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Comparison of Genome Size and Synthesis of Structural Proteins of Hirame Rhabdovirus, Infectious Hematopoietic Necrosis Virus, and Viral Hemorrhagic Septicemia Virus

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Genomic RNA was extracted from purified virions of hirame rhabdovirus (HRV), infectious hematopoietic necrosis virus (IHNV), and viral hemorrhagic septicemia virus (VHSV). The full-length RNA was analyzed using formaldehyde agarose gel electrophoresis followed by ethidium bromide staining. Compared with an internal RNA size standard, all three viral genomic RNAs appeared to have identical relative mobilities and were estimated to be approximately 10.7 kilobases in length or about 3.7 megadaltons in molecular mass. Structural protein synthesis of HRV, IHNV, and VHSV was studied using cell cultures treated with actinomycin D. At 2 h intervals, proteins were labeled with ^{35}S -methionine, extracted, and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The five structural proteins of each of the three viruses appeared in the following order: nucleoprotein (N), matrix protein 1 (M1), matrix protein 2 (M2), glycoprotein (G), and polymerase (L) reflecting both the approximate relative abundance of each protein within infected cells and the gene order within the viral genome.

Hirame rhabdovirus (HRV) is an important virus associated with high mortality among stocks of cultured Japanese flounder (Gorie *et al.*, 1985; Kimura *et al.*, 1986) while infectious hematopoietic necrosis virus (IHNV) and viral hemorrhagic septicemia virus (VHSV) are important pathogens affecting several species of wild and cultured salmonid fish (Pilcher and Fryer, 1980; Wolf, 1988). The virions of IHNV, VHSV, and HRV are comprised of five structural proteins: polymerase (L), glycoprotein (G), nucleocapsid protein (N), and two matrix proteins (M1 and M2) (Lenoir and de Kinkelin, 1975; McAllister and Wagner, 1975; Hsu *et al.*, 1985; Nishizawa *et al.*, in press). All three viruses share biochemical and morphological characteristics typical of members of the *Lyssavirus* genus of the family *Rhabdoviridae* (Kimura *et al.*, 1989).

Rhabdoviruses have a negative sense, single-stranded RNA genome with a sedimentation coefficient (S) of 38-45, corresponding to a molecular mass of approximately 3.5-4.6 megadaltons (md) or about 11-12 kilobases (kb) in length (Banerjee, 1987; Wagner, 1987). The genomic RNAs of five fish rhabdoviruses, including IHNV and VHSV, have been analyzed by sucrose gradient centrifugation and found to have sedimentation coefficients of 38-40S (Hill *et al.*, 1975). Kurath and Leong (1985) used glyoxal-treated RNA and gel electrophoresis in 1% agarose to determine that the IHNV genome had an estimated mass of 3.7 md.

The genes of lyssaviruses are known to occur in the following order in the genome: 3'-N-M1-M2-G-L-5' (Banerjee, 1987; Wagner, 1987). Kurath *et al.* (1985) used R-loop mapping to determine the gene order for IHNV and found

it was typical of lyssaviruses with the exception of a novel gene coding for a non-virion (NV) protein which occurred between the G and L genes (Kurath and Leong, 1985). Bernard and de Kinkelin (1985) used UV transcriptional mapping to determine the gene order of the VHSV genome. The N, M1, and G genes mapped in the order predicted; however, the positions of the L and M2 genes could not be determined by this method.

Rhabdovirus transcription proceeds in a sequential and polar manner, but the five structural proteins are not synthesized in equimolar amounts due to a mechanism whereby transcription is attenuated at each gene junction (Banerjee, 1987; Wagner, 1987). This transcriptional regulation means that, in a general sense, the rhabdovirus gene order reflects the number of molecules of each protein that will be required to build the complete virion (Wagner, 1987). Leong *et al.* (1983) used ^{35}S -methionine to label the five IHNV structural proteins during synthesis in infected cells. They found the N protein was produced first and as early as 2–3 h post-infection. Other IHNV proteins appeared in the order predicted from the gene sequence. Similar results were obtained for VHSV by de Kinkelin *et al.* (1980) using cells treated with actinomycin-D.

