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# Structural Characterization of Virion Proteins and Genomic RNA of Human Parainfluenza Virus 3

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**The virion proteins and genomic RNA of human parainfluenza virus 3 have been characterized. The virion contains seven major and two minor proteins. Three proteins of 195 × $10^3$ molecular weight (195K), 87K, and 67K are associated with the nucleocapsid of the virion and have been designated L, P, and NP, respectively. Three proteins can be labeled with [$^{14}$C]glucosamine and have molecular weights of 69K, 60K, and 46K. We have designated these proteins as HN, $F_0$, and $F_1$, respectively. HN protein has interchain disulfide bonds, but does not participate in disulfide bonding to form homomultimeric forms. $F_1$ appears to be derived from a complex, $F_{1,2}$, that has an electrophoretic mobility similar to that of $F_0$ under nonreducing conditions. A protein of 35K is associated with the envelope components of the virion and aggregates under low-salt conditions; this protein has been designated M. The genome of human parainfluenza virus 3 is a linear RNA molecule with a molecular weight of approximately 4.6 × $10^6$.**

Human parainfluenza virus 3 (HPIV3) causes serious lower respiratory tract infections and is second only to respiratory syncytial virus as a cause of bronchiolitis and pneumonia (8, 27). Furthermore, because HPIV3 infections occur early in life, are readily transmissible, and are likely to recur (8, 12, 27), HPIV3 infections are an important clinical problem. Despite its importance as an infectious agent, little is known about the structural components of the HPIV3 virion.

Sendai virus is the most thoroughly studied parainfluenza virus. Sendai virions contain a large single-stranded RNA genome with a molecular weight of approximately 5 × $10^6$ (1, 2, 16, 18). Virions of Sendai contain at least six major structural proteins from 145 × $10^3$ molecular weight (145K) to 34.7K when grown in cultured cells (20, 26, 31). The envelope fraction of Sendai virus contains the M, HN, and F proteins (20), whereas the nucleocapsid contains the L, NP, and P proteins (25, 38). The biological roles of the major structural proteins have also been ascertained. L, NP, and P proteins have all been shown to be involved in RNA polymerase activity (5, 11, 25, 38). The M protein is located on the inner surface of the lipid bilayer of the viral envelope, where it is probably responsible for maintaining the structural integrity of the virus. The large glycoprotein, HN, has both hemagglutinin and neuraminidase activity (28, 30, 31, 39). The HN protein of Sendai virus is believed to be present on the surface of the virion as a dimer joined by disulfide bonds in the hydrophilic region and by hydrophobic bonds at the base (6, 22, 34). The other glycoprotein is involved in virus-induced hemolysis, membrane fusion, and initiation of infection (13, 14). The active fusion protein ($F_{1,2}$) of Sendai virus is formed by a proteolytic cleavage of an inactive precursor ($F_0$) to form two disulfide-linked proteins, $F_1$ and $F_2$ (6, 30, 32, 33).

Detailed work of this nature has not been carried out on parainfluenza virus type 3 viruses. Virions of bovine parainfluenza virus 3 (BPIV3) contain five proteins of 79K, 69K, 68K, 55K, and 35K (24, 35, 36). Two of these proteins (69K and 55K) are glycosylated (35). In addition, BPIV3 contains RNA with a sedimentation coefficient of 42S and a molecular weight of 4.5 × $10^6$ (35). Preliminary reports on HPIV3 has indicated that it has eight or nine virion proteins, two of which are glycoproteins (10), ranging from 125K to 17K. Structural similarities between parainfluenza 3 viruses and Sendai virus seem to exist. In this report, we describe the structural components of HPIV3 in detail.

## MATERIALS AND METHODS

**Cells and virus.** LLC $MK_2$, a continuous monkey kidney cell line, was obtained from Flow Laboratories and grown in monolayer culture in Dulbecco modified minimal essential medium supplemented with 5% heat-inactivated fetal calf serum. The virus used in this study, HPIV3 strain C-243, was provided by D. A. McLeod of the Laboratory Centre for Disease Control, Ottawa, Canada. The strain C-243 was originally obtained from the American Type Culture Collection (strain 47885).

