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Molecular Characterization of a Rotaviruslike Virus Isolated from Striped Bass (*Morone saxatilis*)†

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The characteristics of a rotaviruslike (SBR) virus isolated from striped bass (*Morone saxatilis*) were examined following purification of viruses from infected cell cultures. Virions had a double-layered capsid of icosahedral symmetry and a diameter of 75 nm. Purified viruses contained five polypeptides ranging in molecular mass from 130 to 35 kDa. None of the structural proteins were glycosylated. Treatment with EDTA did not remove the outer capsid. By using enzymes and a chaotropic agent, it was shown that VP5 was the most external polypeptide. The genome of SBR virus was composed of 11 segments of double-stranded RNA (dsRNA). The electrophoretic pattern of the dsRNA of SBR virus was different from that of reovirus type 1 (Lang) and rotavirus (SA11) dsRNA. The SBR virus was compared with reovirus type 1 and SA11 virus by RNA-RNA blot hybridization. There was no cross-hybridization between any of the genome segments of the SBR, reovirus type 1, or SA11 viruses. Antigenic comparison of SBR virus and SA11 virus by cross-immunoprecipitation and cross-immunofluorescence tests did not show any relationship. These results suggest that SBR virus could represent a new genus within the family *Reoviridae*.

During the past decade, viruses with characteristics of the family *Reoviridae* have been isolated from a number of aquatic animals (1, 2, 11, 12, 16, 20, 21, 24). Some of these viruses were isolated from diseased animals, while others were isolated during the routine examination of normal fish. The original isolates from oysters (20) and golden shiners (21) are of low pathogenicity for their natural hosts; however, in controlled-transmission studies, the oyster isolate kills bluegills and produces hepatitis in rainbow trout (20). An isolate from the tissues of adult chum salmon produces only low mortalities in experimentally infected chum, chinook, and Kokanee salmon (24). Similarly, an isolate from channel catfish (2) exhibits only low virulence for its host.

The physical and biochemical characteristics of this group of viruses have been described (11, 25). The virions are ether resistant, stable at pH 3, and approximately 70 nm in diameter, with a double-layer capsid and a genome composed of 11 segments of double-stranded RNA (dsRNA). These characteristics are similar to those of members of the genus *Rotavirus* in the family *Reoviridae*. Rotaviruses, the single most important etiologic agent of acute infantile diarrhea, have been recovered from a number of species, including humans, cattle, pigs, horses, rabbits, mice, dogs, cats, and birds (13) and some are known to cross species barriers. Although the possibility of transmission of rotavirus to aquatic animals through polluted water exists, this has never been established, nor have antigenic and biochemical relationships between the aquatic and mammalian rotaviruses been described.

In this study, we report the characterization of a rotaviruslike virus isolated from striped bass. We examined the extent of genetic relatedness among the aquatic rotaviruslike virus, reovirus type 1, and simian rotavirus (SA11) as determined by reciprocal RNA-RNA blot hybridization. The striped bass virus and SA11 virus were also compared for antigenic relatedness by cross-immunoprecipitation and cross-immunofluorescence tests.

MATERIALS AND METHODS

Viruses and cells. The aquatic rotaviruslike virus studied (SBR strain) was isolated from a moribund striped bass (*Morone saxatilis*) that was also infected with a *Moraxella* sp. The chinook salmon embryo cell line CHSE-214 was used to propagate the SBR virus strain. Reovirus type 1 (Lang strain) and simian rotavirus (SA11 strain) were provided by R. F. Ramig, Baylor College of Medicine, Houston, Tex. The Lang strain of reovirus type 1 and SA11 virus were grown in mouse L cells and MA104 cells, respectively.

