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Molecular biological characterization of the new virus isolated from abnormally swimming salmonid

-Reverse transcriptase activity-

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A new RNA virus isolated from abnormally swimming behavior has caused mortalities in salmonid fish (Oh *et al.*, 1995 a), A reverse transcriptase (RTase) activity of the virus was determined by using poly r(A) : oligo d(T) as templete : primer. This RTase activity was associated with virus particles of buoyant density of 1.16 g/ml. The virus particles in sucrose fractions were enveloped and were about 85 nm diameter with central electron-dense core. The brain and kidney samples of artificially infected fish showed RTase activity. Virus particle associated proteins about 120, 80, 65, 61, 48, 42, 35, 30, 25, 19, and 15 kDa were observed when examined by polyacrylamide gel electrophoresis analysis. This study showed the presence of a new retrovirus in salmonid fish, which tentatively called RVS (Retrovirus of salmonid).

Key words : Retrovirus, Abnormal swimming, Salmonid, RTase activity, Viral density, RVS

Retrovirus was first discribed 80 years ago as filterable agents that cause cancer in chikens. Today the family of retroviridae is currently classified into 7 genera (Francki *et al.*, 1991). Currently 13 retroviruses or retrovirus-like particles associated with fish were reported (Hetrick and Hedrick, 1993). Recently, a new virus that be able to multiply in some fish cell lines was isolated from salmonid fishes (Oh, 1995 ; Oh *et al.*, 1995 a,b,c). In the comparison with the characteristics of the known fish RNA viruses, the virus was closely related to retrovirus in the morphological, biological and biochemical properties.

All retroviruses have an outer lipid envelope and an inner protein nucleocapsid containning an RNA genome which encodes the RNA-directed DNA polymerase also known as reverse transcriptase (RTase). The enzyme synthesizes a double-stranded DNA copy of the genomic RNA (Temin and Baltimore, 1972). The DNA molecule can then integrate into the host genome and become a functional component of its genetic make up.

I have undertaken survey of the virus strain for RTase activity and detection of particles of retroviral density using sucrose gradient ultracentrifugation.

Materials and Methods

Virus

The cloned virus strains BrCo-9221, isolated from brain of diseased coho salmon (*Oncorhynchus kisutch*), BrRt-9203, from brain of diseased rainbow trout (*O. mykiss*), BrIw-9201, from brain of diseased charr (*Salvelinus pluvius*) and BrAy-9201, from brain of diseased ayu (*Plecoglossus altivelis*) cultured in the CHSE-214 cell line were used in this study. The viruses were replicated with MEM containing 10% FBS (MEM-10) at 15℃ for 7 days.

Virus purification and determination of virion density

Virus was purified and concentrated with methods previously described (Oh *et al.*, 1995a). After centrifugation with continuous sucrose gradient (15 to 60% w/v) at 100,000×g, the linear gradient was fractionated into 200 μl volume. The refractive index of each fraction was determined with a refractormeter (Atago) and the buoyant density was calculated, and then the RTase activity of each fraction was determined.

Artificial infection study

Rainbow trout (30 to 35g body weight) were artificially infected by the waterborne method described in previous paper (Oh *et al.*, 1995b). The virus titer of strain BrCo-9221 was adjusted to the infectivity of $10^{3.5}$ $TCID_{50}$/ml in the aquarium, and rainbow trout were exposed for 2 hrs at 15℃. Twenty fishes were held in tank with flow-through fresh water at about 16℃. Non injected control fish of 20 were similarly maintained. At 4 weeks post infection, brain and kidney from 2 dead, 3 treated but survive and 3 control fish were separately collected for RTase analysis. Brain and kidney from 3 groups were pooled. All tissues to be analyzed were frozen and thawed, then diluted in 0.01 M Tris-Cl, pH 7.5, 1.0 mM EDTA, 0.1 M NaCl, 5% sucrose and homogenized with a Polytron at 4℃. Homogenates were centrifuged for 1 hr at 10,000×g, and the supernatants were collected and centrifuged for 2 hrs at 80,000×g. The pellets were resuspended in 500 μl of 0.01 M Tris-Cl, pH 7.5, 1.0 mM EDTA, 0.1 M NaCl, and overlayed onto sucrose step gradient of 15, 35 and 50% in the TNE buffer and centrifuged at 100,000×g for 2 hrs. The region at the 35~50% sucrose interface was collected, centrifuged at 100,000×g for 1 hr, and resuspended in 500μl of TNE containing 0.01 M dithiothreitol. These samples were used for RTase assay.