**Plaque assay.** Serial dilutions of the virus were inoculated onto monolayer cultures of LLC $MK_2$ cells and allowed to adsorb for 1 h. Cultures were overlaid with Dulbecco modified minimal essential medium containing 0.9% Noble agar, incubated at 37°C for 4 days, and subsequently overlaid with 1 ml of a 1/8,000 dilution of neutral red in phosphate-buffered saline for an additional 24 h before plaques were counted.

**Radiolabeling of virion proteins and virus preparation.** Proteins were labeled with radioactive precursors immediately after the adsorption period of 1 h. Viral proteins were labeled with [$^{35}$S]methionine (10 μCi/ml, 1,295 Ci/mmol; Amersham Corp.), a mixture of $^{14}$C-amino acids (5 μCi/ml, ≃300 mCi/mmol; Amersham), or [$^{14}$C]glucosamine hydrochloride (5 μCi/ml, 309 mCi/mmol; Amersham), and culture medium containing virus was harvested 48 h after infection. The culture medium was clarified by low-speed centrifugation to remove the cellular debris. Virus was then pelleted by high-speed centrifugation (113,000 × g, 2 h, 4°C), suspended in sample buffer (6.25 mM Tris-hydrochloride [pH 6.8], 4% sodium dodecyl sulfate [SDS], 20% glycerol, 0.02% bromophenol blue, and with or without 4% dithiothreitol), and then analyzed by SDS-polyacrylamide gel electrophoresis.

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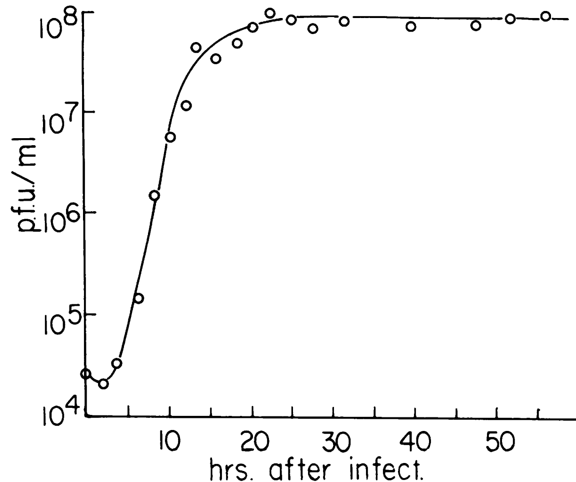


FIG. 1. Growth curve of HPIV3 in LLC $MK_2$ cells. Cell cultures ($7 \times 10^6$ cells) were infected at a multiplicity of infection of 3. Samples were assayed for infectivity by plaque assay with LLC $MK_2$ cells.

**Polyacrylamide gel electrophoresis.** SDS-polyacrylamide gel electrophoresis of the viral proteins was carried out as previously reported (19). After electrophoresis, the proteins were transferred onto nitrocellulose paper by electroblotting (4) and exposed to X-Omat AR X-ray film (Eastman Kodak Co.) at −70°C.

**Triton X-100 fractionation of the virion proteins.** Triton X-100 fractionation of the virus proteins was carried out as previously reported (31, 28, 29).

**Extraction and electrophoresis of HPIV3 RNA.** [$^3$H]uridine was added to the culture medium for the period between 12 and 24 h after infection of LLC $MK_2$ cells to label HPIV3 RNA. Virus was pelleted from clarified culture medium, disrupted with 0.5% SDS, and digested with 200 μg of proteinase K per ml at 37°C for 30 min. Virion RNA was isolated by phenol-chloroform-isoamyl alcohol (25:24:1) extraction and precipitated with ethanol. HPIV3 RNA was then denatured with glyoxal and electrophoresed in 1% agarose gels (23). The gels were impregnated with 2,5-diphenyloxazole, dried, and exposed to X-ray film at −70°C (3).