In this study, we obtained estimates of the molecular weight of the HRV, IHNV, and VHSV genomes by comparing the relative mobility of genomic RNAs in formaldehyde gels. We used ^{35}S -methionine labeling of viral proteins produced in cells treated with actinomycin-D to investigate the rate of structural protein synthesis of the viruses. Our results suggest the three viruses are very similar in the size of the genome and in the order of protein synthesis.

Materials and Methods

Cells and Viruses

Epithelioma papulosum cyprini (EPC) cells (Fijan *et al.*, 1983) were grown at 18°C in Eagle's minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 100 I.U. of penicillin, and 100 µg/ml streptomycin. Hiram rhabdovirus (strain 8401-H), VHSV (Egtved strain

F1), and IHNV (Cultus Lake strain) were used in this study.

Virus Purification

Viral purification was performed as described by Nishizawa *et al.* (1991). Briefly, virus from cell culture fluid was concentrated using polyethylene glycol (PEG-6,000) and purified by centrifugation through both a discontinuous gradient comprised of 50%, 35%, and 20% (W/W) sucrose in STE buffer (20 mM Tris, pH 7.4, 100 mM NaCl, and 1 mM EDTA) and a continuous gradient comprised of 5% to 30% (W/W) sucrose in STE.

Preparation of Viral RNA

Sodium dodecyl sulfate (SDS) and proteinase K were added to the viral suspension at 1% (V/V) and 1 mg/ml, respectively, and the mixture was incubated for 30 min at 37°C. Viral RNA was extracted from the disrupted virions with STE-saturated phenol, followed by STE-saturated chloroform. The aqueous phase was made 0.3 M by addition of sodium acetate (pH 7.4) and the RNA precipitated by addition of 2.5 volumes of ethanol. The viral RNA was pelleted and resuspended in distilled, deionized water.

Electrophoresis of RNA

Formaldehyde agarose gel electrophoresis was used to analyze viral RNA (Sambrook *et al.*, 1989). The purified viral genomic RNA was mixed with an equal volume of sample buffer containing 22 mM of 3-(N-morpholino) propane-sulfonic acid (MOPS, pH 7.0), 5 mM of sodium acetate, 0.1 mM EDTA, 6.3% (V/V) formaldehyde, 7% (V/V) glycerol, and 0.5% (W/V) bromophenol blue (BPB), and incubated for 15 min at 65°C. A 1% (W/V) agarose gel was prepared containing 22 mM MOPS (pH 7.0), 5 mM sodium acetate, 1 mM EDTA, and 7.4% formaldehyde, the running buffer containing 22 mM MOPS (pH 7.0), 5 mM sodium acetate, and 0.1 mM EDTA. A 0.27–9.5 Kb RNA size ladder (BRL Inc., USA) was included in one lane of the gel and used to provide estimates of the relative mobilities of the genomic RNAs. Following electrophoresis at 50 V for 2 h, the gels were stained with ethidium bromide (10 µg/ml) and photographed. The molecular weight of each

virus genome was estimated by interpolation from a plot of the relative mobilities of the RNA species in the size ladder.