Virus purification. Viruses were purified from infected cells by a modified procedure of Ramig et al. (22). When cytopathic effects were extensive, the remaining cells were scraped from the surface of the flask into the medium and the culture fluids were centrifuged for 15 min at 1,000 × *g*. The pellet of cells was suspended in 1 ml of homogenization buffer (0.01 M Tris, 0.25 M NaCl, and 0.01 M 2-mercaptoethanol) per 10^8 infected cells and mixed thoroughly. Sodium deoxycholate was added to a final concentration of 0.1%, and the mixture was incubated at 4°C for 30 min. One-half volume of Freon was added, and the mixture was thoroughly homogenized by sonication. The resultant emulsion was separated into Freon and aqueous phases by centrifugation. Virus was pelleted from the aqueous phase by centrifugation for 90 min at 78,000 × *g*. The virus pellet was suspended in SSC (0.15 M NaCl plus 0.015 M sodium citrate), sonicated for 10 s, and layered on a 15 to 50% sucrose gradient. The gradients were centrifuged for 60 min at 78,000 × *g*. The visible virus band was collected and concentrated by centrifugation for 90 min at 79,000 × *g*. The virus pellet was suspended in distilled water and stored at 4°C.

Electron microscopy. A drop of purified virus in distilled water was placed onto a Formvar-carbon-coated grid. The

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virus particles were negatively stained with 2% phosphotungstic acid (adjusted with NaOH to pH 7.0) and examined with a Hitachi 11-F transmission electron microscope at 75 kV.

Characterization of the genome of SBR virus. The viral nucleic acid type was determined by labeling with [^{3}H]uridine. Monolayers of CHSE-214 cells were infected with SBR virus. Following adsorption, the inoculum was removed and replaced with medium containing 25 μCi of [5,6-^{3}H]uridine (34.4 Ci/mmol; New England Nuclear) per ml. Seven days after infection, cells were processed for virus purification. Purified virus was treated with proteinase K (200 μg/ml) at 37°C for 30 min in the presence of 0.1% sodium dodecyl sulfate (SDS). The treated viruses were mixed with 2× sample buffer (15), boiled for 2 min, and analyzed in a 7.5% polyacrylamide gel.

The strandedness of the RNA segments of SBR virus genome was determined by digestion with RNase A under low-salt and high-salt conditions. Briefly, 10 μg of RNA extracted from purified virus was denatured at 85°C for 5 min in low-salt buffer (50 mM Tris hydrochloride [Tris-HCl, pH 7.5], 5 mM EDTA, 10 mM NaCl) and high-salt buffer (50 mM Tris-HCl [pH 7.5], 5 mM EDTA, 300 mM NaCl). The denatured RNA was allowed to anneal at room temperature for 10 min and digested with RNase A (40 μg/ml) at 30°C for 1 h before analysis in a 7.5% polyacrylamide gel.

Polyacrylamide gel electrophoresis. Polyacrylamide gels for the separation of dsRNA or protein were prepared by the method of Laemmli (15). The dsRNAs extracted from purified virus (14) were analyzed in 7.5% polyacrylamide gels, 1.2 mm thick and 45 cm long, that were run for 24 h at 250 V. The dsRNA bands were visualized after staining with ethidium bromide (10 μg/ml) by using a transmitted UV light source. Viral proteins were analyzed in 12.5% polyacrylamide gels, 1.2 mm thick and 20 cm long, that were run for 18 h at 8 mA. Gels were prepared for autoradiography or fluorography and exposed to X-ray films.

Trypsin treatment. The SBR virus was mixed with either buffer or trypsin (10 μg/ml). The mixtures were incubated at 37°C for 30 min and assayed on CHSE-214 cells by the 50% tissue culture-infectious dose ($TCID_{50}$) technique.

Treatment with EDTA. Purified [^{35}S]methionine-labeled SBR virus particles were incubated for 10 min at 20°C in 50 mM Tris-HCl (pH 7.5) with or without 3 mM EDTA and then pelleted by centrifugation at 72,000 × *g* for 2 h at 4°C. Polypeptides in the viral pellets were then analyzed in a 12.5% polyacrylamide gel.

