 32:1277-1308.