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Characterization of Bovine Parainfluenza Virus Type 3

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Abstract Bovine parainfluenza virus type 3 (PIV-3) has a buoyant density of 1.197. The RNA of PIV-3, like that of Sendai virus, is a single continuous chain which lacks polyadenylic acid sequences and tends to self-anneal to a marked extent. It has a sedimentation coefficient of 42S and a molecular weight of 4.5×10^6, being slightly smaller than Sendai virus RNA (47S, 5.3×10^6).

PIV-3 has 5 main structural proteins, of which 2 are glycoproteins. The molecular weights of protein$_1$, protein$_2$, protein$_3$, glycoprotein$_1$, and glycoprotein$_2$ were estimated to be 79,000, 68,000, 35,000, 69,000, and 55,000, respectively. Protein$_2$ was suggested to be nucleocapsid protein.

Increased informations about Newcastle disease virus, Sendai virus, simian virus 5 and measles virus have made a new concept about the paramyxovirus group with respect to the virion RNA (1, 15, 16, 20, 25, 26) and proteins (6, 8, 16, 18, 19, 22, 23, 31), and therefore, have evoked much interest in researches of other remaining members of the paramyxovirus group.

This communication describes a number of similarities between bovine parainfluenza virus type 3 (PIV-3), another member of the paramyxovirus group, and Sendai virus in their RNA and structural proteins. The most marked difference was that PIV-3, unlike Sendai virus, could form plaques in Vero and MDBK cells without the aid of trypsin incorporated into agar overlay.

MATERIALS AND METHODS

Viruses. Four strains of PIV-3, passaged several times in primary bovine kidney cell cultures, were kindly supplied by Dr. Y. Inaba, National Institute of Animal Health, Kodaira, Tokyo. These strains grew well and formed plaques in Vero cells, a stable cell line derived from an African green monkey kidney, and Mardin-Darby bovine kidney (MDBK) cells. A small amount of trypsin in agar overlay, an essential factor for Sendai virus plaquing in Vero and MDBK cells, did not favor the plaquing of PIV-3 but decreased the number and size of plaques to about one half. Among the 4 strains, Shimane 910N showed homogeneous plaques and therefore was used as a representative strain in this study. The virus received serial passages in

Vero cells and showed neuraminidase activity as well as agglutination and hemolysis of red blood cells of guinea pigs.

Z strain of Sendai virus (HVJ) had been maintained by serial passage in embryonated hen's eggs (27).

Virus preparation. Vero cell monolayers prepared in roller vessels, 10 cm in diameter and 22 cm in length, rotated at 0.06 rpm, were washed with LE (0.5% lactalbumin hydrolysate in Earle's solution) medium and infected with PIV-3 or Sendai virus at multiplicities of infection of about 5 and 25, respectively. After 1 hr adsorption at 37 C, unadsorbed viral inocula were aspirated. Each vessel was then washed with LE medium 3 times, received 30 ml of a maintenance medium consisting of 9 parts of MEM (Eagle's minimum essential medium) and 1 part of tryptose phosphate broth, and was incubated at 37 C. Culture media were harvested at 24 and 72 hr after infection with Sendai virus and PIV-3, respectively.

To label viruses with radioactive precursors, infected cell monolayers in vessels were incubated under a labeling medium for 24 hr before harvest. For RNA labeling, the maintenance medium contained additionally 10 μCi/ml of ^{3}H-uridine-(G) (6.5 Ci/mM) and 0.5 μg/ml of Actinomycin D. For protein labeling, leucine-free MEM mixed with 4 μCi/ml of ^{3}H-L-(4,5)-leucine (38 Ci/mM), or both 2 μCi/ml of ^{14}C-L-leucine-(U) (324 mCi/mM) and 5 μCi/ml of ^{3}H-(6)-D-glucosamine was used. In the case of protein labeling, 1 ml of 200 mM L-leucine and 3 ml of TPB were added to each vessel 4 hr before harvest.