Radiolabeling of SBR virus polypeptides with carbohydrates. Confluent monolayers of CHSE-214 cells in 150-mm plastic flasks were infected with SBR virus stocks. At least 72 h after infection, when greater than 50% of the cells showed syncytium formation, the cell sheets were rinsed with medium lacking glucose and radiolabeled with 100 μCi of either [2-^{3}H]mannose (30 Ci/mmol; New England Nuclear) or [1,6-^{3}H]glucosamine (40 Ci/mmol; Amersham) per ml in medium lacking glucose. After 18 h of radiolabeling, the infected cells were processed for virus purification. The purified viruses were analyzed in a polyacrylamide gel.

In some experiments, the infected cells were starved for 1 h in glucose-free medium and labeled for 3 h with 200 μCi of either [2-^{3}H]mannose or [1,6-^{3}H]glucosamine per ml. At the end of the pulse, the cells were harvested with RIPA buffer (see below), centrifuged to remove nuclei and other insoluble matter, and analyzed directly and after immunoprecipitation in a polyacrylamide gel.

Endo H digestion of purified virus. Purified SBR virus particles radiolabeled with [^{35}S]methionine were denatured by adding SDS to 1% and boiling the mixture for 2 min. The denatured virus was then diluted to 10 mM sodium phosphate (pH 6.0) and 0.2% SDS. This was digested with endo-β-*N*-acetylglucosaminidase H (Endo H; 0.25 U/ml, final concentration; Sigma Chemical Co.) for 1.5 h at 37°C. The reaction was stopped by adding an equal volume of 2× sample buffer and boiling the mix for 2 min before analysis by polyacrylamide gel electrophoresis.

Immunoprecipitation. Hyperimmune guinea pig antiserum prepared with purified SA11 virus was provided by M. K. Estes, Baylor College of Medicine, Houston, Tex. Hyperimmune guinea pig antiserum was prepared against purified SBR virus. Antigens for immunoprecipitation experiments were prepared by labeling SBR virus-infected CHSE-214 cells and SA11 virus-infected MA104 cells with [^{35}S]methionine for 2 h in methionine-free medium. The infected cells were harvested in RIPA buffer (150 mM NaCl, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, 10 mM Tris-HCl [pH 7.2]). The polypeptides were immunoprecipitated with hyperimmune guinea pig antisera. The immune complexes were collected with *Staphylococcus aureus* protein A (14), and the polypeptides were detected following fluorography.

Immunofluorescence staining. CHSE-214 cells grown on cover slips and infected with SBR virus and MA104 cells grown on cover slips and infected with SA11 virus were fixed with 100% ethanol. Uninfected cells were processed in parallel. Cross-indirect immunofluorescence staining was performed with anti-SBR hyperimmune guinea pig antiserum and anti-SA11 hyperimmune guinea pig antiserum.

SVP. The purified SBR virus was mixed with buffer or different concentrations of enzymes and incubated in a 37°C water bath for 30 min. The subviral particles (SVP) were then purified on 15 to 60% linear sucrose gradients as described above. To examine the effect of a chaotropic agent, purified SBR virus was suspended in 5 mM Tris-HCl–50 mM NaCl (pH 8.0) and treated at room temperature with 1 M $CaCl_2$ for 10 min. The mixture was then layered carefully on the top of a 30 to 60% sucrose gradient (the density of 1 M $CaCl_2$ being close to the density of 30% sucrose) and centrifuged for 2 h at 154,000 × *g*. The viral band was recovered from the sucrose gradient as described previously (22). The SVP were suspended in 1× electrophoresis sample buffer and boiled for 2 min before they were loaded onto gels.

