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NOTE

Complete nucleotide sequence of the S10 genome segment of grass carp reovirus (GCRV)*

Tao Qiu, Ren-Hou Lu, Jing Zhang, Zuo-Yan Zhu**

State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China

ABSTRACT: Hemorrhagic disease, caused by the grass carp reovirus (GCRV), is one of the major diseases of grass carp in China. Little is known about the structure and function of the gene segments of this reovirus. The S10 genome segment of GCRV was cloned and the complete nucleotide sequence is reported here. The S10 is 909 nucleotides long and contains a large open reading frame (ORF) encoding a protein of 276 amino acids with a deduced molecular weight of approximately 29.7 kDa. Comparisons of the deduced amino acid sequence of GCRV S10 with those of other reoviruses revealed no significant homologies. However, GCRV S10 shared a putative zinc-finger sequence and a similar distribution of hydrophilic motifs with the outer capsid proteins encoded by Coho salmon aquareovirus (SCSV) S10, striped bass reovirus (SBRV) S10, and mammalian reovirus (MRV) S4. It was predicted that this segment gene encodes an outer capsid protein.

KEY WORDS: Grass carp reovirus (GCRV) · S10 genome segment · Nucleotide sequence

Grass carp reovirus (GCRV), an important fish pathogen involved in hemorrhagic disease (Chen & Jiang 1983), not only infects grass carp *Ctenopharyngodon idellus*, but also was found capable of infecting black carp *Mylopharyngodon piceus*, topmouth gudeon *Pseudorasbora parva* (Ding et al. 1991) and rare minnow *Gobiocypris rarus* (Wang et al. 1994), and historically has resulted in large losses in freshwater fish culture in China. The virions are resistant to chloroform and ether, non-sensitive to acid (pH 3) and alkaline (pH 10) treatment, and stable within a certain range of temperature. The virus belongs to the genus *Aquareovirus*, as a tentative member, and shares the physicochemical properties and morphological characteristics of the family Reoviridae. About 10 isolates reported to date (Li et al. 1999) had different electrophoretic patterns but the same number of genomic dsRNA. The GCRV possesses a double-stranded dsRNA genome consisting of 11 segments packaged into a non-enveloped icosahedral double capsid approximately 55 to 80 nm in diameter (Ke et al. 1990). The genome segments are approximately 25 000 nucleotide pairs in total size deduced by the dsRNA molecular weight and separated into 3 size classes: large (L1, L2, and L3); medium (M4, M5, and M6); and small (S7, S8, S9, S10, and S11) in order of mobility in polyacrylamide gels from the slowest to fastest, respectively.

Many reoviruses capable of infecting aquatic organisms have been identified (Plumb et al. 1979, Hedrick et al. 1984, Ahne & Kölbl 1987, Winton et al. 1987, Varner & Lewis 1991, Neukirch et al. 1999), and using RNA-RNA blot hybridization, 7 different genogroups have been established among at least 45 aquareovirus isolates (Rangel et al. 1999). However, unlike those of mammalian and plant reoviruses in the family Reoviridae, which have been extensively studied, little information is available on the molecular characteristics of the genomes of members of the genus *Aquareovirus*. More recently, the sequences of the 3 largest segments of GCRV were determined (Fang et al. 2000); they are putative guanylyltransferase, viral polymerase and helicase genes. In addition, 2 sequences of other aquareoviruses have been reported. They are genome segment 10 of the Coho salmon aquareovirus (SCSV) and that of the striped bass reovirus (SBRV), which encode major outer capsid proteins (Lupiani et al. 1997a,b). Although these sequences provided some knowledge helpful for determining the diseases derived from aquatic viruses, they are still limited. In order to obtain further information on the GCRV genome and use

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**Corresponding author. E-mail: rhlu@ihb.ac.cn

available data on *Aquareovirus*, the full-length cDNA of GCHV genome segment S10 was cloned and the characteristics of this sequence are documented here.