Purification of virus. Fluids of infected cultures were centrifuged at 16,000 *g* for 20 min. Then fetal calf serum, NaCl, and polyethylenglycol 6,000 were added to the supernatant to final concentrations of 1%, 0.5 M and 6.25%, respectively. After being left at 4 C overnight, precipitates were collected by centrifugation at 4,000 *g* for 15 min and resuspended in a small amount of PBS(0.8% NaCl–0.02% KCl in 0.01 M phosphate buffer, pH 7.2) by homogenization in a tight-fit teflon homogenizer and sonication at 20 Kc for 5 sec. The concentrated virus was layered onto a discontinuous sucrose density gradient column consisting of 60% (2 ml), 52% (2 ml), 47% (3 ml), 35% (3 ml), and 25% (2 ml) sucrose (w/v) in PBS, which was centrifuged at 25,000 rpm for 3 hr at 4 C in a Spinco SW 27-1 rotor. A band formed at the top of the 47% sucrose layer was collected from the bottom as purified virus. The polyethylenglycol method was not applied to egg-borne Sendai virus. Instead, Sendai virus in allantoic fluids was first concentrated onto a 60% sucrose cushion through a 20% sucrose layer by centrifugation at 82,000 *g* for 60 min at 4 C and purified by the same discontinuous sucrose density gradient centrifugation as mentioned above.

Analysis of viral RNA. ^{3}H-uridine-labeled purified virions were sedimented by centrifugation at 189,000 *g* for 60 min at 4 C and subjected to RNA extraction (2). RNA was dissolved in 0.2 ml of re-distilled water and stored at −20 C until use.

For sedimentation analysis, 0.1 ml of the RNA appropriately diluted in NTE (0.1 M NaCl–0.01 M Tris-HCl–0.001 M EDTA, pH 7.5) buffer was layered on a 15 to 30% sucrose density gradient in NTE buffer containing 0.3% SDS and sedimented at 48,000 rpm for 95 min at room temperature in a Spinco SW 50.1 rotor. ^{3}H-

uridine-labeled ribosomal RNA of 18S and 28S species prepared from SR-CDF1-DBT mouse cells (12) was run in a parallel tube as a reference. Fractions were collected from the bottom and assayed for 5% trichloroacetic acid (TCA)-insoluble radioactivity.

The molecular size of viral RNA was determined by electrophoresis in 0.5% agarose–2% polyacrylamide gels (24). The gels were prepared in plastic tubes, 0.6 cm in diameter and 14 cm in length, and pre-run for 30 min with E buffer (0.036 M Tris-HCl–0.03 M Na_2PO_4–0.001 M EDTA, pH 7.6) containing 0.2% SDS and 10% glycerol. Then, 0.05 ml of the RNA in E buffer with 20% glycerol was applied to the gels and they were electrophoresed for 3 hr at 5 mA/gel at room temperature. The gels were sliced, and each slice was incubated with 0.5 ml of 3% ammonia at 50 C overnight for solubilization. Radioactivity was counted in toluen scintillator plus Triton X-100 (2:1).

Self-annealing of RNA was estimated as follows. One-tenth ml of RNA appropriately diluted in 0.4 M NaCl was incubated at 70 C for 24 hr. Before and after the incubation, 0.9 ml of 0.3 M NaCl–0.03 M sodium citrate containing 20 μg RNase A was added to aliquots of the sample, and the mixtures were incubated at 37 C for 30 min. The residual 5% TCA-insoluble radioactivity was expressed as percentage of the label in the original sample.

For detection of Poly(A) sequences in RNA, polyuridylic acid (Poly(U))-fiberglass filters were prepared and the binding ability of radioactive RNA to the filter was determined by the method of Sheldon et al (26).

