

# Chapter 2

## Molecular Biology of Aquareoviruses



Qin Fang, Jie Zhang, and Fuxian Zhang

**Abstract** Aquareoviruses are nonenveloped multiple-shelled particles composed of a genome of 11 double-stranded RNA segments, which encode at least 12 proteins. Among the seven approved *Aquareovirus* species (*Aquareovirus A-G*), the striped bass reovirus in the *Aquareovirus A* (AqRV-A) group and grass carp reovirus (GCRV) in the *Aquareovirus-C* (AqRV-C) group have been well studied. Intact virions are composed of five inner shell proteins (ISPs; VP1–VP4 and VP6) and two outer capsid proteins (OCPs; VP5 and VP7). The inner core proteins contain all endogenous enzymes responsible for viral transcription and replication. During cellular entry, intermediate sub-viral particles or core particles can be generated by the uncoating of the OCPs VP5 and VP7. In addition to the seven structural proteins of the viral particle, the remaining five proteins have been approved as nonstructural (NS) proteins (NS80/NS1, NS38/NS2, NS31/NS3, NS26/NS4, and NS16/NS5), except the newly identified NS12. Three genotypes of GCRVs (GCRV-I, -II, and -III) have been classified from infected grass carp in China. Studies indicate that NS80 and NS38 of GCRV-I are related to viral inclusion body formation during replication and morphogenesis. Furthermore, NS16 and NS12, encoded by the S7 segment, have been found to play critical roles as fusion-associated small transmembrane proteins to induce syncytium formation via cell–cell fusion. However, no such protein, instead of a fiber protein on the particle surface, which is a counterpart of the orthoreovirus  $\sigma$ 1-cell attachment protein, has been found in GCRV-II. Moreover, other NS proteins may be involved in viral replication and pathogenesis via interaction with host cells. This chapter outlines the molecular biology of aquareoviruses.

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## Abbreviations

AHRV	Atlantic halibut reovirus
AqRV	Aquareovirus
ARV	Avian reovirus
BF-2	Bluegill fry
CCRV	Channel catfish reovirus
CHSE	Chinook salmon embryo
CIK	<i>Ctenopharyngodon idella</i> kidney
CoSRV	Coho salmon reovirus
CPE	Cytopathic effect
CSRV	Chum salmon reovirus
FAST	Fusion-associated small transmembrane
FHM	Fathead minnow
GCHV	Grass carp hemorrhage virus
GCRV	Grass carp reovirus
GSRV	Golden shiner reovirus
ISPs	Inner shell proteins
MRV	Mammalian orthoreovirus
MSReV	<i>Micropterus salmoides</i> reovirus
NCR	Non-coding region
NS	Nonstructural
OCPs	Outer capsid proteins
ORF	Open reading frame
p.i.	Post-infection
PB	Polybasic
PRV	Piscine orthoreovirus
RdRP	RNA-dependent RNA polymerase
SBRV	Striped bass reovirus
SMReV	Turbot <i>Scophthalmus maximus</i> reovirus
SVP	Sub-viral particle
TEM	Transmission electron microscopy
TFV	Threadfin reovirus
TM	Transmembrane
TRV	Turbot reovirus
VIBs	Virus inclusion bodies

## 2.1 Introduction

Genus *Aquareovirus* is one of the approved members of the family *Reoviridae* and grouped in the *Spinareovirinae* or “turreted” sub-family [30]. The viruses have been isolated from freshwater and seawater aquatic organisms in a wide range of global water areas. Generally, aquareoviruses (AqRVs) are of low pathogenicity in breeding aquatic animals and often detected by routine examination of seemingly healthy fish and shellfish [32]. However, some members of this genus are important fish pathogens that are capable of causing severe hemorrhagic disease, hepatitis, and pancreatitis, leading to a high mortality rate in some cases [12, 32, 33, 37, 52, 54, 60]. Therefore, these viruses pose a significant threat to global aquaculture.

