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Detection of Aquareovirus RNA in Fish Tissues by Nucleic Acid Hybridization with a Cloned cDNA Probe†

KOTHANDARAMAN SUBRAMANIAN,[1] BLANCA LUPIANI,[1] FRANK M. HETRICK,[2] AND SIBA K. SAMAL*

College of Veterinary Medicine[1] and Microbiology Department,[2] University of Maryland, College Park, Maryland 20742

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A nucleic acid hybridization assay was developed to rapidly detect small quantities of aquareovirus RNAs in infected cells and organs. Cloned cDNA copies were synthesized from the genomic RNA of the SBR strain of aquareovirus. By using cloned cDNA probes, aquareovirus RNAs were detected in spleen and kidney tissues of experimentally infected fish.

The aquareoviruses have recently been classified as a new genus in the family *Reoviridae* (5). They have a genome composed of 11 segments of double-stranded RNA (11). These viruses have been isolated from fish, shellfish, and crustacea from different environments and geographic areas (1, 7, 11). However, they grow slowly in tissue culture, and there is a need for more-sensitive and -rapid techniques for the diagnosis of aquareovirus infections. In this study, we used a dot hybridization assay for the detection of aquareovirus RNAs in infected cells and in tissues from experimentally infected fish.

The biochemical characterization of five aquareoviruses used in this study has been described elsewhere (11). One isolate (SBR virus) was obtained from a Chesapeake Bay striped bass (*Morone saxatilis*) that had numerous external hemorrhagic lesions (1). Two other isolates were obtained from ovarian fluids of different populations of normal Atlantic salmon (*Salmo salar*) that were being routinely sampled, one by us in Maryland (11) and one by Moore and McMenemy (10) in Canada. A fourth strain was isolated from smelt (*Osmerus mordax*) suffering mortalities in New Brunswick, Canada (8). The fifth isolate was isolated in Spain from cultured turbot (*Scophthalmus maximus*) (7). Infectious pancreatic necrosis virus, a birnavirus, and infectious hematopoietic necrosis virus, a rhabdovirus, were used to determine the specificities of the probes.

In order to synthesize cDNA clones, the genomic double-stranded RNA was extracted from purified SBR virus as described previously (11). First-strand cDNA was synthesized with reverse transcriptase and random primer (3). Second-strand cDNA was synthesized by the procedure of Gubler and Hoffman (6). The double-stranded cDNA was blunt ended with T4 DNA polymerase, and *Eco*RI linkers were ligated with T4 DNA ligase. *Eco*RI-ended cDNA was ligated to *Eco*RI-digested plasmid pUC19, and this preparation was used to transform *Escherichia coli* JM109 cells.

To determine the identity of viral inserts, the cloned fragments were excised from representative plasmids and radiolabeled by the random primer method (4). Each fragment was hybridized to a Northern (RNA) blot of polyacrylamide gel electrophoresis-separated double-stranded RNA (2). Northern blot hybridization indicated that sequences from all SBR virus genes were synthesized. The sequence conservation of three different cDNA clones representing large, medium-sized, and small RNA segments (Fig. 1) was examined by hybridization to dot blots of double-stranded RNA from five different aquareovirus strains. All three