Labeling of Viral Proteins

Monolayers of EPC cells were infected with

virus at a multiplicity of infection of 10 and incubated for 1 h at 18°C to allow for adsorption. The monolayers were washed twice using Hanks' balanced salt solution (HBSS) and incubated at 18°C with MEM containing 1.0 µg/ml of actinomycin D. At 2 h intervals after

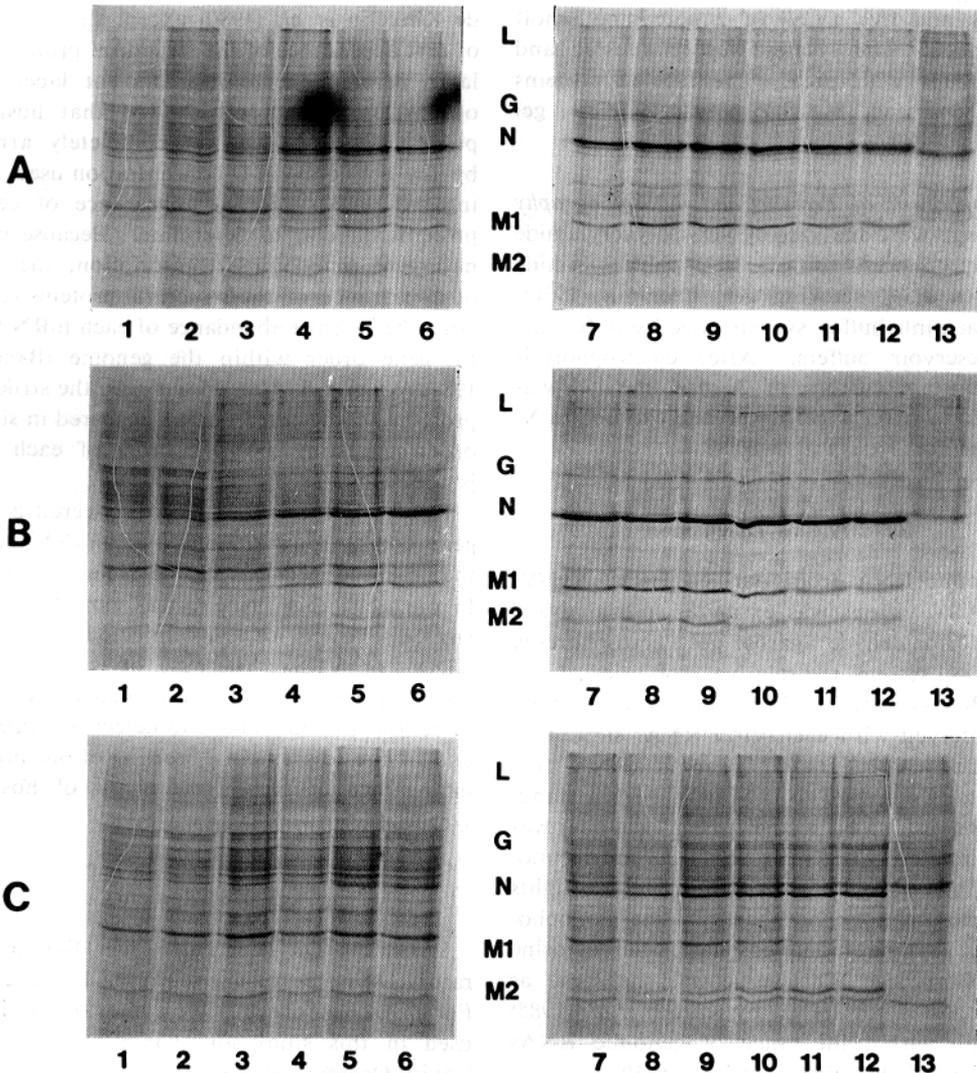


Fig. 1. Autoradiogram of SDS-polyacrylamide gel electrophoresis of viral proteins labeled with ³⁵S-methionine at 2 h intervals after virus adsorption. Cultures of EPC cells were infected with hirame rhabdovirus (panel A); viral hemorrhagic septicemia virus (panel B); and infectious hematopoietic necrosis virus (panel C). Lane 1, extracts of cells labeled with ³⁵S-methionine at 0-2 h after viral adsorption; lane 2, labeled at 2-4 h; lane 3, labeled at 4-6 h; lane 4, labeled at 6-8 h; lane 5, labeled at 8-10 h; lane 6, labeled at 10-12 h; lane 7, labeled at 12-14 h; lane 8, labeled at 14-16 h; lane 9, labeled at 16-18 h; lane 10, labeled at 18-20 h; lane 11, labeled at 20-22 h; lane 12, labeled at 22-24 h; and lane 13, mock infected cells labeled at 22-24 h.

