Characteristics of a New Reovirus from Channel Catfish (Ictalurus punctatus)

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SUMMARY

The characteristics of a reovirus (CRV) recently isolated from channel catfish (Ictalurus punctatus) were examined following purification of virions from infected cell cultures. Virions had double capsids, the inner and outer with diameters of 55 nm and 75 nm respectively. Complete virions had a density of 1.36 g/ml in CsCl gradients and contained seven polypeptides of 132000, 130000, 110000, 68000, 56000, 43000 and 32000 mol. wt. The nucleic acid labelled with [5-3H]uridine had a density of 1.56 g/ml in CsSO₄ gradients, which suggested that it is double-stranded RNA. The genome was composed of 11 segments that ranged in mol. wt. from approximately 0.4 × 10⁶ to 2.5 × 10⁶. Serum cross-neutralization comparisons of CRV to reoviruses isolated from chum salmon (Oncorhynchus keta) and golden shiners (Notemigonus crysoleucas) indicated that each was distinct although some crossreactions were observed. These viruses seem to represent three serotypes of a new genus within the family Reoviridae.

INTRODUCTION

Following recent outbreaks of channel catfish (Ictalurus punctatus) virus (CCV) disease in three fish farms in Southern California a stock surveillance programme was established. The purpose of this was to determine whether farm disinfection and restocking aimed at eradicating CCV had been successful. During the course of that survey a new reovirus was isolated from catfish at two different farms. The physical characteristics of this virus have recently been described (Amend et al., 1984). The virus has a morphology and size similar to members of the genus Orthoreovirus in the family Reoviridae. There are two easily identifiable capsids surrounding a genome suspected to be RNA from inhibitor studies with 5-bromo-2'-deoxyuridine (Amend et al., 1984; R. P. Hedrick et al., unpublished data). As a result, the virus has tentatively been designated catfish reovirus (CRV). Antisera made to CRV were shown to neutralize partially two previously described reoviruses from fish, namely golden shiner (Notemigonus crysoleucas) virus, GSV (Plumb et al., 1979) and chum salmon (Oncorhynchus keta) virus, CSV (Winton et al., 1981). In this study we have further characterized the antigenic similarity of CRV to GSV and CSV. In addition, the protein and nucleic acid components of CRV were analysed. The results indicate that CRV is related to and probably represents a third serotype of a novel genus of reoviruses that infect fish.

METHODS

Viruses. CRV isolated from diseased channel catfish (Amend et al., 1984) was plaque-purified three consecutive times prior to use in serological and biochemical tests. GSV and CSV used in cross-neutralization studies have been described previously (Plumb et al., 1979; Winton et al., 1981).

Cell lines. The channel catfish ovary (CCO) cell line (Bowser & Plumb, 1980) was used to propagate CRV for serological and biochemical studies. The chinook salmon embryonic (CHSE-214) cell line described by Nims et al. (1970) supported the replication of both GSV and CSV. Both the CCO and CHSE-214 cell lines were grown in...
MEM supplemented with Earle's salts (Flow Laboratories), 10% foetal calf serum (Sterile Systems, Logan, Utah, U.S.A.), 100 U penicillin, 100 μg/ml streptomycin and 0-292 mg/ml glutamine (Gibco). The concentration of serum was decreased to 5% (MEM-5) when the cells were infected with viruses. The CCO and CHSE-214 lines were propagated at 30 °C and 22 °C respectively. The cells used for virus propagation were incubated at 25 °C for CRV and GSV and 20 °C for CSV.

**Virus assays.** The concentrations of virus in neutralization studies and in selected fractions following purification in CsCl gradients were determined by TCID₅₀ assays. The titres were determined in 96-well plates (Linbro) as described by Okamoto *et al.* (1983) and the endpoints determined according to Reed & Muench (1938).

**Cross-neutralization tests.** The serological relationships of CRV to GSV and CSV were determined by cross-neutralization tests using rabbit sera prepared to purified viruses. Hyperimmune rabbit serum was prepared according to Okamoto *et al.* (1983) and virus neutralizations were made by the same method as described by Okamoto *et al.* (1983).

The extent of cross-neutralization (1/r) was calculated according to Archetti & Horsfall (1950) by the formula \( r = \sqrt{r_1 \times r_2} \) where \( r_1 \) and \( r_2 \) represent the titre ratios (heterologous titre divided by the homologous titre) of each respective antiserum. A 1/r of 1.0 indicates serological identity of two viruses, while increasing values represent greater antigenic differences.