RNA-RNA hybridization. The dsRNA probes of SBR virus, reovirus type 1, and SA11 virus were prepared by standard protocols. Briefly, dsRNA was extracted from each of these viruses, and 1 to 2 μg was 3′-end labeled with 50 μCi of [5′-^{32}P]pCp (6). The method of Bodkin and Knudson (3) was used to transfer viral RNA to a GeneScreen Plus membrane. Samples of genomic RNA were analyzed on 7.5% polyacrylamide gels. Gels stained with ethidium bromide were trimmed to size under UV light. The gels were soaked in 0.1 M NaOH for 20 min and then in 4× TAE (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA [pH 7.4]) twice for 20 min each and in 1× TAE once for 20 min. The GeneScreen Plus membrane was soaked for 5 min in 1× TAE prior to transfer. The dsRNA was transferred to GeneScreen Plus membrane with a Trans-blot cell (Hoefer) with a current of 1.6 A for 4 h. Membranes were baked for 1 h at 80°C in vacuo. Blots were prehybridized and hybridized under conditions similar to those described by Bodkin and Knudson (3). Hybridization took place overnight at 52°C in the presence of 50% formamide. Blots were washed twice for 30 min each at room temperature in 2× SSC–0.1% SDS

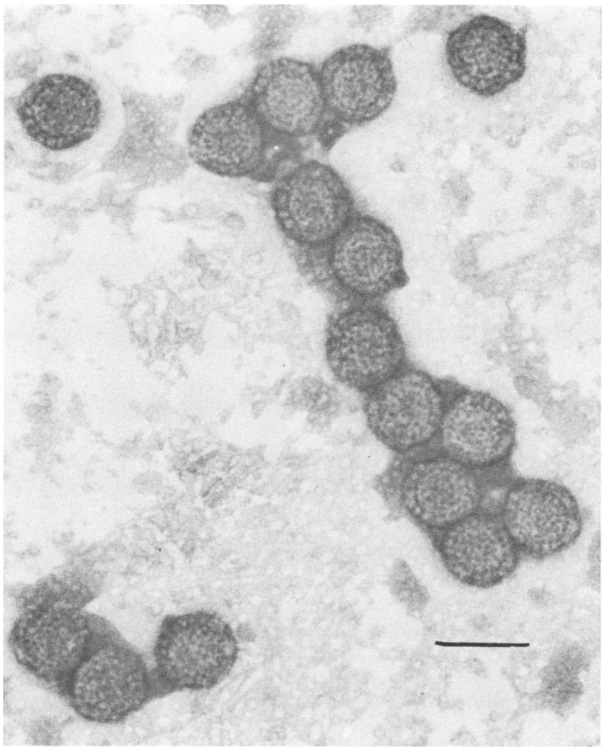

FIG. 1. Electron micrograph of purified striped bass (SBR) virus negatively stained with 2% phosphotungstic acid. Note the double capsid structure. Bar, ca. 100 nm.

and once for 1.5 h in 0.1× SSC–0.1% SDS at 50°C. Hybridization was visualized by autoradiography.

RESULTS

Electron microscopy. Negatively stained preparations of the virions revealed particles of sharply defined outer edges with icosahedral symmetry and a double capsid structure (Fig. 1). The diameter of the virions was approximately 75 nm, and both complete and empty (without nucleic acid) particles were seen. The morphologic features of the SBR virus were different from those of the reoviruses and similar but not identical to those of rotaviruses.

Growth characteristics of SBR virus. The host range of SBR virus was determined in different cell lines. The SBR virus replicated in cells of fish origin at low temperatures (16°C) and produced well-defined syncytia of fused cells. Attempts to grow SBR virus in mammalian cells (MA104, bovine kidney, and HEp-2) produced no cytopathic effect (CPE). Similarly, SA11 virus failed to produce CPE in several fish cell lines.

To determine whether treatment with proteolytic enzymes would result in enhanced infectivity, the SBR virus was treated with trypsin and the preparations were assayed for infectivity. The SBR virus did not respond to trypsin treatment with enhanced infectivity. A control virus suspension that was treated with buffer had a titer of 2.7×10^7 $TCID_{50}$/ml. The trypsin-treated virus suspension had a titer of 1.4×10^7 $TCID_{50}$/ml.