Materials and methods. ***Virus strain, purification and RNA extraction:*** The CIK cell strain (Zuo et al. 1986) was used to propagate GCRV-873 (Ke et al. 1990). Virus was purified from a continuous sucrose gradient (30 to 60% sucrose in NTE [NaCl-Tris-EDTA] buffer) and centrifuged at 100 000 × *g* for 2 h. Genomic dsRNA was extracted from the purified virus particles by 1% SDS and 10 µg ml^{-1} Proteinase K at 37°C for 3 h, as well as by phenol-chloroform extraction. The purified dsRNA was treated with RQ1 RNase-free DNase (Promage) at 37°C for 1 h to remove the contamination of cell DNA, and displayed on 12.5% polyacrylamide gel (Fig. 1A).

Cloning of the S10 gene: The method described by Lambden et al. (1992) for amplification of the rotavirus dsRNA genome segments was used, with modification, to clone the GCRV S10 gene. The oligodeoxyribonucleotide primer 1 (5′-PO_4-ATTTACCGCCGAGCCT-GACTT-NH_2-3′), which was ligated to both ends of the dsRNA genome segment, was chemically synthesized and modified (Sango) to prevent self-ligation and subsequent concatenation during the RNA ligase (Life Technologies) reaction, as described by Lambden et al. (1992). The primer 1-tailed dsRNA was denatured by heating to 94°C for 5 min in 62% DMSO in the presence of primer 2 (5′-AAGTCAGGCTCGGCGG-TAAAT-3′ complementary to primer 1) (Sango) and cooled rapidly on ice. The synthesis of cDNA was carried out in reverse transcriptase reaction mix (200 ng dsRNA, 50 mM Tris-HCL pH 8.3, 3 mM $MgCl_2$, 75 mM KCL, 1 mM DTT, 0.5 mM each deoxynucleoside of dNTP [Promega], 40 U RNAsin [SABC], and 200 U reverse transcriptase [Gibco BRL]). The mixture was incubated at 37°C for 1 h and the reaction was stopped by addition of EDTA to finial 20 mM. The RNA was removed by NaOH and the cDNA was allowed to anneal. After extraction by phenol-chloroform and precipitation by ethanol, the annealed partial duplexes were filled in using DNA Polymerase I Large Fragment (Promage) and purified using a Glassmilk DNA purifying kit (BioStar). The amplification of cDNA was accomplished by PCR with primer 2 only using a PE9600, consisting of a denaturation step at 94°C for 3 min followed by 30 cycles of 30 s at 94°C, 35 s at 60°C, and 2 min at 72°C. Amplified DNA products were separated on agarose gel and the 900 bp fragment, which corresponded in size to that calculated from the GCRV dsRNA molecular weight for S10, was excised and purified using the Glassmilk DNA purifying kit. The purified S10 was ligated directly into pGEM-T vector and transformed into DH5α strain of *Escherichia coli* (Gibco BRL).

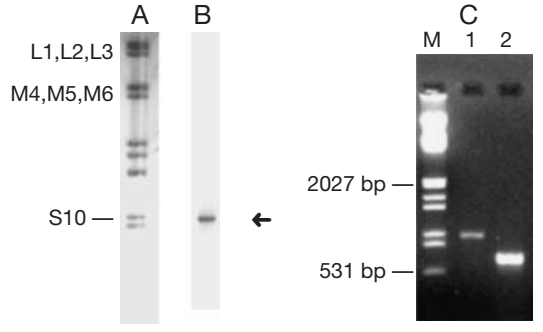

Fig. 1. Analysis of cloned S10 segment of GCRV. (A) PAGE profile of dsRNA genome of GCRV. (B) Result of Northern blotting with S10 cDNA as probe. The position of the S10 segment is indicated with ←. (C) Results of RT-PCR amplification of dsRNA of GCRV. M: Lambda DNA/EcoR I+ *Hin*d III marker; Lane 1: PCR product amplified with Primer 2; Lane 2: RT-PCR product amplified with Primer 3 + Primer 4

S10 gene sequence determination and analysis: The recombinant plasmids containing the full-length cDNA of S10 were identified according to the size of inserted segment by PCR using 2 M13 primers on pGEM-T and were purified using a plasmid DNA purification miniprep kit (Viogene). Two clones that had the full-length cDNA of S10 were used for sequencing. The nucleotide sequence of S10 was determined by Taq dye primer cycle sequencing on an ABI 310 Genetic Analyzer (Perkin Elmer). The sequence of S10 was analysed using Lasergene sequence analysis package (DNAStar).