## RESULTS

**Growth of HPIV3 in LLC $MK_2$ cells.** A difficulty encountered when working with HPIV3 has been low virus yields from cultured cells (10). To overcome this problem, we have grown virus in LLC $MK_2$ cells. Figure 1 shows the growth curve of HPIV3 in LLC $MK_2$ cells after infection at a multiplicity of 3 PFU/cell. A titer of $9 \times 10^7$ PFU/ml was generated within 24 h postinfection. With a multiplicity of infection of 0.1 PFU/cell, $2 \times 10^8$ and $6 \times 10^8$ PFU/ml have been attained. It should be noted that once the maximum titer of virus was achieved, it was maintained for at least 55 h and for up to 72 h postinfection. The formation of polykaryocytes was first observed in these cultures at 12 h postinfection. Cell destruction became prominent at 50 to 70 h postinfection.

**Structural proteins of HPIV3.** Virus was labeled with [$^{35}$S]methionine, harvested at 48 h postinfection, and analyzed by SDS-polyacrylamide gel electrophoresis (Fig. 2, lane 2). Proteins of known molecular weights ($^{14}$C-labeled high-molecular-weight standards; Bethesda Research Laboratories, Inc.) were electrophoresed in the same gel to determine the molecular weights of virion proteins (Fig. 2, lane 1). The virions of HPIV3 contained seven major proteins of 195K, 87K, 69K, 67K, 46K, 44K, and 35K and two minor proteins of 60K and 20K. Three proteins of 69K, 60K, and 46K were labeled with [$^{14}$C]glucosamine and are considered to be glycoproteins (Fig. 2, lane 4). The electrophoretic profile of HPIV3 was similar to that of BPIV3 (24) and Sendai virus (30, 31). On this basis we have tentatively designated six of the major proteins as L, P, HN, NP, $F_1$, and M. The nature of 44K protein is unknown. However, cellular actin, with a similar molecular weight, has commonly been reported to be associated with virions of other paramyxoviruses (7, 9, 21, 24). We believe that the minor 60K protein labeled with [$^{14}$C]glucosamine may correspond to the $F_0$ protein of the other paramyxoviruses. We have not been able to designate a protein that corresponds to $F_2$; however, a likely candidate seems to be the 20K protein.

**Native disulfide bonding of HPIV3 virion proteins.** To examine HPIV3 proteins that participate in native disulfide bonding, [$^{35}$S]methionine- or [$^{14}$C]glucosamine-labeled virion proteins were separated by SDS-polyacrylamide gel electrophoresis under either reducing or nonreducing conditions. On the basis of band intensity, it appeared that the NP band migrated to the same position under both reducing (Fig. 3a, lane 2) and nonreducing conditions (Fig. 3a, lane 1, band c). This suggests that intrachain disulfide bonding does not play a major role in the structure of NP protein. Intrachain bonding must occur in the M and P proteins because they did not migrate to the same positions under nonreducing conditions (compare Fig. 3a, lanes 1 and 2). It is likely that M protein runs faster under nonreducing conditions and corresponds to band e (Fig. 3, lane 1). The migration of P protein under nonreducing conditions had not been firmly established. P protein may migrate faster under nonreducing conditions and correspond to band b or one of the faint bands between bands b and e (Fig. 3, lane 1). It is likely that P does not form a dimer and migrate under nonreducing conditions as band a because band a is glycosylated (Fig. 3a, lane 1, and Fig. 3b, lane 1).