Characterization of the genome of SBR virus. Analysis by polyacrylamide gel electrophoresis of the nucleic acid extracted from SBR virus grown in the presence of [^{3}H]uridine

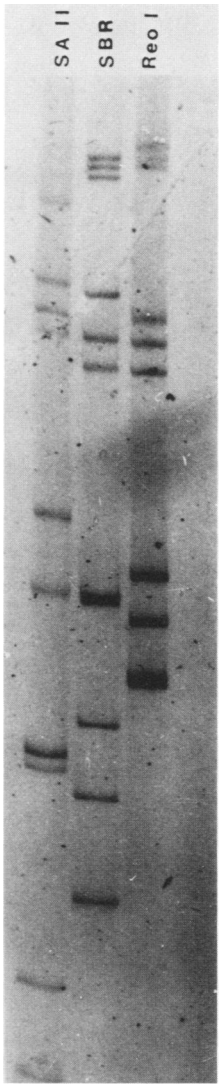

FIG. 2. Genome RNAs of simian rotavirus SA11, striped bass virus, and reovirus type 1. Genomic dsRNAs were extracted from purified viruses and subjected to electrophoresis in a 7.5% Tris–glycine-buffered gel, 1.2 mm thick and 45 cm long. Electrophoresis was for 24 h at 250 V. The RNA segments were detected by staining with ethidium bromide.

demonstrated 11 distinct bands (Fig. 2) which were resistant to RNase A under high-salt conditions but sensitive under low-salt conditions. These results indicate that the genome of SBR virus is composed of 11 segments of dsRNA. The molecular masses of these RNA segments fell into three size classes that ranged from 2.4 to 0.4 MDa. There were three large, three medium, and five small segments. The electrophoretic pattern of the dsRNA of SBR virus was intermediate between the short pattern of reovirus type 1 and the long pattern of SA11 virus.

Structural polypeptides of SBR virus. The SBR virus had at least five structural proteins ranging in molecular mass from 130 to 35 kDa (Fig. 3). There were two large (VP1 and VP2), one medium (VP3), and two small (VP4 and VP5) virion proteins. The two large proteins were difficult to resolve in most gels. Of the five structural proteins, VP3 and VP5 were major proteins, VP1 and VP4 were minor proteins, and VP2

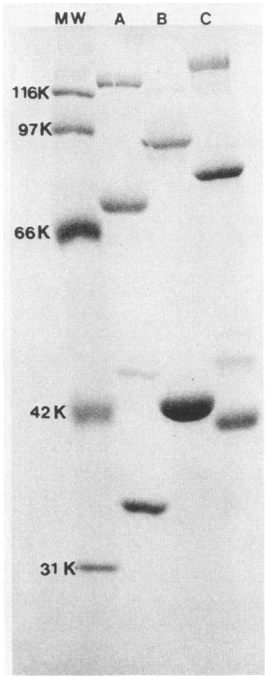

FIG. 3. Electrophoresis of the proteins of striped bass virus, simian rotavirus SA11, and reovirus type 1. Purified virions were lysed into sample buffer and subjected to electrophoresis in 12.5% Tris-glycine buffered gels (1.2 mm thick and 20 cm long) that were run for 18 h at 8 mA. Virion proteins were detected by staining with Coomassie brilliant blue. Lane A, Striped bass SBR virus; lane B, simian rotavirus SA11; lane C, reovirus type 1. MW, Molecular weight markers (in thousands).