SDS-PAGE of viral structural proteins. Purified virions were pelleted as described above and disrupted by boiling for 3 min in a small amount of 0.1 M phosphate buffer of pH 7.2 containing 1% SDS, 0.0125% dithiothreitol, 0.625 M sucrose, and 0.00125% bromophenol blue. In the case of PIV-3, a part of purified virus was suspended in 5 ml of PBS contained 1 M KCl and 1% Triton X-100. This was sonicated at 20 Kc for 60 sec and centrifuged at 215,000 *g* for 2 hr. The resulting pellet will be referred to as Triton-ppt. The remaining proteins in the supernatant were precipitated by treatment with 5% TCA followed by centrifugation at 1,200 *g* for 20 min. To remove TCA and Triton X-100, the precipitates were washed serially with 5 ml of aceton plus one drop of liquid ammonia, 2 ml of aceton, 5 ml of *n*-butanol (22) and finally 2 ml of ether, each time resuspending the pellet in the solvent and centrifuging it at 1,200 *g* for 20 min. The precipitates will be referred to as Triton-sup. Triton-ppt and Triton-sup were then disrupted with SDS and dithiothreitol as mentioned above.

These disrupted samples were applied to slab gels prepared between 2 glass plates. Our PAGE method essentially followed the continuous system of Maizel et al (14). The gels were 7.5% polyacrylamide and electrophoresed at 30 mA/gel for 17 hr. After electrophoresis, the gels were fixed with 5% acetic acid–5% methanol–2% glycerol (AMG) plus 5% TCA, stained with 0.05% Coomasie brilliant blue in AMG and destained by repeated washing in AMG. The stained gels were scanned for optical density at 570 nm. For determination of radioactivity, the gels were dried under vacuum onto a Whatmann 3MM paper and cut into small slices which

were then burnt in Packered TRI-CARB sample oxidizer model 306 to separate ^{14}C- and ^{3}H-radioactivity.

RESULTS

Buoyant Density of PIV-3

PIV-3 labeled with ^{3}H-leucine was concentrated from culture fluids by polyethylenglycol precipitation, as described in Materials and Methods, and sedimented through a linear sucrose density gradient (20 to 55% w/v in NTE buffer) at 25,000 rpm for 16 hr at 4 C in a Spinco SW 27-1 rotor. As presented in Fig. 1, a sharp peak of infectivity coincided with the peak of hemagglutinating activity and that of TCA-insoluble radioactivity, being located at the density of 1.197 which was very similar to the value reported by Tsai and Thomson (32). An electron microscopic study of the peak revealed roughly spherical virus particles carrying spikes on the surface, sharing common features with other viruses of the paramyxovirus group (Fig. 2) and resembling closely the electron micrographs of PIV-3 presented by Waterson et al (33). The size of particles ranged from 80 nm to 300 nm in diameter with a mean value of 130 to 140 nm. The virus in the peak formed a band at the top of the 47% sucrose layer in the discontinuous sucrose density gradient used for virus purification. Occasionally PIV-3 formed another minor band between the 47% and 52% sucrose layers (lower band), depending on each virus preparation. The lower band

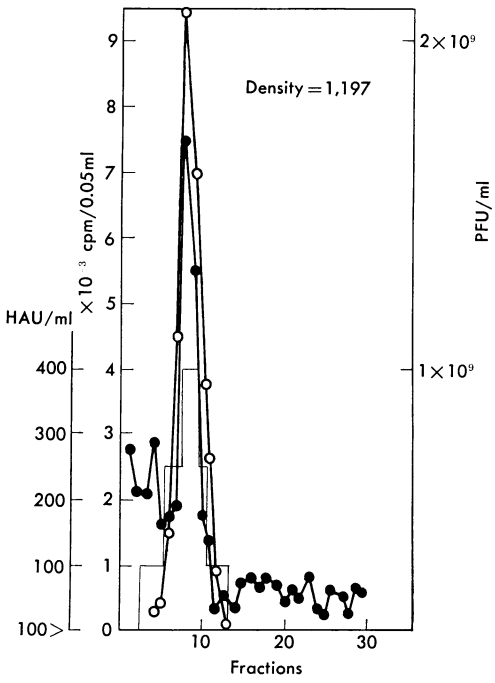

Fig. 1. Buoyant density of PIV-3. ^{3}H-leucine-labeled PIV-3 concentrated from culture media was sedimented through 20 to 55% sucrose density at 25,000 rpm for 16 hr in a Spinco SW 27–1 rotor. Each fraction was assayed for plaque titer (○), hemagglutinin (column) and TCA-insoluble radioactivity (●). Sedimentation was from right to left.