Northern blotting and RT-PCR: Assignment of the cDNA inserts to S10 was confirmed by alkaline blotting analyses as described by Li et al. (1987). In brief, the genome dsRNA was separated in 1.0% agarose gel in Tris-acetate-EDTA buffer and transferred to a nylon memberane (Hybond) in 0.2 N NaOH for 1 h at room temperature. Transferred RNA was hybridized with a cDNA probe labeled with digoxigenin-dUTP, followed by an immunodetection using Dig High Primer Labeling and Detection Starter Kit (Boehringer Mannheim). Two primers for RT-PCR, Primer 3 (5′-CCCCGATCA-TCACCACGAT-3′) (from nucleotide 14 to 32) and Primer 4 (5′-CGCGTTCGCTGATGTAAGG-3′) (from nucleotide 693 to 711), were synthesized according to the cDNA sequences of the cloned S10. The reverse transcription was carried out following the procedure described by Li et al. (1997).

Results and discussion. Comparing with the GCRV genome dsRNA in Fig. 1A, the clone containing the cDNA of S10 was confirmed by Northern blotting as shown in Fig. 1B, in which the cloned cDNA hybridized to genome segment S10 rather than the other genome segments. Fig. 1C indicates that an expected, approximately 700 bp DNA band was ampli-

fied in the result of RT-PCR using 2 primers within the nucleotide sequence of S10. No product was found in negative control of RT-PCR with genomic DNA of CIK cell as template (data not shown).

The complete nucleotide sequence and deduced amino acid sequence of the S10 genome segment of GCRV are shown in Fig. 2. The nucleotide sequence was obtained from 2 different recombinant plasmids and constitutes a consensus sequence. The S10 gene of GCRV is 909 nucleotides long and contains a large open reading frame (ORF) preceded by a 30 bp untranslated region and followed by a 48 bp untranslated downstream sequence. The ORF starts with an ATG codon at nucleotides 31 to 33 and ends with a TGA at nucleotide 859 to 861. No additional ORF of significant length was detected in either the plus or minus strand RNA. Three AUG triplets, the first (ACGATGC) located at 31, the second (CACATGA) at 43, and the third (GCTATGG) at 67, were recognized in the same reading frame and near the 5′ end of the mRNA strand. Since in most cases the AUG nearest the 5′-terminal cap is exclusively used for initiation for protein synthe-

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  1                GTTATTTCTGAGCCCCCGATCATCACCACGATG CCA CTT CAC ATG ATT CCG CAA GTC GCC     60
  1                                                M   P   L   H   M   I   P   Q   V   A     10

 61   CAC GCT ATG GTG CGT GCA GCC GCT GCA GGA CGC CTT ACC TTA TAC ACA AGA ACT AGA ACT    120
 11    H   A   M   V   R   A   A   A   A   G   R   L   T   L   Y   T   R   T   R   T     30

121   GAG ACC ACC AAC TTT GAT CAC GCT GAG TAC GTC ACC TGC GGG CGG TAC ACC ATC TGC GCC    180
 31    E   T   T   N   F   D   H   A   E   Y   V   T   C   G   R   Y   T   I   C   A     50

181   TTC TGC CTT ACG ACT CTG GCT CCC CAC GCC AAC GTC AAG ACC ATT CAA GAC TCC CAC GCT    240
 51    F   C   L   T   T   L   A   P   H   A   N   V   K   T   I   Q   D   S   H   A     70

241   TGT TCA CGT CAA CCA AAT GAA GCC ATT CGC TCA TTA GTC GAA GTG AGT GAC AAA GCG CAG    300
 71    C   S   R   Q   P   N   E   A   I   R   S   L   V   E   V   S   D   K   A   Q     90

301   ACC GCC CTC GTC GGT AGC CGT ACT GTA GAC TAT CAC GAA TTG GAT GTG AAA GCT GGG TTC    360
 91    T   A   L   V   G   S   R   T   V   D   Y   H   E   L   D   V   K   A   G   F    110