Reverse transcriptase activity assay

The RTase assayes were performed as described by Tomely *et al.* (1983) with the following exceptions : poly r(A) : oligo $d(T)_{12-18}$ (Pharmacia LKB Biotechnology Inc., USA) was used as the template and primer for RTase activity, [^{3}H] TTP was used for labelling of the product, 0.2% Nonidet P-40 (final concentration) was added to in the cocktail (0.1 M Tris-Cl, pH 8.3, 0.5 M KCl, 1.0 mM $MnCl_2$, 4.0 mM dithiothreitol, and 20μCi/ml [^{3}H]TTP), and the Watman GF/C filter disks containing the samples were washed for 5 min in each of the following : 10% TCA, 5% TCA, 5% TCA in 1% sodium dodecyl sulfate, and 95% ethanol prior to determining the acid-precipitable cpm.

The RTase activity was determined by substrac-

ting the acid-precipitable cpm of a sample at the start of the incubation (T_0) from the cpm of the sample after 60 min incubation (T_{60}). An identical procedure was conducted using the primer poly d(A) : oligo d(T) to provide a measure of DNA-dependent DNA polymerase activity. The ratio of the cpm at T_{60}-T_0 using the poly r(A) primer and poly d(A) was calculated {T_{60}-T_0 [poly r(A)] : T_{60}-T_0 [poly d(A)]}. A ratio ≥4 was concidered positive for RTase activity and not due to DNA-dependent DNA polymerase activity. The RTase assay performed on the gradient fractions were only reported as the T_{60}-T_0[poly r(A)] values.

Polypeptide analysis of purified virus

Purified virus was mixed with an equal volume of denaturing buffer (166mM Tris-Cl, pH 6.8 ; 5.3 % SDS ; 13% 2-mercaptoethanol) and heated at 100℃ for 3 min. The virus proteins were analyzed with polyacrylamide gel electrophoresis (Laemmli, 1970). After separation, the gels were stained with silver nitrate and blotted with BrCo-9221 polyclonal antibody by the western blotting (Nishizawa *et al.*, 1991).

Electron Microscopy

Infected fish tissues were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 hr, post fixed with 1% OsO_4 in 0.2 M sodium cacodylate buffer for 30 min, embadded in Epon plastic, sectioned, and stained with 2% uranyl-acetate and lead citrate. Gradient fractions were resuspended in TE buffer, and negatively stained with 2% uranyl-acetate on collodion coated grids. All sample grids were examined in a Hitachi H-7000 transmission electron microscope.

Results

RTase assays were performed with 4 purified virus strains and pooled brain and kidney tissue for artificially infected fish. An overall comparision of RTase activity with T_{60}-T_0[poly r(A)] : T_{60}-T_0 [poly d(A)] ratios is shown in Table 1. The purified virus showed RTase activity with T_{60}-T_0[poly r(A)] : T_{60}-T_0[poly d(A)] as high as 11.1. On the study of RT activity for crude virus samples of artificially infected fish, both of brain and kidney samples showed RT activity, with T_{60}-T_0 [poly r(A)] : T_{60}-T_0[poly d(A)] ratio up to 17.5, whereas the negative control fish did not.

The RTase activity from virus strain BrCo-9221 was associated with a buoyant density of about 1.15 to 1.17 g/ml in sucrose, and no RTase activity was associated with any fraction from the negative samples (Fig. 1).

Electron microscopic examination of the fractions with highest enzyme activity revealed the

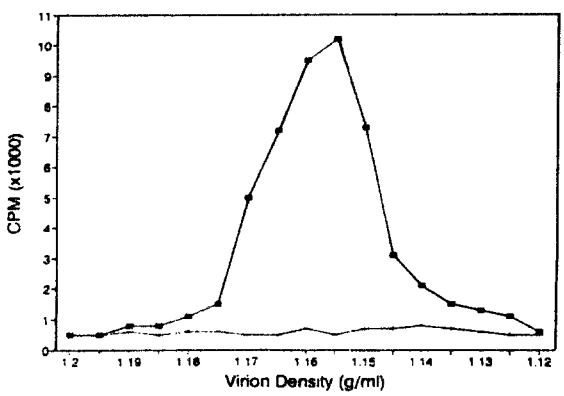

Fig. 1. Buoyant density and RTase activity associated with sucrose gradient fractions from cultured virus strain BrCo-9221. The RTase activity as the ratio of the acid-precipitable cpm at T_{60} and T_0 following an RTase assay. ■, BrCo-9221 ; +, MEM 10.