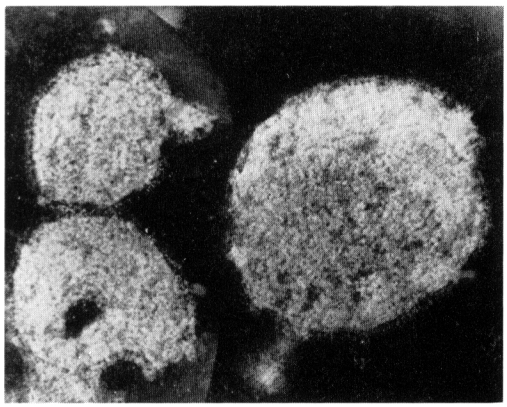

Fig. 2. Electron micrograph of PIV-3 negatively stained with phosphotungstic acid. The viruses were recovered from the peak fractions presented in Fig. 1. $\times$ 112,000.

was revealed by electron microscopy to contain virions identical in size and shape to the one described above and little contaminating cellular debris. However, the infectivity and hemagglutinin titer per mg of protein of the lower band were about one-tenth those of the band at the top of the 47% sucrose layer (data not shown). Therefore, virions in the lower band were not analyzed in subsequent experiments.

RNA of PIV-3

Sedimentation profiles of ^{3}H-uridine-labeled RNA of PIV-3 and Sendai virus are presented in Fig. 3, where the radioactivity of PIV-3 RNA was mainly located at 42S, while that of Sendai virus RNA at 47S.

In order to examine whether or not PIV-3 RNA was a single continuous nucleotide chain, it was incubated with 95% dimethylsulfoxide at 37 C for 5 min so that double-stranded RNA would be denatured. Even after the treatment, most of the radioactivity was found at 41S (not shown in the figure), which was approximate to the value of native PIV-3 RNA. This indicated that PIV-3 RNA, like Sendai virus RNA, was a single continuous chain of RNA.

To determine the size of RNA, PIV-3 and Sendai virus RNA were electrophoresed parallely in 0.5% agarose–2% polyacrylamide gels. PIV-3 RNA migrated faster than Sendai virus RNA as a homogeneous peak (Fig. 4). On the basis that molecular weights of 28S and 18S ribosomal RNA were 1.71 and 0.70×10^6 daltons (13), the molecular weights of PIV-3 and Sendai virus RNA, as averaged from results of 3 experiments, were determined to be 4.5 ± 0.3 and $5.3 \pm 0.3 \times 10^6$ daltons, respectively. The sedimentation rate and molecular weight of Sendai virus RNA presented here are in good agreement with the sedimentation rate determined relative to ribosomal RNA (9) and the molecular weight determined by length measurement in the electron microscope (11).

Virion RNA of Sendai virus has been shown to self-anneal from 16 to 60% (10, 20, 21). It was therefore of interest to examine if PIV-3 RNA also partially self-annealed. ^{3}H-uridine-labeled PIV-3 RNA and Sendai virus RNA, before and after

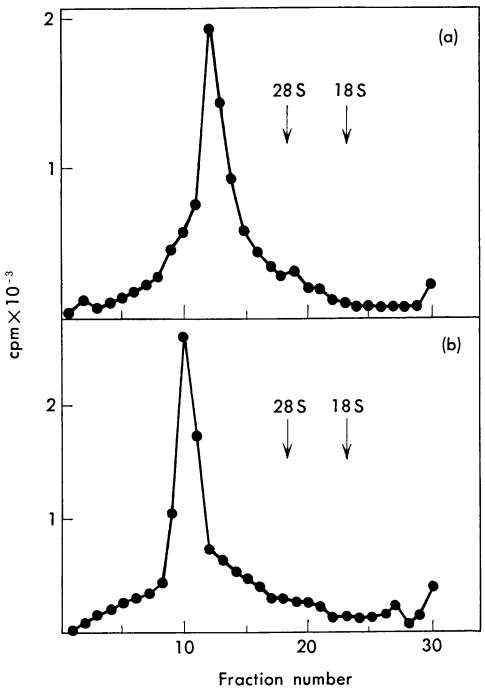

Fig. 3. Sedimentation of RNA extracted from ^{3}H-uridine-labeled virions of PIV-3 (a) and Sendai virus (b) through 15 to 30% sucrose density gradient containing 0.3% SDS at 48,000 rpm for 95 min at 24 C in a Spinco SW 50.1 rotor. Reference ribosomal RNA was sedimented in a parallel centrifuge tube and its positions (28S and 18S) are shown by arrows. Fractions were analyzed for TCA-insoluble radioactivity.