Similar to other members of the *Reoviridae* family, AqRVs contain 11 double-stranded RNA (dsRNA) genome segments that are packaged into two well-organized concentric protein shells, called the inner or core shell and outer capsid. The viral particles are nonenveloped icosahedral particles with a diameter of approximately 75 nm, which has been determined by negative staining in transmission electron microscopy (TEM) analysis [27, 32, 46, 67]. AqRVs, belonging to the *Spinareovirinae* group, have relatively large spikes or turret structures situated at the 12 icosahedral vertices of either the intact virion or the core particle [2, 17]. Morphologically, these viruses resemble mammalian orthoreoviruses (MRVs), while the 11 genomic segments are similar in composition to those in the members of the “non-turreted” genus *Rotavirus* [30]. Based on the morphological and genomic properties of AqRVs isolated from aquatic animals, these were initially referred to as reovirus-like or rotavirus-like agents [36, 46, 54]. However, no cross-reactions are detected between turreted MRVs and non-turreted rotaviruses according to the antigenicity and reciprocal RNA–RNA dot-blot hybridization analyses in earlier studies [9, 32, 38, 52]. Recent nucleotide sequence analysis and comparisons between *Aquareovirus* and other genera in the family *Reoviridae* have suggested that there is a close evolutionary relationship between members of the genera *Aquareovirus* and *Orthoreovirus* [1, 2, 13], which has been further supported by three-dimensional structural reconstruction studies (in Chap. 3). Seven *Aquareovirus* species (*Aquareovirus A-G*) have been approved by the International Committee on Taxonomy of Viruses [30, 39].

Since the first AqRV, golden shiner reovirus (GSRV), reported in the 1970s [46], a large number of AqRVs have been discovered [11, 27, 32, 36, 39, 45, 54, 60, 64, 71], and some pathogenic isolates have complete genome sequences characterized. However, their pathogenic roles in aquaculture remain to be fully elucidated. This chapter summarizes the molecular characteristics of several well-characterized AqRVs, especially grass carp reovirus (GCRV) and striped bass reovirus (SBRV), thus providing a general understanding of the natural biology and molecular properties of AqRVs. For GCRV, the strain GCRV-873 (GCRV-I), originally named as grass carp hemorrhage virus (GCHV), has been suggested as the prototype strain of GCRV species, and hence, it will be referred to as GCRV throughout this chapter.

## 2.2 Particle-Based Properties

### 2.2.1 *Virus Proliferation and Sample Preparation*

Unlike non-fusogenic orthoreoviruses, most AqRV isolates, such as GSRV, American oyster reovirus (strain 13p2), chum salmon reovirus (CSRV), channel catfish reovirus (CCRV), SBRV, GCRV, threadfin reovirus (TFV), and Atlantic halibut reovirus (AHRV), can efficiently replicate in permissive cell lines and induce syncytia as a typical cytopathic effect (CPE). The mature virions can be efficiently released from infected cells by cell lysis; hence, they are fusogenic. Cell lines such as chinook salmon embryo (CHSE), fathead minnow (FHM), bluegill fry (BF-2), *Ctenopharyngodon idella* kidney (CIK), and others are the most commonly used cell lines for plaque assays and proliferation of AqRVs for viral purification [11, 27, 32, 36, 39, 45, 54, 60, 64, 67, 71]. In addition, SBRV has been reported to replicate well in permissive mammalian cell lines (such as CV-1, HeLa, and Vero) and form typical syncytia [53]. In general, AqRV-infected cell cultures are harvested 3–6 days post-infection (p.i.) or when the CPE is extensive enough for virus purification and identification.