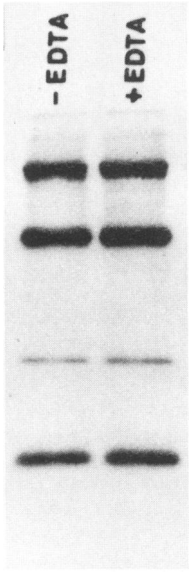

FIG. 4. Effect of EDTA treatment on the polypeptides of purified striped bass SBR virus labeled with [^{35}S]methionine. Purified viruses were treated with or without EDTA as described in Materials and Methods and analyzed by electrophoresis in a 12.5% polyacrylamide gel and fluorography.

was present only in small quantity. The electrophoretic pattern of the SBR virus proteins was quite different from that seen with reovirus type 1 or SA11 virus.

To investigate whether the proteins of SBR virus were glycosylated, we attempted to label viral polypeptides with [^{3}H]mannose and [^{3}H]glucosamine. Labeling periods of up to 18 h failed to show either mannose or glucosamine incorporation into viral polypeptides (results not shown). Immunoprecipitation experiments also failed to show incorporation of either [^{3}H]mannose or [^{3}H]glucosamine in the immunoprecipitated proteins. To further investigate glycosylation of SBR virus proteins, [^{35}S]methionine-labeled SBR virus particles were digested with Endo H. Following digestion, analysis of viral polypeptides by polyacrylamide gel electrophoresis showed that none of the polypeptides decreased in molecular mass, indicating that they were not glycosylated.

To determine whether any of the proteins of SBR virus can be removed by treatment with EDTA, [^{35}S]methionine-labeled purified SBR virus particles were treated with buffer in the presence or absence of 3 mM EDTA. Following treatment, virus samples were centrifuged at 72,000 × *g* for 2 h, and the proteins remaining in the pelleted virus particles were analyzed by polyacrylamide gel electrophoresis. None of the polypeptides of SBR virus was removed by treatment with EDTA (Fig. 4).

Antigenic relationship between SBR virus and SA11 virus. To antigenically compare the polypeptides of SBR virus and SA11 virus, we used cross-immunoprecipitation and cross-immunofluorescence tests. Neither cross-immunoprecipitation nor cross-immunofluorescence was noted between the SBR and SA11 viruses (data not shown). The anti-SBR hyperimmune serum and anti-SA11 hyperimmune serum immunoprecipitated and immunofluoresced in their homologous systems. The results indicate that no antigenic relationships exist between the SBR and SA11 viruses.

Immunoprecipitation of SBR virus-infected cell extracts with the anti-SBR virus hyperimmune serum prepared with purified virus in guinea pigs precipitated only VP3 and VP5, suggesting that these two proteins may be exposed on the virus particle.

Alteration of purified SBR virus by enzymes and a chaotropic agent. Treatment of SBR virus with trypsin at a concentration of 1 μg/ml caused complete digestion of VP5 and partial digestion of VP3. The cleavage product of VP3 was found to be associated with the particles recovered (Fig. 5). Higher concentrations of trypsin (10 μg/ml) caused complete digestion of VP3 and VP5. When chymotrypsin was used, similar results were observed except that there was no cleavage of VP3 at the 1-μg/ml concentration of chymotrypsin. V8 protease had no effect on the SBR virus under similar conditions. Electron microscopy of virus particles treated with 10 μg of trypsin per ml revealed featureless small round particles of about 50 nm in diameter. Treatment of SBR virus with 1 M $CaCl_2$ caused complete solubilization of VP5 (Fig. 5).

Genetic relatedness of SBR virus to other members of the *Reoviridae*. The genomes of SBR virus, reovirus type 1, and SA11 virus were examined by reciprocal RNA-RNA blot hybridization performed at 52°C in the presence of 50% formamide (Fig. 6). Under these conditions, hybridization will occur with up to an 8% base mismatch (23). Each dsRNA hybridized only to itself and not to the dsRNAs of the other viruses. Cross-hybridizations were also carried out in 15% formamide at 52°C (data not shown), a hybridization condition of minimum stringency sufficient to stabilize hybrids with up to 27% average base mismatch. There was no cross-hybridization between any of the genome segments of the SBR, reovirus type 1, and SA11 viruses.