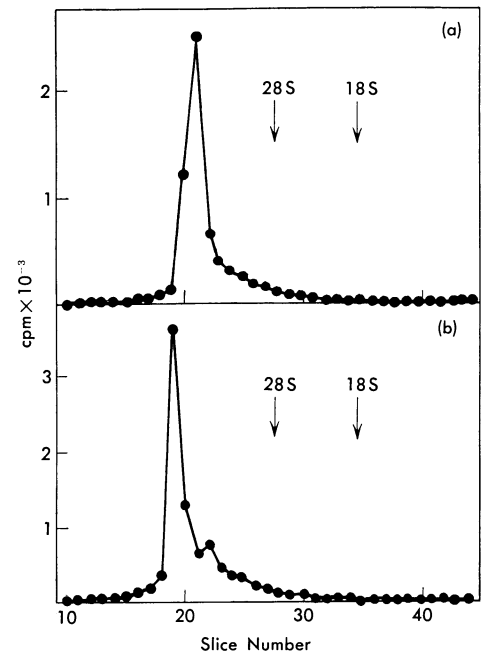

Fig. 4. Electrophoresis of ^{3}H-uridine-labeled PIV-3 RNA (a) and Sendai virus RNA (b) in 0.5% agarose-2% polyacrylamide gel. Arrows indicate the positions of 28S and 18S ribosomal RNA run on a parallel gel. Migration was from left to right.

Table 1. RNase resistance of ^{3}H-uridine-labeled PIV-3 RNA and Sendai virus RNA before and after self-annealing

RNA sample	Percent resistance to RNase	
	Before self-annealing	After self-annealing
PIV-3	4.2	14.8
Sendai	6.4	14.5

Table 2. Retention of ^{3}H-uridine-labeled viral RNA by Poly(U)-fiberglass filters

RNA sample	Percent retention	
	Native	Heated[b]
PIV-3	3	1
Sendai	3	1
MHV[a]	21	24

[a] Mouse hepatitis virus.
[b] RNA was heated at 100 C for 2 min in the presence of 1 μg/ml of non-labeled Poly(A).

self-anneal treatment, were assayed for RNase resistance. As revealed in Table 1, 4% of PIV-3 RNA was resistant to RNase before self-annealing, but the resistant fraction was 15% after that. This result indicated that PIV-3 and Sendai virus RNA were alike in this point.

PIV-3 and Sendai virus RNA were tested for Poly(A) sequences. When ^{3}H-uridine-labeled PIV-3 or Sendai virus RNA was filtered through a Poly(U)-fiberglass filter, only 3% of the radioactivity was retained on the filter (Table 2). In contrast, about 21% of mouse hepatitis virus (a member of the coronavirus group) RNA, possessing a Poly(A) sequence (34), was retained on the filter. The relatively low extent of binding of mouse hepatitis virus RNA was difficult to explain but a similar low efficiency of Poly(U)-filter binding was observed also with another Poly(A)-containing viral RNA (30). To exclude the possibility that PIV-3 and Sendai virus RNA contained Poly(A) hybridized with Poly(U), they were incubated at 100 C for 2 min in the presence of 1 μg/ml of non-labeled Poly(A) and then heat-denatured RNA was applied to Poly(U)-fiberglass filters. The result is presented also in Table 2 which testified that no appreciable amount of RNA was retained on the filters. It is suggested from these results that PIV-3 and Sendai virus RNA do not contain Poly(A) any sequence and consequently PIV-3, like Sendai virus, has virion-associated RNA polymerase.