**Virus purification.** CRV was purified from CCO cells in an identical manner to that described by Hedrick *et al.* (1983) for infectious pancreatic necrosis virus (IPNV). In experiments requiring radiolabelled virus, [5-H]-uridine (New England Nuclear) at a concentration of 5 μCi/ml of MEM-5 was added following a 1 h adsorption of CRV to monolayers of CCO cells. Virus was concentrated from culture fluids using polyethylene glycol (15000 to 20000 mol. wt.; Baker Chemical Co., Philipsburg, N.J., U.S.A.), and from the cell pellet by extraction with Genetron (1,1,2-trichloro-1,2,2-trifluoroethane; Eastman Kodak Co.). The virus band was then withdrawn by side puncture and dialysed against TNE overnight at 4 °C prior to use.

**Electron microscopy.** Small drops of partially purified virions extensively dialysed against TNE were placed on Formvar (Electron Microscopy Sciences, Fort Washington, Pa., U.S.A.) coated grids. A drop of 2% phosphotungstic acid was then added and the excess moisture removed with filter paper. Negatively stained virions were then viewed with a Philips EM 400 electron microscope.

**Density of virions and RNA.** The density of purified virions labelled with [3H]uridine was determined following centrifugation at 130000 g for 22 h at 4 °C in a three-step CsCl gradient. Fractions collected from the bottom of the centrifuge tube were analysed for their refractive index and then for radioactivity by adding Aquasol EP (Beckman) to 10 μl aliquots. The infectivity of selected fractions was determined by TCID₅₀ analysis on CCO cells following 1:100 dilutions into MEM-5.

The density of purified RNA was determined following treatment of purified virus with 200 μg/ml proteinase K (Sigma) and extraction with phenol saturated with TE buffer (0-01 M-Tris–HCl, 0-001 M-EDTA, pH 8-0). The RNA was precipitated overnight in cold ethanol and collected following centrifugation at 2000 g for 1 h. The RNA, resuspended in TNE buffer was then analysed in polyacrylamide gels or in two-step Cs₂SO₄ gradients (2-0 ml of 1-75 g/ml and 2-0 ml of 1-40 g/ml) and centrifuged for 40 h at 130000 g. The refractive index of selected fractions was used to determine the density of the RNA (Ludlum & Warner, 1965). The amount of radioactivity in each fraction was determined by placing 10 μl directly into Aquasol EP (Beckman).

**Polyacrylamide gel electrophoresis.** The same gel concentrations (9% acrylamide) and buffer systems (Laemmli, 1970) were used to analyse the polypeptides and RNA segments of CRV. Purified CRV was pelleted by centrifugation at 130000 g for 1 h and resuspended into sample buffer. The polypeptides were stained with 1% Coomassie Brilliant Blue in 50% TCA after electrophoresis at 12 mA for 6 h. The molecular weights of the virion polypeptides (VP) were determined by their relative mobilities compared to the following protein standards (Bio-Rad) in the same gel: myosin 200000, β-galactosidase 116250, phosphorylase B 92500, bovine serum albumin 66200, ovalbumin 45000, carbonic anhydrase 31000 and soybean trypsin inhibitor 21500.

The molecular weights of the RNA segments of CRV were estimated by a comparison of their relative mobilities to RNA extracted from reovirus 3 (Dearing strain) and CSV in the same gel. The RNA was stained with silver as described by Merrill *et al.* (1981) following electrophoresis at 9 mA for 20 h.

**RESULTS**

**Cross-neutralization tests**

Cross-neutralization tests indicated that CRV was clearly distinguishable from two reoviruses previously isolated from fish (Table 1). The method of Archetti & Horsfall (1950) to compare
**Table 1. Antigenic relationships of CRV, GSV and CSV by serum cross-neutralization tests**

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>CRV</th>
<th>GSV</th>
<th>CSV</th>
</tr>
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<tbody>
<tr>
<td>CRV (150)*</td>
<td>1024‡ (1·0)‡</td>
<td>8 (84)</td>
<td>16 (95)</td>
</tr>
<tr>
<td>GSV (154)</td>
<td>150</td>
<td>8038 (1·0)</td>
<td>114 (114)</td>
</tr>
<tr>
<td>CSV (100)</td>
<td>36</td>
<td>28</td>
<td>5120 (1·0)</td>
</tr>
</tbody>
</table>

* Concentration of virus used in each test (TCID$_{50}$).
† Reciprocal of the highest dilution of serum at which 50% of the test cultures were protected following infection with each virus.
‡ Represents $1/r$ where $r = \sqrt{r_1^2 + r_2^2}$, giving a numerical value to the extent of crossreaction considering titre ratios (heterologous titre divided by homologous titre) of each respective antiserum $r_1$ and $r_2$ (Archetti & Horsfall, 1950).

Viruses considering both homologous and heterologous reactions showed that all three viruses were essentially equivalent in their antigenic distance from each other ($1/r = 84$ to $114$). Partial neutralizations in heterologous reactions indicated the viruses share some antigenic characteristics although each virus was clearly distinct ($1/r > 84$).