361   GTC GCC CCA ACT GCC GAT GAA ACA ATA GCC CCC TCT AAG GAT ATC GTC GAA CTT CCG TTT    420
111    V   A   P   T   A   D   E   T   I   A   P   S   K   D   I   V   E   L   P   F    130

421   CGC ACC TGT GAC TTG GAC GAT TCC TCT GCT ACC GCT TGC GTC CGA AAT CAC TGC CAG GCC    480
131    R   T   C   D   L   D   D   S   S   A   T   A   C   V   R   N   H   C   Q   A    150

481   GGT CAC GAC GGC GTT ATC CAC CTC CCG ATC CTT TCT GGA GAT TTC AAA TTG CCT AAC GAG    540
151    G   H   D   G   V   I   H   L   P   I   L   S   G   D   F   K   L   P   N   E    170

541   CAT CCC ACC AAA CCG TTG GAC GAT ACG CAT CCC CAC GAC AAG GTG CTG ACT CGC TGC CCC    600
171    H   P   T   K   P   L   D   D   T   H   P   H   D   K   V   L   T   R   C   P    190

601   AAG ACT GGT CTC CTC CTC GTC CAT GAC ACT CAC GCA CAC GCC ACC GCC GTA GTT GCC ACC    660
191    K   T   G   L   L   L   V   H   D   T   H   A   H   A   T   A   V   V   A   T    210

661   GCT GCT ACG AGA GCT ATC CTC ATG CAC GAC CTC CTT ACA TCA GCG AAC GCG GAT GAC GGC    720
211    A   A   T   R   A   I   L   M   H   D   L   L   T   S   A   N   A   D   D   G    230

721   CAT CAA GCA CGT TCC GCT TGC TGC GGT CCA GCG TTT AAC AAC CTG ACC TTC GCT TGC CAC    780
231    H   Q   A   R   S   A   C   C   G   P   A   F   N   N   L   T   F   A   C   H    250
                                                       *   *   *   *
781   TCC ACC TGT GCT TCA GAT ATG GCT CAC TTC GAC TGC GGC CAG ATC GTT GGA CTC GAC TTG    840
251    S   T   C   A   S   D   M   A   H   F   D   C   G   Q   I   V   G   L   D   L    270
                                                   #   #   #   #   #   #
841   CAT GTG GAG CCA TCC GAT TAAGCACCGGCCTACCCACCCGGTGCGGGTGTAGGGGTCTCTCGTTTCATC        909
271    H   V   E   P   S   D   .                                                        276
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Fig. 2. Complete nucleotide sequence (presented in the cDNA form) and deduced amino acid sequence of S10 RNA segment of GCRV. The 5′- and 3′-terminal nucleotide sequences are **bold** and indicated with a grey background and the inverted repeats are **bold**. Three ATG triplets near the 5′end of nucleotide sequence are underlined. In the amino acid sequence, the putative zinc-finger motif (amino acids 49–71) is also indicated with a grey background. Possible N-glycoslation site (*) and N-myristoylation site (#) are indicated

sis in a 'scanning model' (Kozak 1980), the putative methionine start codon should be the first triplet rather than the others. Interestingly, the third triplet also appeared to be potentially suitable as a functional initiator because it was consistent with the strong initiation sequence of RNNATGG (R = purine nucleoside, N = any nucleoside) (Kozak 1981).

The GCRV S10 genome segment displays the terminal sequences 5′ GUUAUU and CAUC 3′, which were recognized to be conservative in the GCRV RNA segments (authors' unpubl. data). Moreover, the 3′-terminal sequence CAUC 3′ was found to be the same as that of segment S10 of SBRV (Lupiani et al. 1997b), another member of *Aquareovirus*. In addition, a putative inverted repeat sequence was identified adjacent to the terminal sequence. The 5′ end sequence, GAGCCCCC at 10 to 17, and its 3′ end inverted repeat sequence, GGGGTCTC at 892 to 899, are almost completely complementary.