PAGE of PIV-3 Structural Proteins

The viral structural proteins were analysed by PAGE using PIV-3 doubly labeled with ^{14}C-leucine and ^{3}H-glucosamine. Figure 5 presents stained gels showing the proteins of PIV-3 grown in Vero cells and egg-borne Sendai virus simultaneously electrophoresed. Figure 6a demonstrates distribution of radioactivities of ^{14}C-leucine

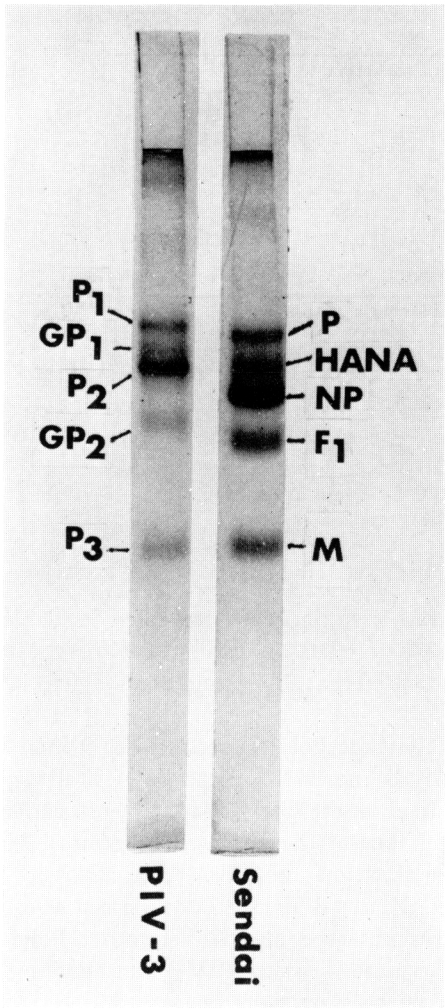

Fig. 5. Gel stained by Coomasie brilliant blue showing patterns of structural proteins of PIV-3 and egg-borne Sendai virus separated by PAGE; P, polymerase; HANA, hemagglutinin-neuraminidase; NP, nucleocapsid protein; F, fusion protein; M, membrane protein.

and ^{3}H-glucosamine in another gel. These figures show that PIV-3 has at least 5 proteins, of which 2 are glycoprotein. We designated the PIV-3 proteins as P_1, P_2, P_3, glycoprotein$_1$ (GP_1), and GP_2. Analysis of Triton X-100-treated samples electrophoresed in other slots of the gel identical to that of Fig. 6a revealed that the radioactivity of ^{3}H-glucosamine of GP_1 remained entirely in Triton-sup (see Materials and Methods) (Fig. 6b) and did not sediment with Triton-ppt (Fig. 6c), whereas Triton-ppt showed a single sharp peak of ^{14}C-radioactivity of P_2 (Fig. 6c). These facts suggested that GP_1 was an envelope-associated glycoprotein and P_2 nucleocapsid protein (22). The insufficient recovery of GP_2, P_1, and P_3 after Triton X-100 treatment could not be explained but was supposed to be due to loss of these proteins dur-