**Electron microscopy**

Three bands were observed in CsCl gradients with partially purified preparations of CRV. An examination of each band indicated that virions found at $\rho = 1·29$ g/ml were most often lacking the internal capsid (Fig. 1a). Virions with a characteristic double-shelled capsid were found at $\rho = 1·36$ g/ml (Fig. 1b). The average diameter of these virions was 75 nm. Particles of $\rho = 1·38$ g/ml had variable diameters and typically possessed four prominent points (Fig. 1c).

**Density of virions and RNA**

Density determinations on $[^3]$H]uridine-labelled purified virions indicated that most radioactivity was associated with virions at $\rho = 1·36$, as was infectivity as shown by TCID$_{50}$ analysis of selected fractions on CCo cells (Fig. 2).

The density of CRV RNA labelled with $[^3]$H]uridine and extracted from purified virions was determined by equilibrium centrifugation in Cs$_2$SO$_4$ gradients. The density of viral RNA was found to be $1·56$ g/ml following centrifugation for 40 h at 130000 g (Fig. 3).

**Polyacrylamide gel electrophoresis of virion RNA and polypeptides**

An analysis of RNA extracted from purified virions in polyacrylamide gels indicated the segmented nature of the genome (Fig. 4a). Eleven segments were found with estimated molecular weights ranging from $0·4 \times 10^6$ to $2·5 \times 10^6$ (Table 2). Although segments L1 and L2 migrated together in routine gels, they were separable upon extended electrophoresis. Segments S4 and S5 were also often difficult to separate in our gels. An electrophoretic examination of the apparently empty virions confirmed the absence of RNA as suggested by electron microscopy. These particles did however contain polypeptides similar to those in complete virions following Coomassie Brilliant Blue staining (data not shown). The virion polypeptides ranged in mol. wt. from 132000 to 32000 (Table 2). The five major polypeptides were VP1, 2, 4, 6 and 7. Two minor polypeptides, VP3 and VP5, were also observed in gels when loading concentrations were increased fivefold.

**DISCUSSION**

Viruses with double-stranded (ds) RNA genomes have been isolated from many species of cultured finfish (Hill, 1982). However, these have most often been shown to be similar to infectious pancreatic necrosis virus (IPNV), originally described by Wolf et al. (1960). IPNV is the type strain of the newly proposed Birnaviridae family which includes viruses characterized by a genome of two segments of dsRNA surrounded by a single capsid (Dobos et al., 1979).

Plumb et al. (1979) were the first to describe a dsRNA virus from golden shiners (GSV) which
Fig. 1. Electron micrographs of CRV particles partially purified by centrifugation in CsCl gradients. (a) Empty capsids ($\rho = 1.29$ g/ml); (b) complete virions ($\rho = 1.36$ g/ml); (c) particles presumed to be cores ($\rho = 1.38$ g/ml). Bar marker represents approx. 100 nm.

was clearly different from the birnaviruses both in particle morphology and serological characteristics (Schwedler & Plumb, 1980). A subsequent report by Winton et al. (1981) showed that a reovirus from chum salmon (CSV) shared physical properties with GSV, suggesting the two viruses were related. Furthermore, a biochemical analysis revealed that CSV contains 11 segments of dsRNA (Winton et al., 1983). A reovirus (CRV) recently isolated from channel catfish and reported by Amend et al. (1984) shares physical and morphological properties with both GSV and CSV. In addition, Amend et al. (1984) found that antisera to CRV partially neutralizes both CSV and GSV, suggesting the three viruses are related members of a new group of reoviruses clearly different from the previously reported birnaviruses.
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A more comprehensive examination of the serological relatedness of these viruses by cross-neutralization tests supported this proposition (Table 1). A comparison of the 1/r values obtained from considering both homologous and heterologous neutralization of two viruses (Archetti & Horsfall, 1950) showed that all three viruses are serologically distinct. In these tests, crossreactions indicated CRV and GSV are more closely related than either was to CSV (Table 1). If a value of 1/r > 20 is used to distinguish serotypes (Okamoto et al., 1983) each virus represents a unique serotype.

The size and morphology of CRV is similar to that reported for GSV (70 nm) by Plumb et al. (1979) and identical to that observed for CSV (75 nm) by Winton et al. (1981). All three viruses resemble members of the genus Orthoreovirus of the family Reoviridae (Palmer & Martin, 1982) in size and morphology but clearly differ by possessing a genome consisting of 11 segments of dsRNA rather than 10. The core-like structures of CRV (Fig. 1c) generated in sufficient quantities to yield visible bands in CsCl may have resulted from our purification procedure as these were not observed by Winton et al. (1983) with CSV. They were able to generate subviral particles by α-chymotrypsin treatment which more closely resembled reovirus 3 cores (Smith et al., 1969) than core-like particles of CRV.