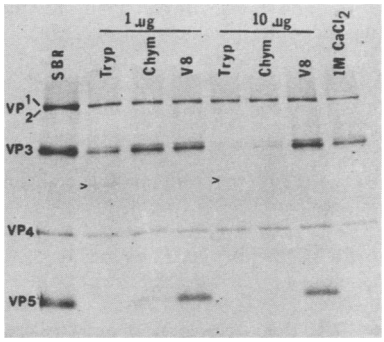

FIG. 5. Effect of protease treatment on the polypeptides of purified striped bass SBR virus labeled with [^{35}S]methionine. Purified viruses were treated with various proteases (Tryp, trypsin; Chym, chymotrypsin; V8, V8 protease) and a chaotropic agent ($CaCl_2$) as described in Materials and Methods and analyzed by electrophoresis in 12.5% Tris–glycine-buffered polyacrylamide gels and fluorography. Arrows indicate cleavage product.

DISCUSSION

We have antigenically and biochemically characterized a rotaviruslike virus isolated from striped bass. The SBR virus contains a genome composed of 11 segments of dsRNA. Viruses containing dsRNA segments have been reported previously from aquatic animals such as fish and shellfish (2, 20, 21, 24). The pathogenicity of SBR virus is not well known, but in preliminary experiments, SBR virus caused mortality in rainbow trout. All these isolates are members of the family *Reoviridae* and probably will form an important group of pathogens for fish; however, their relationship to other members of the family has not been established.

In this study, we have examined the antigenic and genetic relationship of an aquatic rotaviruslike virus with a mammalian reovirus and rotavirus. The SBR virus was quite different from the *Orbivirus*, *Fijivirus*, and *Phytoreovirus* genera of the *Reoviridae* in morphology and physicochemical characteristics. Morphologically, the SBR virus had a clearly defined double capsid structure similar to that of members of the genus *Rotavirus*. Like other rotaviruses, the SBR virus had a genome of 11 dsRNA segments. However, the electrophoretic patterns of the RNA and proteins of SBR virus were quite different from those of reovirus type 1 and rotavirus (SA11).

The growth characteristics of SBR virus were different from those of a simian rotavirus. The SBR virus grew only in cells of fish origin at low temperatures (16°C) and produced well-defined syncytia of fused cells. Attempts to grow SBR virus in mammalian cells (MA104, bovine kidney, and HEp-2) yielded no CPE. Similarly SA11 virus produced no CPE in several fish cell lines. Trypsin enhancement of SBR virus infectivity, a distinctive feature of rotavirus growth in cell culture (8, 9), also was examined. It was found that unlike rotaviruses, SBR virus did not respond to trypsin treatment with enhanced infectivity.

Attempts to radiolabel SBR virus proteins with sugar precursors were unsuccessful, indicating that the SBR virus proteins are not glycosylated. This was further supported by failure to digest SBR virus proteins with Endo H. However, the presence of glycosylated nonstructural (NS) proteins in SBR virus cannot be ruled out. In SBR virus-infected cell extracts, it was difficult to distinguish viral NS proteins from cellular proteins due to poor inhibition of cellular proteins by the SBR virus. Work is in progress to characterize the NS proteins of SBR virus. Absence of any structural glycosylated protein in SBR virus is in agreement with the members of the *Reovirus* and *Orbivirus* genera, in which none of the proteins is glycosylated, but is different from the members of the genus *Rotavirus*, in which some of the structural and NS proteins are highly glycosylated (7, 10, 18).