To facilitate the identification of the glycoprotein species, both a two-dimensional nonreducing-reducing gel system

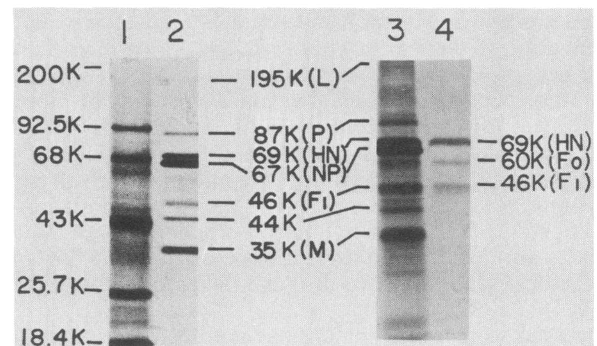


FIG. 2. Polyacrylamide gel analysis of HPIV3 proteins. $^{14}$C-amino acid-labeled myosin (200K), phosphorylase B (92.5K), bovine serum albumin (68K), ovalbumin (43K), chymotrypsinogen (25.7K), and β-lactoglobulin (18.4K) (lane 1), [$^{35}$S]methionine-labeled HPIV3 proteins (lanes 2 and 3), and [$^{14}$C]glucosamine-labeled proteins (lane 4) were analyzed by electrophoresis in 15% polyacrylaminde gels containing 0.1% SDS.

(37), and one-dimensional gels were used. Under nonreducing conditions, HPIV3 glycoproteins resolved into three bands (Fig. 3b, lane 1) labeled band a and a double band d, which contained a slower-migrating predominant protein and a faint faster-migrating protein. In the two-dimensional gel system (Fig. 4), band d migrated in the reducing dimension as a predominant T-shaped spot and two fainter faster-migrating spots. The molecular weight of the T-shaped spot corresponded to that of HN. Furthermore, the two fainter spots migrated to the same position as $F_0$ and $F_1$ under reducing conditions (Fig. 4). These findings indicate that band d (Fig. 3a and b) is composed of HN, $F_0$, and $F_{1,2}$, with HN being the predominant slower-migrating protein. This implies that both $F_0$ and the cleaved $F_{1,2}$ proteins are present on HPIV3 virions. It also indicates that HN is not present as a disulfide-linked multimer on the virion. The nature of the high-molecular-weight material (Fig. 3b, lane 1, band a) is unknown. It is unlikely that this material is disulfide-linked HN because no material with the molecular weight of HN or $F_0$ is derived from it in our two-dimensional gels (Fig. 4). Furthermore, in our two-dimensional gels, band a is located on the diagonal, which indicates that disulfide bonds do not affect the migration of this protein.

**Location of the virion proteins.** The envelope proteins of Sendai virus, simian virus 5, and Newcastle disease virus have been separated from nucleocapsid proteins by disrupting virions with Triton X-100 in the presence of 1 M KCl (13, 20, 28, 29). A similar procedure has been used to determine the location of each of the proteins in the HPIV3 virion. Figure 5, lane 1, shows the electrophoretic pattern of virion proteins of HPIV3 labeled with [$^{35}$S]methionine. To solubilize the envelope components, virions were disrupted with 2% Triton X-100 and 1 M KCl. After ultracentrifugation, proteins associated with the soluble portion or the pellet were compared by SDS-polyacrylamide gel electrophoresis. Proteins in the pellet (Fig. 5, lane 2) resembled those found in intact virus, except there was a large reduction in the amounts of HN and M relative to other proteins. Both $F_0$ and $F_1$ proteins were associated with the pelletable nucleocapsid material and not the soluble envelope fraction. The predominant protein bands in the soluble portion (Fig. 5, lane 3) were the HN, 44K, and M protein bands. Some NP protein was found in the soluble fraction (Fig. 5, lane 3), but it was greatly reduced in amount compared with the pelletable material and the total viral NP protein. The envelope components were further separated by sucrose velocity sedimentation. The upper peak fraction (Fig. 5, lane 4) had a predominance of the HN protein and little M protein. The lower peak fraction contained predominantly the M protein, with a trace amount of HN and NP proteins (Fig. 5, lane 5). When the lower peak proteins were pooled, dialyzed to remove the salt, and ultracentrifuged, only the M protein was pelleted; no HN protein remained (Fig. 5, lane 6).