There are many different methods used for AqRV particle isolation and purification from infected cells or diseased fish tissues [16, 52, 67]. Despite their respective advantages in virion isolation, the traditional centrifugation method is commonly used. For general purification, some chemical and physical methods have been used on infected cells in the early stages. Sonication has been used to break virus-infected cells; thus, nascent virus particles can be efficiently released from infected cells. Next, using deoxycholate and Freon extraction, the virus particles are disassociated from the infected cell culture mixtures [52, 67]. In addition, owing to its fusogenic nature, AqRV can be disassociated from cell lysates using physical methods with several freeze-thaw cycles [14–16, 54]. After obtaining the virus-cell suspension, centrifugation at several different speeds generally needs to be performed to remove cell debris extensively. The first low-speed centrifugation is used to remove cell debris from the infected culture supernatant, followed by ultracentrifugation to precipitate viral particles. The virus pellet is suspended in  $1\times$  SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.4) or  $1\times$  phosphate-buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 8.1 mM  $\text{Na}_2\text{HPO}_4$ , 1.5 mM  $\text{KH}_2\text{PO}_4$ , pH 7.5). To obtain viral components for viral protein analyses or reconstruct three-dimensional images of viral particles, a CsCl equilibrium density or sucrose gradient purification is required for isolating various virus particle components from pelleted virus-cell preparations [15, 16, 67]. After obtaining a uniform viral layer in the centrifugation tube, harvested virions are either extensively dialyzed against TM buffer (50 mM Tris-HCl, pH 7.6, 10 mM  $\text{MgCl}_2$ ) or further centrifuged to remove sucrose or salts. The concentrations of the purified viral particles can be determined from optical density measurements at 260 nm, as previously described [15, 16]. For freshly purified virions, short-term storage at 4 °C is the best for further TEM analysis. For long-term

storage or maintenance of the structural integrity of purified AqRV virions, storage temperatures of  $-30\text{ }^{\circ}\text{C}$  or  $-80\text{ }^{\circ}\text{C}$  are recommended.

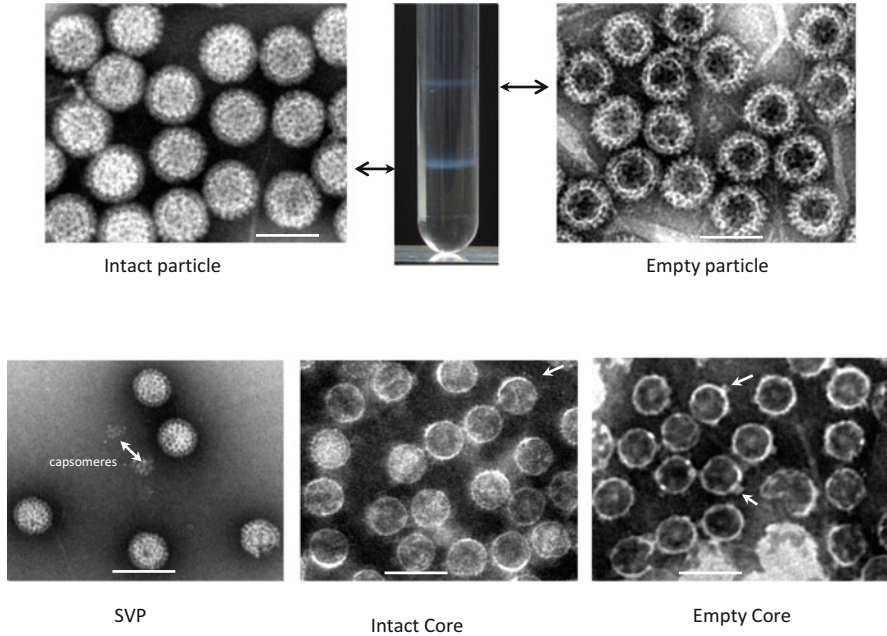
### ***2.2.2 Different Particle Components Extracted from Purified Virus Preparations***

Intact virions are the predominant form of mature particles released from infected cell cultures or extracted from aquatic animal tissue homogenates. Intact virions of AqRV can readily undergo particle uncoating to yield intermediate or infectious sub-viral particles (SVPs) and core particles. This phenomenon is consistent with the orthoreoviruses or MRVs, wherein the SVP or the core also appears naturally in the course of infection and plays specific roles in the early and late replication stages [16, 43]. In addition, SVPs or cores can be generated in vitro by protease treatment of purified virions. Chymotrypsin or trypsin is commonly used to convert reovirus virions into infectious SVPs or cores in vitro [16, 35, 40].