The conserved terminal sequences are broadly reported in members of the family Reoviridae. They may be important in sorting and packing functions of the virus (Anzola et al. 1987). Analysis of the terminal nucleotide sequences is of interest in relation to understanding mechanisms of transcription and replication. The same 3′ terminal sequence between GCRV S10 and SBRV S10 implied relatively closer relations between them. In addition, like other reoviruses, a domain of inverted repeat adjacent to the 5′ and 3′ terminus always plays an important role in distinguishing this genome segment from other segments (Anzola et al. 1987).

The ORF is considered to encode a protein of 276 amino acids with a deduced molecular weight of approximately 29.7 kDa. The possible modification sites, an N-glycosylation site present at 244 to 247 and an N-myristoylation site at 263 to 268, were indicated and near the C terminus of the polypeptide (Fig. 2). Using the BLAST programs (Altschul et al. 1997), we were unable to find substantial similarity with either the nucleotide or the amino acid sequence of S10 and sequences of characterized animal reovirus and plant-infecting reovirus. However, some similarities in GCRV S10, SCSV S10 and SBRV S10 were observed. They not only have similar segment lengths, but the molecular weights of their deduced peptides are also analogous. Moreover, they are all segment 10 of the viruses in the same genus, i.e. *Aquareovirus*. We compared the deduced amino acid sequence of GCRV S10 with those of SCSV S10 and SBRV S10 using the Lasergene program (DNAStar). The predicted protein encoded by GCRV S10 showed a similarity index of 17.1 (Lipman & Pearson 1985) with SCSV S10 in a 173 amino acid overlap, and of 30 with SBRV S10 in only a 20 amino acid overlap.

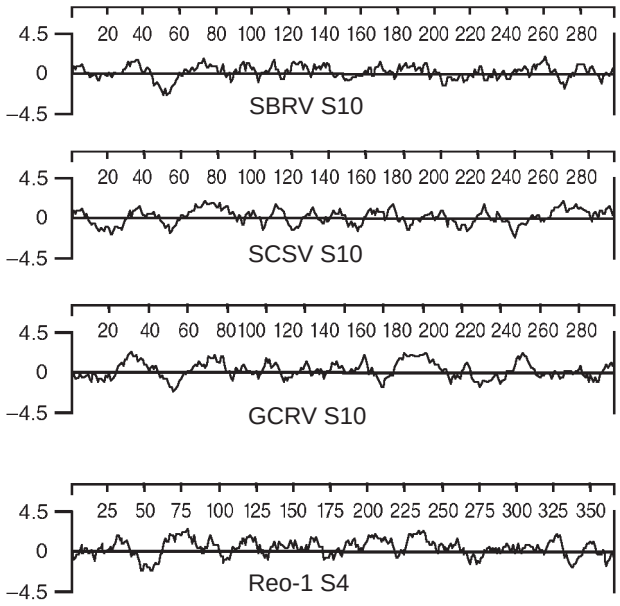

Fig. 3. Hydrophilic plots of deduced proteins encoded by SBRV S10, SCSV S10, GCRV S10 and Reo-1 S4

Although it can be proposed that GCRV S10 may correspond to SCSV S10 to a low extent and may not be related to SBRV S10 with respect to amino acid sequences, the hydrophilic plots (Fig. 3) produced according to the method of Kyte & Doolittle (1982) significantly showed that the predicted proteins encoded by GCRV S10, SCSV S10 and SBRV S10 had very similar profiles, particularly in the regions of the N-terminal half. Thus, it can be predicted that their amino acid sequences may have an analogous molecular conformation. Furthermore, it is very likely that this analogous conformation leads to functional similarities among the deduced proteins of the S10 segments of these aquareoviruses.