An incorporation of [5-3H]uridine into particles banding at a density of 1.36 g/ml coincided with peak infectivities determined by TCID50 analysis. Phenol extractions, following protease K digestion of virions at this density showed that the 3H label was associated with 11 segments of RNA separable in polyacrylamide gels (Fig. 4a). An analysis of this RNA in Cs2SO4 equilibrium gradients showed its density to be 1.56 g/ml, an identical value to that reported for CSV RNA by Winton (1981). These values are close to 1.60 g/ml as expected for dsRNA (Iglewski & Franklin, 1967; MacDonald & Yamamoto, 1977) but well above the 1.42 to

Fig. 2. Density of CRV in CsCl gradients following centrifugation of purified virions for 22 h at 130000 g. The radioactivity (△), density (●) and infectivity expressed as log TCID50 (○) of selected fractions were determined following collection of the gradient.

Fig. 3. Density of CRV RNA in Cs2SO4 gradients following centrifugation for 40 h at 130000 g. The density (△) and radioactivity (●) of selected fractions were determined following collection of the gradient.

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1.46 g/ml of dsDNA (Price, 1982). These data combined with DNA inhibitor studies on CRV (R. P. Hedrick et al., unpublished data) suggest that the genome is indeed dsRNA.

The mobilities of the 11 genome segments of CRV in polyacrylamide gels compared to those reported for CSV (Winton et al., 1983) showed the viruses to have three size classes (L, M and S) of dsRNA. The mobilities of the individual segments in these size classes, however, is quite different between CSV (Winton et al., 1983), CRV (Fig. 4a) and GSV (J. R. Winton et al., un-
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Table 2. Molecular weight estimates of RNA segments and polypeptides from purified virions of CRV

<table>
<thead>
<tr>
<th>Virion polypeptides</th>
<th>Mol. wt. (× 10^-3)</th>
<th>RNA segments</th>
<th>Mol. wt. (× 10^-6)</th>
</tr>
</thead>
<tbody>
<tr>
<td>VP1</td>
<td>132</td>
<td>L1</td>
<td>2.5</td>
</tr>
<tr>
<td>VP2</td>
<td>130</td>
<td>L2</td>
<td>2.5</td>
</tr>
<tr>
<td>VP3</td>
<td>110</td>
<td>L3</td>
<td>2.4</td>
</tr>
<tr>
<td>VP4</td>
<td>68</td>
<td>M1</td>
<td>1.5</td>
</tr>
<tr>
<td>VP5</td>
<td>56</td>
<td>M2</td>
<td>1.4</td>
</tr>
<tr>
<td>VP6</td>
<td>43</td>
<td>M3</td>
<td>1.2</td>
</tr>
<tr>
<td>VP7</td>
<td>32</td>
<td>S1</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S2</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S3</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S4</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S5</td>
<td>0.4</td>
</tr>
</tbody>
</table>

published results) and resemble comparisons between the genome segments of reovirus types 1, 2 and 3 (Fields, 1982).

The genome of CRV is surrounded by a capsid composed of at least seven structural polypeptide species (Table 2). A similar polypeptide profile was observed for whole, empty and core-like particles of CRV (data not shown). We anticipated that certain polypeptides would be absent in both the empty and core-like particles when compared to complete virions, as has been shown with reovirus 3 (Joklik, 1981). If each genome segment codes for a polypeptide as shown with the reoviruses (Fields, 1982) then a further four polypeptides were either not detected or are non-structural in nature. Alternatively, the core-like structures may be degenerating forms of the virus produced by our purification methods. Gene coding assignments and an examination of the intracellular protein synthesis of CRV-infected cells will be required to distinguish between these possibilities.

The five major polypeptides (VP1, 2, 4, 6 and 7) of CRV are of similar molecular weight to those reported for CSV by Winton et al. (1983); they found five major (137000, 126000, 72000, 44000 and 34000 mol. wt.) and five minor polypeptides. Although the two viruses share five major polypeptides of similar size, they can be distinguished by the relative mobilities and number of minor polypeptides and the mobilities of the RNA segments (Fig. 4a).

The biochemical and serological characteristics of CRV reported in this study show that it is clearly distinct from viruses previously described from fish (Wolf & Mann, 1980). This virus does, however, share several properties (including common antigens) with both CSV and GSV, two previously described viruses with segmented dsRNA genomes from fish. A fourth virus, isolated from American oysters (Crassostrea virginica) using a fish cell line, has been described by Meyers (1979). This virus (13p2) was later found to be pathogenic in at least one fish species (Meyers, 1980) and may also represent another member of this new group of reoviruses.

REFERENCES


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