MRV virions are relatively stable in natural environments and maintain infectivity for years when stored below  $4\text{ }^{\circ}\text{C}$  [43]. Similarly, AqRVs retain relatively high infectivity when stored at or below  $4\text{ }^{\circ}\text{C}$  [16, 51]. However, unlike the relatively stable MRV particles, the AqRV SVPs can easily be generated after few days of storage at  $22\text{--}26\text{ }^{\circ}\text{C}$  or  $4\text{ }^{\circ}\text{C}$  in laboratory conditions without undergoing any external treatment with proteases [16, 40]. In most cases, various viral components (including empty and core particles) can be observed from purified sample preparations by TEM.

#### **2.2.2.1 Intact Virions and Empty Particles**

During AqRV purification, two viral component layers with a clear opalescent appearance are often obtained after CsCl density gradient centrifugation, which correspond to approximately  $1.31$  and  $1.37\text{ g/cm}^3$  in buoyant density from the top of the tube to the bottom. The upper layer (top component) largely comprises double-shelled empty particles, while the lower layer contains intact virions or native virions (Fig. 2.1, upper panel). Electron micrograph of negatively stained purified virus preparations shows that an AqRV appears as a nonenveloped icosahedral particle with a double-layered capsid and is approximately  $75\text{ nm}$  in diameter with 5:3:2 symmetry. The particles viewed along the fivefold axis of symmetry exhibit 20 capsomeres on the periphery of the outer capsid shell. Consistent with MRVs, purification of AqRV particles from infected cells often yields a substantial proportion of particles that lack genomic dsRNA [16, 67]. These empty particles have a lower buoyant density in the CsCl gradient and their respective layer is closer to the top of the gradient in comparison with intact particles (containing dsRNA genome), and hence, the empty particle is termed as the “top component.” Both intact and



**Fig. 2.1** Electron micrographs of negatively stained GCRV particles showing various particle components. Intact (left in upper panel) and empty particles (right in upper panel) are isolated from CsCl gradient centrifugation tube (medium in upper panel). Intermediate SVP, intact and empty cores are shown in lower panel. The SVP, intact and empty cores are generated by treatment with 100 mg/ml trypsin at 28 °C for 30 and 120 min, respectively. Double arrowhead in SVP image (left in lower panel) indicates some outer viral capsomeres removed from GCRV particle. Arrowheads indicate pentonal turrets situated at fivefold vertices in intact and empty core images (lower panel). The scale bars represent 100 nm

incomplete (empty) particles are observed in most purified AqRV preparations [11, 14–16, 45, 54, 71]. Moreover, the morphology of AqRV particles is strikingly the same as that of MRVs [15, 67]. The top component particles appear similar to intact virions in their morphology, but the central region of these particles can be penetrated by the stain, reflecting the absence of genomic dsRNA in the interior region of the top component particles [16].

#### 2.2.2.2 SVP and Virus Core

For a detailed understanding of the particle components of AqRV, different forms of GCRV particles have been isolated from mock-treated and protease-treated virion preparations using an established CsCl gradient centrifugation procedure and then examined under an electron microscope. A visual inspection of the gradient centrifugation showed that the different particles form homogeneous layers with an opalescent appearance [16]. The four distinct layers, including two top component

layers, one medium, and one bottom layer, correspond to approximately 1.30, 1.31, 1.38, and 1.44 g/cm<sup>3</sup>, respectively, in buoyant density from the top of the tube to the bottom. TEM observations revealed that after CsCl gradient centrifugation, the viral top components, including the empty double-layered capsids and empty single core shells, are located in the two top layers, and the intact virions and intact cores stand in the medium and bottom layers, respectively. Here, it should be noted that the intact virion with 1.38 g/cm<sup>3</sup> buoyant density might contain a few intermediate SVPs [16], which is very similar to naturally degraded particles of SBRV [40]. This result suggests that parts or some residues of the outermost protein VP7 may fall off from native particles in the virus preparations.