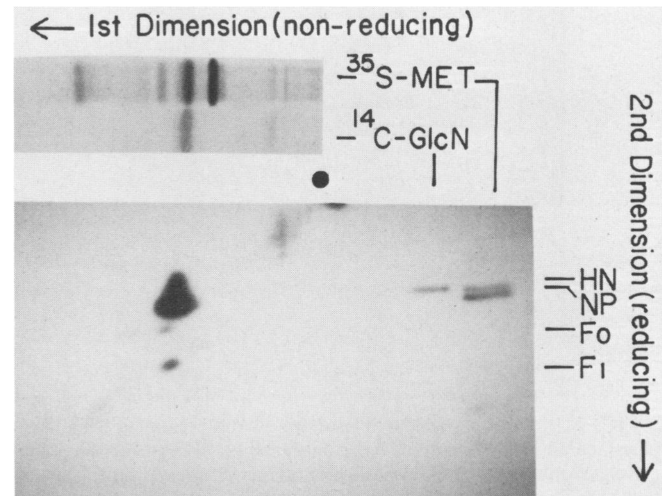


FIG. 4. Two-dimensional polyacrylamide gel electrophoresis of [$^{14}$C]glucosamine-labeled HPIV3 proteins. Radioactively labeled virion proteins were electrophoresed in the first dimension (horizontal) under nonreducing conditions. A single lane was cut from the gel, soaked in buffer containing 0.5 M Tris-hydrochloride (pH 6.8)–0.4% SDS–2% mercaptoethanol for 30 min, positioned on a 15% polyacrylamide gel containing 0.1% SDS, and electrophoresed in the second dimension (vertical). The black dot indicates the origin. The upper gel shows the position of proteins after electrophoresis under nonreduced conditions in the first dimension. Marker lanes in second-dimension gel indicate the position of proteins after electrophoresis under reduced conditions. Both [$^{14}$C]glucosamine- and [$^{35}$S]methionine-labeled proteins have been used as markers.

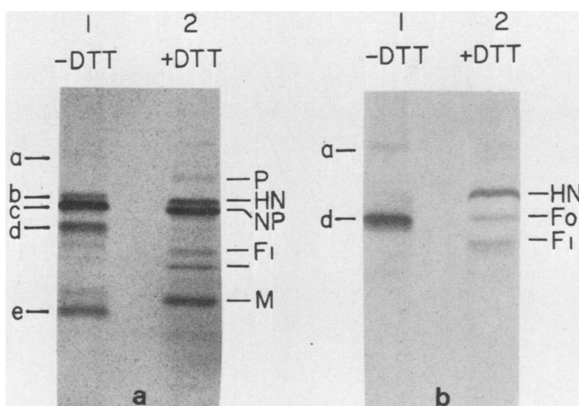


FIG. 3. Analysis of HPIV3 proteins in the presence or absence of a reducing agent. (A) [$^{35}$S]methionine-labeled proteins. (B) [$^{14}$C]glucosamine-labeled proteins. Lanes 1 contained proteins electrophoresed without dithiothreitol (DTT) in the sample buffer (nonreducing conditions). In lanes 2, dithiothreitol was added to sample buffer (reducing conditions). Predominant bands in the nonreduced samples have been designated a through e. The glycoproteins $F_0$ and HN have been assigned to band d on the basis of the two-dimensional gels (see Fig. 4).

**HPIV3 RNA.** To extend our examination of the structural components of HPIV3, we have also characterized its genomic RNA. It has been shown that measles virus genomic RNA is readily degraded, whereas Sendai virus genomic RNA is stable. To determine the nature of HPIV3 genomic RNA, it was labeled with [$^{3}$H]uridine between 12 and 24 h postinfection. Virion RNA was isolated by phenol-chloroform-isoamyl alcohol extraction and analyzed in a 1% agarose gel after glyoxal denaturation (Fig. 6). The molecular weight of the genomic RNA was determined by using rRNAs and vesicular stomatitis virus genomic RNA as markers. The RNA genome of HPIV3 appeared to be relatively easy to isolate in an intact form and had a molecular weight of approximately $4.6 \times 10^6$.