Table 1. RTase and DNAP directed activity in the purified strains and tissue samples

Sample	Synthethic template (cpm)[a]		r(A) : d(A)
	poly r(A)[T_{60}-T_0]	poly d(A)[T_{60}-T_0]	
Purified virus[b]			
BrCo-9221	14,305	1,527	9.4
BrRt-9203	15,003	1,350	11.1
RrIw-9201	12,960	1,204	10.8
BrAy-9201	10,639	1,226	8.8
Artificial infection study[c]			
Brain of died fish	10,586	1,487	7.1
Kidney of died fish	13,348	986	13.5
Brain of recipient fish	12,690	1,659	7.7
Kidney of recipient fish	20,935	1,198	17.5
Brain of negative control	1,442	873	1.7
Kidney of negative control	1,564	954	1.6

[a] Samples were processed and analyzed for RTase and DNAP activity as described in text using 20 μg of poly r(A) : oligo d(T) or poly d(A) : oligo d(T) primer, 50 μl samples, and 50 μl cocktail (0.1 M Tris-Cl, pH 8.0, 0.5 M KCl, 1.0 mM $MnCl_2$, 4.0 mM dithiothreitol, 0.2% Nonidet P-40, and 20 μCi/ml [^{3}H]TTP). The acid precipitable cpm were determined from each sample at time 0 (T_0) and after 60 min incubation at 22℃ (T60). A T_{60}-T_0[poly r(A)] : T_{60}-T_0[poly d(A)] ratio ≥4 is considered positive for RTase.

[b] Purified virus prepared due to sucrose gradient centrifugation method as described in the text.

[c] Brain and kidney tissue pooled. Virus strain BrCo-9221 infection to rainbow trout with waterborne method.

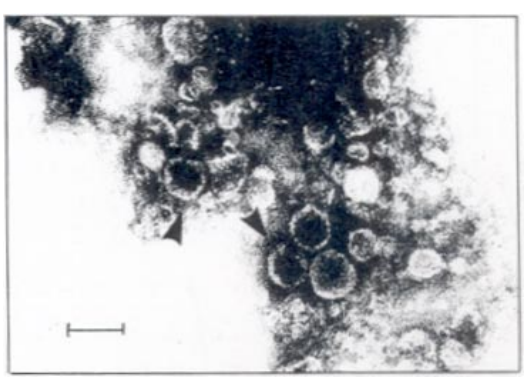

Fig. 2. Pellet from purified, RTase active fractions from sucrose density gradient Negatively stained viral particles are present. Viral particles have a circular and central electron-dense core surrounded by a wide electron-licent space. Bar, 100 nm.

presence of numerous, often clustered virus particles with envelope and 85 nm in diameter (Fig.2).

The virus contaning fractions were analyzed by SDS-PAGE and western blotting. As shown in Fig. 3A, proteins with molecular weights of 120,000, 80,000, 65,000, 61,000, 48,000, 42,000, 35,000, 30,000, 25,000, 19,000 and 15,000 were detected. Western blot analysis showed positive immunoreactivity to the same protein bands with the anti BrCo-9221 serum (Fig. 3B).

Discussion

Retroviruses are widespread in nature, and the hundreds of isolates can be classified into groups according to biological properties, morphology and

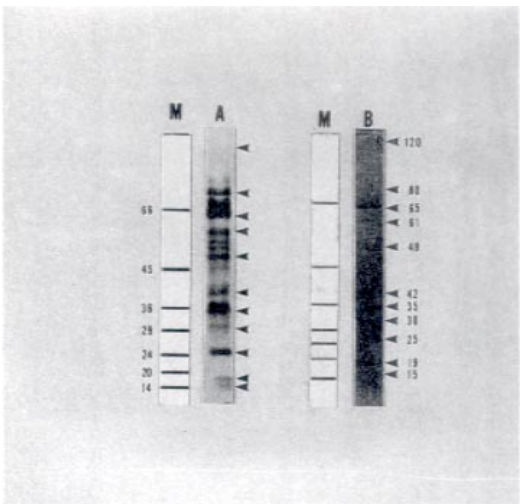

Fig. 3. SDS-polyacrylamide gel electrophoresis and western blotting pattern of the structural polypeptides of isolated virus.
M : Maker, Sigma, $\times 10^3$ dalton
A : Virus polypeptides stained with silver nitrate
B : Western blotting of the virus polypeptides.