adsorption, intracellular proteins were labeled by removing the tissue culture medium and replacing it with MEM containing 50 $\mu\text{Ci/ml}$ of ^{35}S -methionine and 1.0 $\mu\text{g/ml}$ of actinomycin D. At the end of the 2 h labeling period at 18°C, the medium was removed, and 100 μl of SDS-lysis buffer (166 mM Tris, pH 6.8, 5.3% (W/V) of SDS, and 13% (V/V) of 2-mercaptoethanol) was added. The lysates were harvested and made 8.0 M with urea. The labeled proteins were separated by SDS-polyacrylamide gel electrophoresis.

Electrophoresis of Proteins and Autoradiography

Proteins were analyzed by SDS-polyacrylamide gel electrophoresis using a 10% polyacrylamide gel with 2.5% stacking gel (Laemmli, 1970). The Laemmli buffer system was used for gel and reservoir buffers. After electrophoresis at a constant current of 30 mA, the gel was dried for 2 h on a gel dryer and exposed to X-ray film for 3 days at -80°C.

Results and Discussion

Genomic RNA from purified HRV, VHSV, and IHNV virions was extracted and analyzed using formaldehyde agarose gel electrophoresis. In this gel system, the relative mobilities of the three RNAs appeared identical. A single band was observed with each viral RNA, suggesting that full-length, intact, genomic RNA was present. Compared to the internal RNA standard, the estimated size of each genome was approximately 10.7 kb or about 3.7 md in molecular mass using the average molecular weights of adenosine monophosphate, uridine monophosphate, guanine monophosphate, and cytosine monophosphate. This value was the same as that reported for IHNV by Kurath *et al.* (1985) and falls within the range of genomic RNAs typical of rhabdoviruses (Wagner, 1987).

A comparison of structural protein synthesis of HRV, IHNV, and VHSV was conducted by adding ^{35}S -methionine to cultures of infected EPE cells at two hour intervals following virus adsorption (Fig. 1). In cells infected with HRV, the N, M1, M2, G, and L proteins appeared at 6-8, 6-8, 8-10, 8-10, and 14-16 h, respectively. In the cells infected with VHSV, the N, M1, M2,

G, and L proteins appeared at 4-6, 6-8, 8-10, 8-10, and 12-14 h, while in cells infected with IHNV, the N, M1, M2, G, and L proteins appeared at 8-10, 12-14, 12-14, 16-18, and 18-20 h after adsorption. Our results were generally analogous to those already reported for IHNV by Leong *et al.* (1983) and for VHSV by de Kinkelin *et al.* (1980) except that the time of first appearance of the structural proteins was later, perhaps due to less efficient labeling in our system. Our gels showed that host cell protein synthesis was not completely arrested by the actinomycin-D concentration used, making the time of initial appearance of certain proteins difficult to determine. Because of the nature of rhabdovirus transcription, the order of appearance of the structural proteins reflects both the relative abundance of each mRNA and the gene order within the genome (Banerjee, 1987; Wagner, 1987). In our gels, the structural proteins of the three viruses appeared in similar order suggesting the gene order of each virus is the same.

Kurath and Leong (1985) discovered a novel gene coding for a 12 Kd nonvirion (NV) protein that mapped between G and L genes of IHNV. In our gels, this protein was not detected in labeled extracts of cells infected with HRV, IHNV, or VHSV. Because of the position of the NV gene, the relative abundance is expected to be low and our failure to detect it is probably due to the less efficient labeling of our proteins in the face of incomplete arrest of host cell synthesis.

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