Treatment of AqRVs with proteases (chymotrypsin and trypsin) can generate intermediate SVPs and cores, similar to those reported in other AqRV isolates, such as SBRV [35, 40]. It has been reported that the digestion of the outermost capsid protein (OCP) VP7 of SBRV is associated with increased infectivity [35]. Examination of purified viruses treated with chymotrypsin showed that intact core particles are about 50–60 nm in diameter, which appeared to lack outer capsid capsomeres/proteins in comparison with intact virions of approximately 75 nm. Similarly, infectious SVPs of GCRV can be obtained by treating intact particles with protease (chymotrypsin or trypsin) for a short time. Long protease treatment removes all of the OCPs and exposes prominent turrets, while the core particle structure remains intact (Fig. 2.1, lower panel). Negatively stained TEM images reveal that the core particles exhibit projections at each fivefold vertex and some indistinct projections can be visualized on the empty core shell.

Of note, a few empty single outer capsid shells, empty double outer capsid shells, or single core particles are often found in preparations of natural viral particles without protease treatment, during the purification process or during long-term storage of viral stock at 4 °C. This indicates that the AqRV (or GCRV-873) particle structures are not stable during purification or long-term storage at 4 °C. The related structural architecture of GCRV-873 has been elucidated by three-dimensional image reconstruction [7, 15, 66], and it is described in Chap. 3.

In fact, similar to MRVs, different components of AqRVs (such as SVPs, empty particles, and cores) are also commonly observed in infected cells or tissue sample preparations, except for purified virus preparations that have been treated with chymotrypsin or trypsin under controlled conditions *in vitro* or virion disassembly during infection of cells [11, 16, 45, 50, 67]. The characteristics and properties of the various AqRV particle components are shown in Table 2.1.

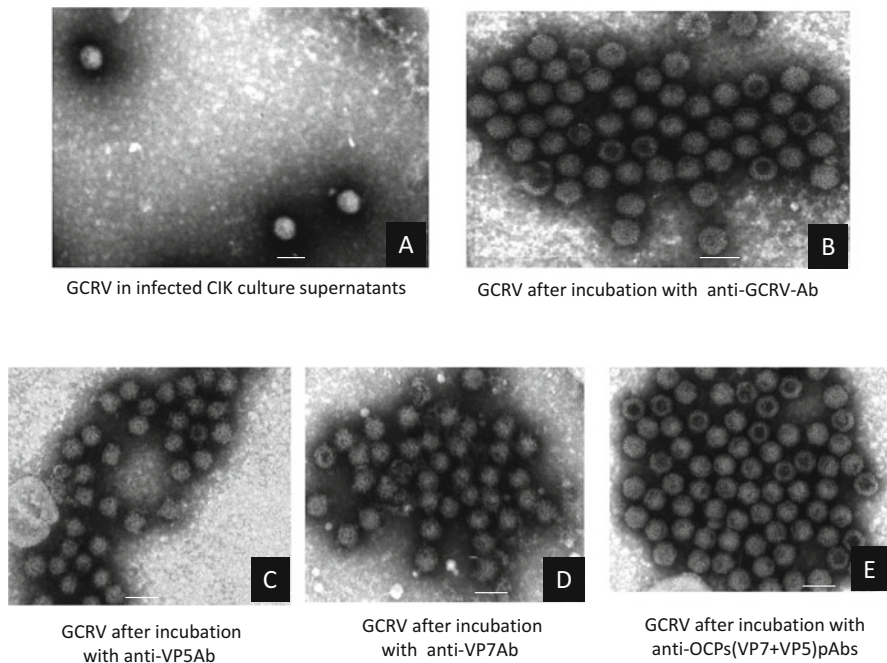
### 2.2.3 *The Virus-Antibody Immunocompound of GCRV*

The immunocompound particle of GCRV, which includes its antibody, has been obtained by directly inoculating virions in infected cell culture supernatants with prepared anti-GCRV polyclonal antibodies [14]. In particular, an excellent aggregation of GCRV–antibody immune complexes has been visualized clearly by direct