genome structure. The entire family is characterized by the presence of the enzyme reverse transcripase (RTase) in the virions. RTase is required for the unique retroviral type of multiplication. After entering the cell, the uncoated viral RNA is transcribed into a double-stranded DNA copy by reverse transcription. This copy is then integrated into the cellular DNA as a provirus. The provirus is transcribed by the cellular DNA-dependent RNA polymerase II into the viral messenger RNA. We have previously demonstrated the transmissibility of RVS, and detected viral particles having a morphology similar to those of retro-like virus. The virus replicated with IUdR and BVdU, and inclusion bodies in the infected cells were stained with acridine orange. Density of the purified virus was 1.155 g/ml and the viral RNA was 7.3 kb in length (Oh, 1995). Retrovirus have about 8 structural proteins and 7 to 10 kb RNA. The virion density is between 1.16 and 1.18 g/ml in sucrose, and they have RTase activity (Coffin, 1990). Our study showed the new virus that isolated from abnormally swimming salmonid fish is retrovirus, which we are tentatively calling RVS.

All of the purified virus from cell culture had RTase activity. In addition, this RTase activity was associated with virus particles of retroviral buoyant density and morphology. The artificial infection study showed that the virus is the etiological agent of the disease. Although the activities of RTase assays were low to moderate, the lack of any significant activity was not due to DNA-dependent DNA polymerase, but was due to RTase. Reverse transcriptase activities as low or lower than those observed with RVS have been reported with retroviruses isolated from fish (Papas *et al.*, 1976), dogs (Tomley *et al.*, 1983), rabbit (Bedigian *et al.*, 1976), baboons (Benveniste *et al.*, 1974) and humans (Manzari *et al.*, 1987). We are currently characterizing the RTase activity of RVS with respect to requirements of $MnCl_2$ concentrations, temperature and incubation period in an attempt to optimize the assay. Many retroviruses contain polypeptides with molecular weight similar to those associated with RVS in this study (Burny *et al.*, 1978 ; Deshayes *et al.*, 1977). The RVS proteins of 120~42 kDa may present structural proteins for the virus. They may also represent capside and capsid-associated precursor polypeptides, as retroviruses often have such proteins in theses molecular weight ranges. The 30~15 kDa proteins may represent the capside and capsid-associated proteins of RVS, as numerous retroviruses have such structural proteins of 24~30

kDa and 11~20 kDa ranges, respectively.

In conclusion, the RTase activity is associated with particles of RVS which have buoyant density of 1.16 g/ml in sucrose. Work is currently under way to develop more rapid diagnostic procedures using PCR and ELISA method.

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異常遊泳 症狀을 나타내는 연어과 魚類에서 分離된 새로운 바이러스의 分子生物學的 特性
-역전사효소 활성-

吳 明 柱

국립수산진흥원 병리과

이상유영 증상을 나타내는 연어과 어류에서 분리된 새로운 RNA 바이러스의 역전사 효소활성을 조사하였다. 정제바이러스는 레트로바이러스 특유의 유전자전환 효소인 역전사효소 (RTase) 활성을 가지고 있었다. Sucrose 밀도구배 초원심법으로 바이러스 밀도를 조사한 결과 1.16 g/ml에서 밴드를 형성하고, 그 부분의 역전사효소 활성이 최고를 나타내었으며, 전자현미경 관찰결과 외막을 가진 크기 85 nm의 레트로바이러스 형태를 확인할 수 있었다. 인위감염 어류의 뇌 및 신장 조직내에 감염되어있는 바이러스를 대상으로 RTase 활성을 조사한 결과 높은 활성치를 확인할 수 있었다. 정제 바이러스의 구성 단백질은 전기영동법으로 확인한 결과 11종이었다. 이상의 결과, 새롭게 분리된 바이러스는 레트로바이러스의 일종인 것이 확인되었으므로 그 이름을 RVS (Retrovirus of salmonid)로 부를 것을 제안한다.

Key words : Retrovirus, Abnormal swimming, Salmonid, RTase activity, Viral density, RVS