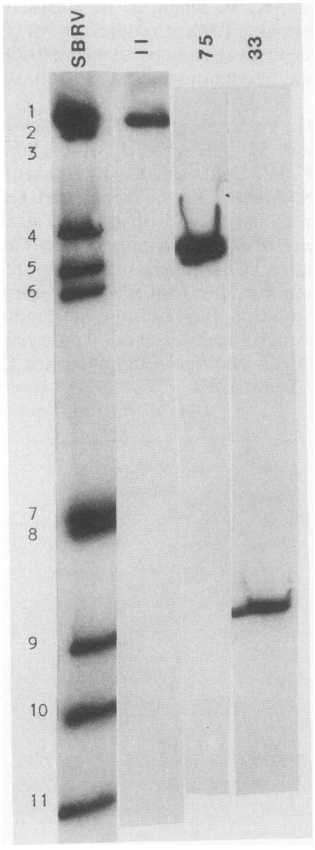

FIG. 1. Identification of cloned SBR virus (SBRV) genes by Northern blot hybridization. cDNA inserts (numbered lanes) were labeled with ^{32}P by the random primer method, and each insert was hybridized to a strip of the membrane that contained all 11 double-stranded RNA segments. The positions of the 11 genome segments on these strips are indicated on the left.

* Corresponding author.

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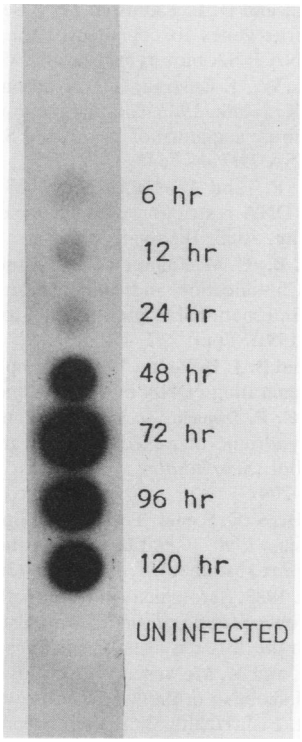

FIG. 2. Time course of SBR virus RNA replication. Chinook salmon embryo cells were infected with SBR virus, and samples were collected at the times indicated on the right. Total RNA from 10^3 cells was spotted onto a nylon membrane and hybridized to a ^{32}P-labeled probe prepared from clone 33 cDNA.

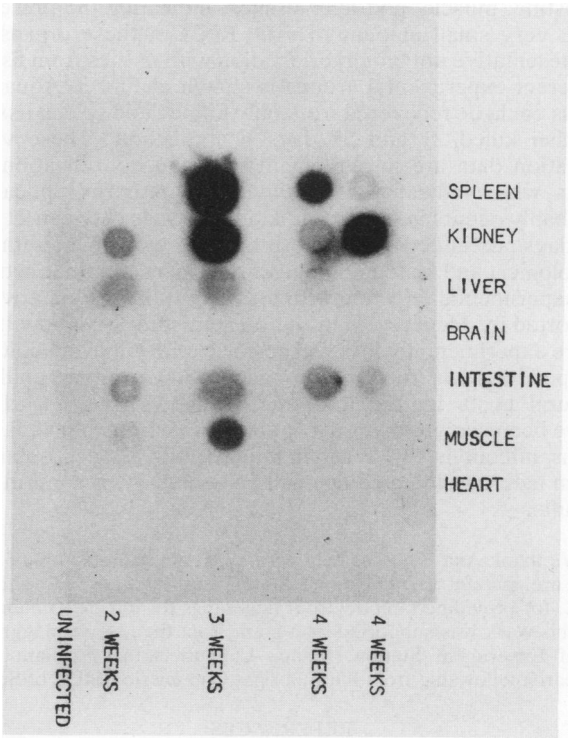

FIG. 3. Representative sample of results of dot blot hybridization used to detect SBR virus RNA in organs of experimentally infected rainbow trout fingerlings. ^{32}P-labeled probes from the clone 33 insert were hybridized with nucleic acids extracted from different organs as described in the text. Nucleic acids extracted from organs of one representative fish sacrificed 2 weeks postinfection, one fish sacrificed 3 weeks postinfection, and two fish sacrificed 4 weeks postinfection were used to show viral RNAs in spleen and kidney tissues. Nucleic acids extracted from organs of one sham-inoculated fish after 4 weeks were used as a control.

cDNA clones hybridized well with four North American isolates but not with the isolate from Europe. These results confirm our previous finding that North American isolates are genetically different from the European isolate (11). Clone 33, representing 90% of segment 9, was used as a diagnostic probe in further studies.