**Table 2.1** Characteristics of various aquareovirus particles and components

Particle forms	Buoyant density (g/cm <sup>3</sup> in CsCl)	Proteins	Genome	Size (TEM/cryo-EM)
Intact virions	1.37–1.38	VP1–VP7	+	75~85
Intact Core	1.44	VP1–VP4, V6	+	55~65
SVP <sup>a</sup>	1.38–1.39	VP1–VP6/ and Partial VP7	+	70~75
Empty particle	1.30	VP1–VP7	–	~75
Empty core	1.31	VP1–VP4, V6	–	50~60

<sup>a</sup>Mark indicates that partial VP7 fragment may remain in SVP [40, and unpublished data]



**Fig. 2.2** TEM analysis of the immunocomplex of GCRV. (a) TEM image of mock-treated GCRV-CIK culture supernatants. (b, c, d, e) TEM image of GCRV after incubation with anti-GCRV-Ab, VP5Ab, VP7Ab, and OCPs (VP5 + VP7)Abs. The scale bars represent 100 nm

immune electron microscopy after inoculating virus-infected cell supernatants with either anti-VP5 and/or VP7 polyclonal antibody (VP5Ab and VP7Ab) or intact GCRV antibody without performing purification (Fig. 2.2). These results clearly indicate that individually prepared antibodies of outer shell proteins (either VP5Ab or VP7Ab) effectively bind with GCRV particles in cultured cell suspensions, and the binding effect is mostly similar to that of the antibody generated using intact GCRV particles as an antigen. In the case of the control, only a few dispersed



particles can be found in GCRV-infected cell supernatants. In fact, the GCRV immunocompound derived from anti-VP7Ab is observed more easily by TEM than that derived from anti-VP5Ab (statistical data for each field not shown here). Furthermore, this is consistent with a previous study showing that enhanced GCRV-neutralizing capacity can be obtained by incubation of GCRV-infected CIK cell culture supernatants with the combination of anti-OCPs (VP5 and VP7) Abs [20, 57]. The excellent neutralizing ability of anti-VP5Ab and VP7Ab may be related to the structure and confirmation of the AqRV VP5-VP7 heterodimer complex [7, 15]. It has been reported that the  $\sigma 1$  cell attachment protein of MRV is a major antigen that is able to neutralize reovirus [43]. Despite the lack of  $\sigma 1$  cell attachment protein in GCRV, GCRV VP5 and VP7 proteins represent excellent antigenic epitopes, suggesting that the AqRV VP5 and VP7 proteins have the potential to be used as antigens for vaccines.

### ***2.2.4 Recombinant GCRV Particles***

Reoviruses, such as MRV, rotavirus, and bluetongue virus, can automatically assemble into virions by expression of major structural proteins *in vitro*. As it is known that the AqRV particle is composed of the inner core capsid and the outer shell, an *in vitro* viral assembly model could be established for AqRVs. Using the baculovirus-based Bac-to-Bac expression system for dual expression of the OCPs VP5 and VP7 or mutant VP5<sup>N42A</sup> and VP7, which are then combined with purified cores, an *in vitro* assembly of the recovered native and mutant GCRV intact particles (R-GCRV, R-VP5<sup>N42A</sup>) has been obtained [70]. Recoated GCRV (R-GCRV, VP5<sup>N42A</sup>/VP7 R-GCRV) particles closely resemble native GCRV (N-GCRV) in terms of particle morphology and protein composition.

Furthermore, infectivity assays indicate that the recoated particles display infectious properties similar to those of native virions; R-GCRVs with a VP5<sup>N42A</sup> mutation have been shown to be defective in infectivity and progeny protein expression in infected cells, indicating that a VP5<sup>N42A</sup> substitution almost completely blocks the autocleavage of the VP5 N-terminus. This observation is consistent with a previous study on MRVs, wherein cores recoated with  $\sigma 3$  and  $\mu 1$  bearing the N42A mutation have been shown as defective in infectivity [4, 42, 44]. These data indicate that the autocleavage of GCRV VP5 is required for efficient AqRV infection [70].