## DISCUSSION

We have studied the structural components of HPIV3. The isolation of the virion components in sufficient quanti-

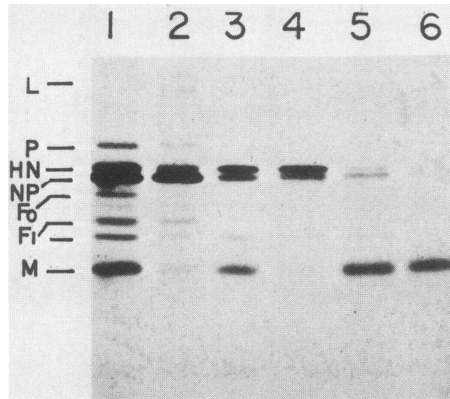


FIG. 5. Location of HPIV3 proteins in the virion. [$^{35}$S]methionine-labeled HPIV3 proteins were analyzed by SDS-polyacrylamide gel electrophoresis after various treatments. Lanes: 1, total virion proteins; 2, pelleted nucleocapsid proteins after treatment with 2% Triton X-100 and 1 M KCl; 3, soluble envelope components after treatment with 2% Triton X-100 and 1 M KCl; 4, upper peak fraction after 10 to 25% sucrose gradient velocity ultracentrifugation; 5, lower peak fraction after sucrose velocity sedimentation; 6, pelletable low-salt aggregate from lower peak fraction.

ties was made possible by the growth of HPIV3 to high titers in LLC $MK_2$ cells (Fig. 1). HPIV3 contains at least seven major and two minor structural proteins (10) (Fig. 2) ranging from 195K to 20K (Fig. 2). On the basis of physical characteristics and comparison to other paramyxoviruses, we have assigned seven HPIV3 proteins as L, P, HN, NP, $F_0$, $F_1$, and M. In addition, we have determined the size of the HPIV3 genome.

The L protein of HPIV3 was designated on the basis of its high molecular weight (195K, Fig. 2), its association with the nucleocapsid (Fig. 5), and its lack of abundance in the virion (Fig. 2 and 3). In Sendai virus, L also has a high molecular weight (>160K) and is the least abundant protein in the virion, being present in virion with an average of 40 molecules (21). The P protein of HPIV3 is the second largest protein in the virion (Fig. 2), and it also is associated with the nucleocapsid (Fig. 5). Intrachain disulfide bonding affects the migration of P protein in nonreducing gels (Fig. 3a). The P proteins of Sendai virus, simian virus 5, and Newcastle disease virus all have been shown to be associated with the nucleocapsid. Work with BPIV3 indicates that an 88K protein, designated P, is associated with the nucleocapsid (24, 35). Sendai virus P protein has also been shown to participate in native disulfide bonding to form multimeric forms (22). The P protein of HPIV3 does not appear to participate in disulfide bonding to form multimers.

We have designated the 69K protein of HPIV3 as the HN protein on the basis of its glycosylation, abundance (Fig. 2, lane 1), and association with envelope components after Triton X-100 solubilization in high salt (Fig. 5). These characteristics correspond to the properties of the 69K HN glycoprotein of Sendai virus (31, 32) and to the 69K glycoprotein of BPIV3 (35, 36). A major difference between HPIV3 HN protein and the HN proteins of the other paramyxoviruses seems to be in their native disulfide bonding. The HN protein of Newcastle disease virus is mainly present on the virion as a disulfide-linked dimer; for Sendai virions, HN is found primarily as tetramers or dimers, with only a small amount not found disulfide linked (22). We have shown that HN protein is not found on the surface of HPIV3 as a disulfide-linked dimer or multimer because it migrates with an approximate molecular weight of $60 \times 10^3$ (Fig. 3b, band d) under nonreducing conditions. This result suggests that intrachain disulfide bonds affect the migration of HN under nonreduced conditions. The nature of the high-molecular-weight material (band a in Fig. 3a and b and at the top of Fig. 4) has not been determined. However, it probably does not represent HN dimers.