The specificity of the gene 9 probe was examined with nucleic acids extracted from infectious pancreatic necrosis and infectious hematopoietic necrosis viruses, which commonly infect fish. Infected cells were lysed in TNE (10 mM Tris [pH 7.4], 100 mM NaCl, 1 mM EDTA) buffer containing 0.5% Nonidet P-40. The cell lysates were extracted with phenol and precipitated with ethanol. The RNA pellets were dissolved in TE buffer (10 mM Tris [pH 7.4], 1 mM EDTA), denatured in 10 mM methyl mercuric hydroxide, and dotted onto a nylon membrane. The hybridization and washing conditions were similar to those described by Bodkin and Knudson (2). The probe did not hybridize to nucleic acids extracted from infectious pancreatic necrosis and infectious hematopoietic necrosis viruses. The probe was sensitive enough to detect viral RNA from 10^3 cells as early as 48 h after infection (Fig. 2) and to detect as little as 5 ng of RNA from purified virus (data not shown).

The gene 9 clone was evaluated for hybridization with SBR virus-specific RNA in tissues from experimentally infected fish. Twenty rainbow trout fingerlings (4 to 6 in. [ca. 10 to 15 cm] long) were experimentally infected each with 0.5 ml (10^6 50% tissue culture infective doses of SBR virus) by the intramuscular route. Six fish received 0.5 ml of cell culture medium and were housed in a separate tank as negative controls. Tissues from three infected fish were collected on days 7, 14, 21, and 28 postinfection. Each tissue sample was homogenized in 0.5 ml of 1× SSC (0.15 M NaCl, 0.015 M sodium citrate), Nonidet P-40 was added to a final concentration of 0.5%, and the mixture was incubated on ice for 30 min. The homogenate was centrifuged at 1,000 × *g* for 10 min to yield a clear supernatant. Sodium dodecyl sulfate and proteinase K were added to final concentrations of 0.1% and 2 mg/ml, respectively, and the mixture was incubated at 37°C for 1 h. The lysate was extracted with phenol, and the nucleic acids were precipitated with ethanol, dried, and dissolved in TE buffer. RNA extracted from tissue specimens was denatured in 10 mM methyl mercuric hydroxide and dotted onto a nylon membrane. The hybridization and washing conditions were as described previously (2).

No gross pathological lesions were observed in internal organs of any fish. Hybridization of the gene 9 probe to tissue samples from fish after 7 days of infection was not detected. Weak hybridization was detected in the kidney, liver, and intestine tissues of two fish after 14 days of infection. Strongly positive signals were obtained with the spleen and kidney tissues of two fish killed 21 days after infection. Only kidney tissue of the third fish in the same group showed hybridization. Of the three fish killed after 28 days of infection, only kidney tissue in two fish and spleen tissue in the third fish showed strong hybridization. Weaker signals were also visible with nucleic acids extracted from

intestine, muscle, and liver tissues, indicating the presence of a very small amount of viral RNA in these organs. A representative dot blot hybridization with tissues from fish of different experimental groups is shown in Fig. 3. Aquareovirus could be recovered from only kidney and spleen tissues of fish killed 21 and 28 days postinfection. These virus isolation data are in agreement with the hybridization results, which indicate that maximum aquareovirus replication probably occurs in spleen and kidney tissues between 21 and 28 days postinfection. Our results are in agreement with the serological and histopathological responses of rainbow trout to experimental infection with the 13_{p2} strain of aquareovirus reported by Meyers (9). In the present study, rainbow trout were experimentally infected because of the unavailability of striped bass fingerlings. Although rainbow trout are unlikely natural hosts for aquareovirus, rainbow trout fingerlings have been shown to be susceptible to experimental aquareovirus infections (9). Further studies with tissues obtained from naturally infected fish will be necessary to confirm our results.

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