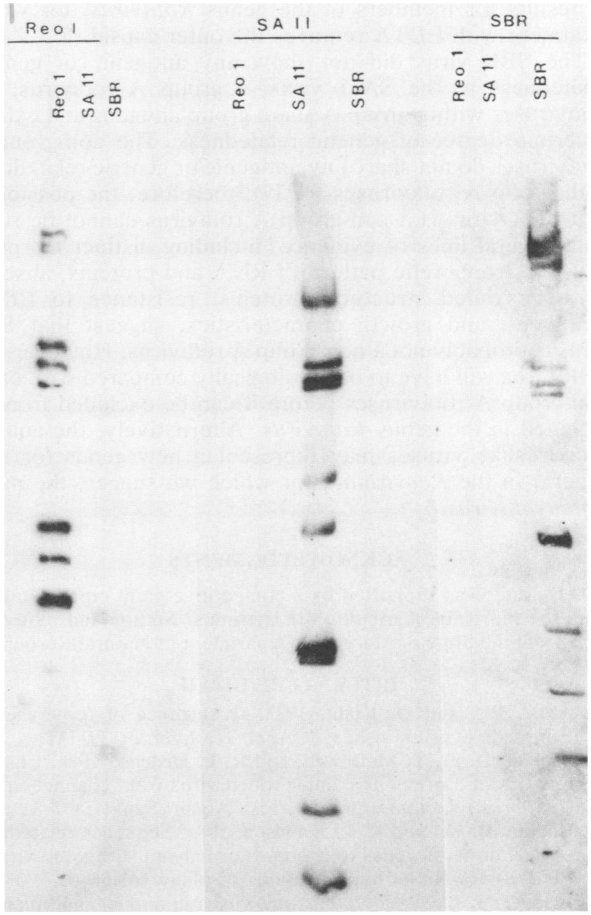

FIG. 6. Sequence homology between reovirus type 1, simian rotavirus SA11, and striped bass SBR virus genomes. Genomic dsRNAs were extracted from purified viruses, subjected to electrophoresis in 7.5% Tris–glycine-buffered gels, and transferred to GeneScreen Plus membranes with a Trans-blot cell. Total genomic dsRNAs of striped bass virus, reovirus type 1, and simian rotavirus SA11 were end labeled with [5′-^{32}P]pCp and hybridized separately to a membrane containing genome profiles of the three viruses. Hybridization took place at 52°C in the presence of 50% formamide.

The localization of SBR virus proteins in the virus architecture was studied by treatment with enzymes and a chaotropic agent. The results suggest that VP3 and VP5 constitute the outer layer, which could be removed by trypsin and chymotrypsin treatment. This was further suggested by the results of the immunoprecipitation test, in which only VP3 and VP5 were precipitated by the guinea pig hyperimmune antiserum. Treatment with 1 M $CaCl_2$ selectively solubilized VP5, the most exposed protein of SBR virus. It should be noted that this procedure of removal of VP5 constitutes a simple method for the purification of the outermost protein of the virion without the use of a detergent.

Treatment of SBR virus with EDTA did not result in the removal of any outer capsid protein. This result is in contrast

to results for members of the genus *Rotavirus*, for which treatment with EDTA removes the outer capsid (5).

The SBR virus did not show any antigenic or genetic relatedness to the SA11 virus, a group A rotavirus. All rotaviruses within group A share group antigens and exhibit a certain degree of genetic relatedness. The non-group A rotaviruses do not share any antigenic or genetic relatedness with group A rotaviruses (4, 17); therefore, the possibility that SBR virus is a non-group A rotavirus cannot be ruled out. Several lines of evidence, including distinct morphology, electrophoretic pattern of RNA and proteins, absence of glycosylated structural proteins, resistance to EDTA treatment, and growth characteristics, suggest that SBR virus is probably not a non-group A rotavirus. However, the SBR virus will have to be serologically compared with other non-group A rotaviruses before it can be excluded from or included in the genus *Rotavirus*. Alternatively, the aquatic rotaviruslike viruses may represent a new genus (or new genera) in the *Reoviridae*, for which we suggest the name *Aquarrotavirus*.

ACKNOWLEDGMENTS

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