Nucleocapsid protein of Sendai virus, simian virus 5, and Newcastle disease virus is the most abundant protein in the virion (21, 25). We found that the 67K protein of HPIV3 is the most abundant protein in the virion (Fig. 2). Furthermore, this protein is predominantly found associated with the pelletable nucleocapsid (Fig. 5, lane 2). Accordingly, we have assigned 67K protein as NP.

We believe that HPIV3 contains two glycosylated forms of the fusion protein, $F_0$ and $F_{1,2}$. Under nonreducing conditions, these two proteins migrate together with an apparent molecular weight of approximately $60 \times 10^3$ (Fig. 3b, lane 1, and Fig. 4). This suggests that intrachain disulfide bonds are affecting the migration of $F_0$ under nonreducing conditions. Identification of a $F_2$ subunit in HPIV3 would have confirmed this hypothesis; however, we do not yet have conclusive evidence for the existence of $F_2$. It is apparent (Fig. 5, lane 2) that both $F_0$ and $F_1$ proteins pellet with the nucleocapsid material. The nature of the association between these two glycosylated proteins and the nucleocapsid is not known.

The 44K protein may well be cellular actin, since this band disappears when the virus is grown in actinomycin D and cycloheximide-pretreated cells (data not shown). The 44K protein is associated with the HN protein of HPIV3 (Fig. 5, lane 4). This is in contrast to the cellular actin found in association with the M protein of Sendai virus (9).

The M protein of HPIV3 has a molecular weight of $35 \times 10^3$, is associated with the envelope of the virion, and aggregates under low-salt conditions, and intrachain disulfide bonds affect its migration pattern in nonreduced SDS-polyacrylamide gel electrophoresis. These characteristics correspond to those of the M proteins of Sendai virus and BPIV3 (20, 31, 35).

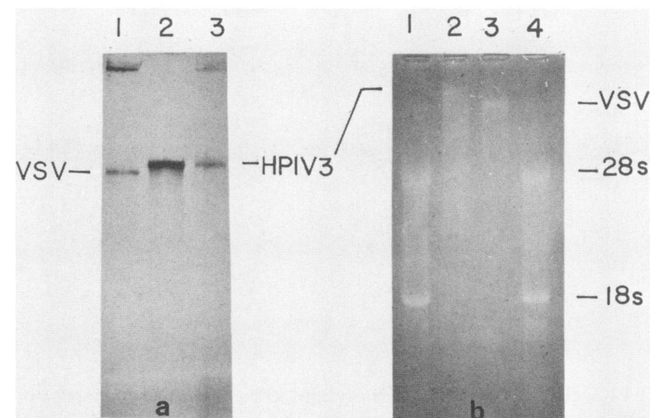


FIG. 6. Agarose gel electrophoresis of genomic RNA of HPIV3 after glyoxal denaturation. (a) Autoradiograph of [$^{3}$H]uridine-labeled HPIV3 genomic RNA isolated from two different preparations of virus (lanes 2 and 3) and vesicular stomatitis virus (VSV) genomic RNA (lane 1). (b) Ethidium bromide-stained gel. Lanes: 1 and 4, ribosomal markers; 3, vesicular stomatitis virus genomic RNA; 2, HPIV3 genomic RNA.

The genomic RNA of HPIV3 (molecular weight, $4.6 \times 10^6$) compares favorably in size to that of the other paramyxoviruses. Measles virus RNA has a molecular weight of $4.8 \times 10^6$ (40), and respiratory syncytial virus RNA has a molecular weight of $5 \times 10^6$ (15). The more closely related Sendai virus genome has a molecular weight of approximately $5 \times 10^6$ (17, 18, 35). Shibuta et al. (35) found that the BPIV3 genomic RNA has an approximate molecular weight of $4.5 \times 10^6$ and a sedimentation value of 42S.

In conclusion, we found that HPIV3 structural components correspond well with the previously reported components of the other parainfluenza viruses.

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