The deduced amino acid sequence of GCRV S10 possesses a $CX_2CX_{16}HX_1C$ sequence within the N terminus, from residues 49 to 71, which is analogous to the zinc-finger domain (CCHC) identified within the mammalian reovirus (MRV) σ3 protein encoded by genome S4 gene (Mabrouk & Lemay 1994). Comparable in position, the zinc-finger sequence $CX_2CX_{15}HHX_1C$ is from amino acids 51 to 73 within the MRV σ3 protein. Notably, we also recognized the zinc-finger domains within deduced amino acid sequences of SCSV S10 ($CX_2CX_{16}HX_1C$) and SBRV S10 ($CX_2CX_{15}HX_1C$) (Fig. 4), although they had not been indicated in the original reports (Lupiani et al. 1997a,b). Moreover, they are located at the same or a position very similar to that of GCRV S10, namely, at residues from 50 to 71 and from 49 to 71, respectively. Thus, the $CX_2CX_{15-16}HX_1C$ was found to be the special zinc-finger

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          45        55        65        75
SBRV S10  GAYVICACCFKVLLNWPGPPIHITHECHDSHGVCR
               *  *                 * *

          45        55        65        75
SCSV S10  GQYQLCALCLKQVCSYHVTEPCYYPHECHHGQATR
               *  *                 * *

          45        55        65        75
GCRV S10  RYTICAFCLTTLAPHANVKTIQDSHACSRQPNEAI
               *  *                 * *

          45        55        65        75
Reo-1 S4  CGGAVVCMHCLGVVGSLQRKLKHLPHHRCNQQIRH
               *  *                 ** *
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Fig. 4. Sequences of the putative zinc-binding sites in deduced amino acid sequences of GCRV S10, SBRV S10, SCSV S10, and Reo-1 S4. *Residues of the putative zinc fingers. Numbers refer to the amino acid positions

sequence shared by the deduced peptide of the S10 segments of these aquareoviruses and MRV σ3. Since the most probable functional motif was identified as being a form $CX_2CX_{15}HX_2C$ (Mabrouk & Lemay 1994), the function of the zinc-finger motifs indicated here requires further study. In previous reports, the zinc-finger domains may be required by some proteins to maintain proper conformation (McIntyre et al. 1993) and may also be involved in protein-protein interactions (Cunningham et al. 1991).

Since the comparable zinc-finger domain exists in the MRV σ3 protein, the hydrophilic plot of the reovirus serotype-1 (Reo-1) S4 (Seliger et al. 1992), as an ex-ample of MRV, was given together with those of aquareoviruses. Surprisingly, many profiles were also similar to those of aquareoviruses, although MRV belongs to another genus, *Orthoreovirus*. In particular, the N-terminal 160 amino acids of Reo-1 S4 are very like those of GCRV S10, and the C-terminal is analogous to those of GCRV S10 and SBRV S10, suggesting that the deduced amino acid sequences of the aquareovirus S10 may share some functional similarity with MRV σ3 protein. In addition, it was notable that the terminal sequences of MRV S4, 5′ GCUAUU and CAUC 3′ are very similar to that of GCRV S10. From an evolutionary standpoint, it is likely that the GCRV S10 and the MRV S4 have evolved from a common ancestral precursor.

Since SCSV S10, SBRV S10, and Reo-1 S4 were previously revealed to encode major outer capsid proteins (Atwater et al. 1986, Lupiani et al. 1997a,b), and since similarities have been shown between the zinc-finger sequences and hydrophilic plots of GCRV S10 and the above viral segments, it can be predicted that the deduced peptide of GCRV S10 is an outer capsid protein. In addition, the smallest segment of the GCRV genome, S11, has already been assigned to encode nonstructural proteins (authors' unpubl. data), suggesting that the deduced peptide encoded by GCRV S10 may be the smallest outer capsid protein.

We compared the molecular weight of the deduced peptide of GCRV S10 with that of the proteins isolated from GCRV virions as described by Ke et al. (1992). In that report, the smallest outer capsid protein of GCRV is 27 kDa, slightly smaller than the 29.7 kDa protein deduced. Considering previous studies on aquareoviruses (Winton et al. 1987, Hsu YL et al. 1989, Subramanian et al. 1994) which reported that the smallest structural proteins all were approximately 31 to 36 kDa, it is possible that a deviation from reading data on the SDS-PAGE existed in the Ke et al. report. Moreover, the same protein identified in another report (Wang et al. 1990) was 31 kDa, which approximates the size of deduced product of S10. Our study will serve as a preliminary for future investigations. In order to learn more about the structure and function of the protein encoded by GCRV S10 and its similarities with other proteins, additional work including biochemical assays and immunological analysis should be performed.

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