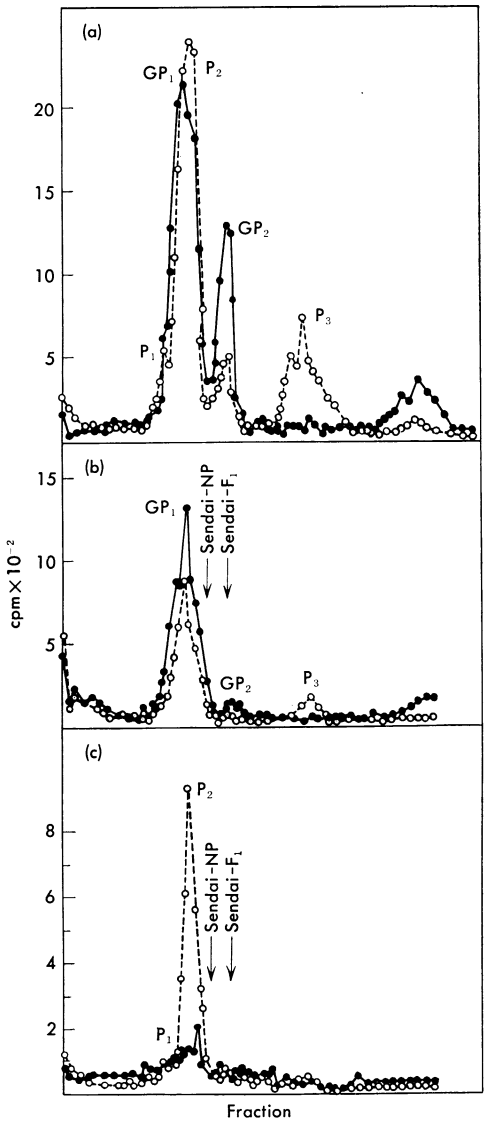

Fig. 6. Distribution of ^{14}C-leucine (○) and ^{3}H-glucosamine (●) of PIV-3 proteins in PAGE. (a), Whole virion. (b), Triton-sup (see Materials and Methods). (c), Triton-ppt. Migration was from left to right.

ing repeated washing with solvents. Periodic acid-Schiff (PAS) staining of gels confirmed that GP_1 and GP_2 were glycoproteins whereas P_1, P_2, and P_3 were not (not shown).

From the results, the molecular weights of PIV-3 structural proteins were calculated, comparing their migration rates with those of Sendai virus proteins of known molecular weights (8). Thus, P_1, P_2, P_3, GP_1, and GP_2 of PIV-3 were estimated to be 79,000, 68,000, 35,000, 69,000, and 55,000 daltons, respectively.

DISCUSSION

The results presented here give us some additional informations about the basic nature of PIV-3.

The RNA of PIV-3 was a single continuous one with a molecular weight of 4.5×10^6 daltons which had a tendency to self-anneal to a high extent and lacked Poly(A) sequence, showing similarities to the RNA of Sendai virus. These observations suggest that PIV-3 RNA is a negative stranded and therefore the virus carries virion-associated RNA-polymerase. In fact, we have found the enzyme activity of PIV-3 and studies on its nature are now in progress.

PIV-3 was shown in PAGE to have at least 5 main structural proteins of which 2 were glycoproteins. The finding that P_2 protein was recovered from the sediment of a high speed centrifugation of Triton X-100–1 M KCl-disrupte virions (Fig. 6c) suggests that P_2 is the nucleocapsid protein (22). The facts that PIV-3 grown in Vero cell culture has hemolytic activity and PIV-3 does not require, in contrast to Sendai virus, the addition of trypsin for plaque formation suggest that PIV-3 grown in Vero cell culture has a cleaved form of the fusion protein. PIV-3 is different in this respect from Sendai virus (4–7, 23, 27, 28), parainfluenza virus type 4 (17) and some strains of Newcastle disease virus (19). PIV-3 is known to have neuraminidase activity (3) and conceived to carry RNA-polymerase as stated above. Thus, we tried to tentatively identify structural proteins of PIV-3 by comparing them to egg-borne Sendai virus proteins (Fig. 5) as follows: P_1 is associated with RNA-polymerase activity, GP_1 hemagglutinin-neuraminidase protein, GP_2 fusion protein, and P_3 membrane protein. Of course substantiation of this relationship needs further study. It also remains to be investigated whether or not the neuraminidase activity of PIV-3 is associated with hemagglutinin like simian virus 5 (22) and Sendai virus (31). The total molecular weight of PIV-3 main structural proteins was calculated to be 306,000 which is very similar to that of Sendai virus main structural proteins, 302,000, showing a reflexion of similarity between PIV-3 RNA and Sendai virus RNA in size.

In a parallel study (29), we found that PIV-3, like Sendai virus, induce hydrocephalus in mice when administrated intracerebrally to new born mice. This finding means that PIV-3 and Sendai virus share another common characteristic in a biological behavior.

In summary, this study revealed that PIV-3, as a member of the paramyxovirus group, shares a number of common characteristics with egg-borne Sendai virus in viral structural components.

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