### ***2.2.5 Surface Labeling of AqRV Particles***

Methods of fluorescent labeling of virus particles and cellular structures have been developed to monitor virus trafficking in live infected cells. For enveloped viruses, fluorescent labeling has been successfully applied to trace virus entry and

interactions with host cell factors. For nonenveloped viruses, surface coat proteins modified with fluorescent materials can also be realized. Studies have shown that some reoviruses, such as fluorescent-labeled MRV and tetracysteine-tagged blue-tongue virus, can retain their biological properties after modification in vitro [76]. To elucidate the AqRV entry into live cells and its interactions with host cell organelles, GCRV virions have been modified in vitro via modification of the surface proteins with biotinylation, followed by conjugation with streptavidin-quantum dots. The GCRV particles modified by quantum dots retain their native biological functions and infectivity. In addition, the quantum dot-labeled GCRV particles show intact, and hence, quantum dots are suitable for use as fluorescent markers to study the molecular mechanisms of viral entry. Moreover, using quantum dot-labeled GCRV, the caveolae/raft-mediated endocytosis viral entry pathway has been identified for efficient AqRV infection [76].

### ***2.2.6 Infectivity Assays of Purified or Modified Virions and SVPs***

AqRV infectivity is less affected by particle purification and modification. Studies indicate that the intermediate SVPs of SBRV and GCRV are more infectious than the intact virion and core particle [16, 35]. Several experiments indicated that treatment with 200 $\mu$ g/mL of trypsin or chymotrypsin for approximately 5, 15, or 30 min at 37 °C increases infectivity by more than 2–3 logs in viral titer compared with that of the mock-treated viral particles. However, longer treatments of AqRVs with proteases consistently decrease viral titers because the AqRV core is less infectious, similar to that of the MRV or rotavirus [35], suggesting that AqRVs share common infectivity characteristics with other reoviruses. Plaque assays determining the infectivity of purified and quantum dot-labeled AqRVs have shown that these retain excellent infectivity similar to that of native GCRV [76].

## **2.3 AqRV Structural Protein Profile**

### ***2.3.1 Identification of AqRV Particle Proteins***

Following established purification methods, highly purified virions of AqRV have been obtained. The structural protein profiles of AqRVs purified through sucrose gradient show protein components and gel-mobility types similar to those obtained by separating proteins from the purified virion preparation via CsCl gradient centrifugation and analyzed using vertical slab polyacrylamide gels (SDS-PAGE) of 10–15% concentration [15, 16, 52, 54, 67].

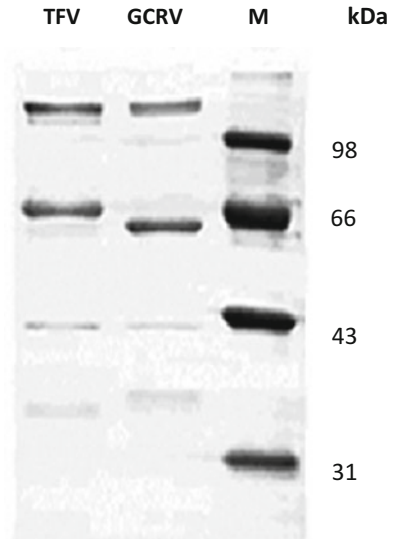
CSRV structural proteins were first characterized in 1983 [38]. Five major structural proteins with molecular weights of 137, 126, 72, 44, and 34 kDa have been identified by SDS-PAGE analysis. The two proteins with molecular weights of approximately 72 and 34 kDa have been shown to be present at higher concentrations compared with the other identified proteins. Moreover, SDS-PAGE analysis of purified CSRV has shown five major (132, 130, 68, 43, and 32 kDa) and two minor (110 and 56 kDa) structural proteins [32]. In addition, few minor polypeptides with molecular weights of 110, 94, 80, 62, and 31 kDa have also been detected [32, 67]. In 1984, similar findings were reported for CCRV by Hedrick et al. [21].

In 1987, the first comparative analysis of structural proteins between four AqRV isolates (GSRV, 13p2, CCRV, and CSRV) was performed [67]. Five major structural proteins were compared using SDS-PAGE. Two large polypeptides (approximately 135 and 125 kDa), one medium-sized polypeptide of approximately 70 kDa, and two small polypeptides of 45 and 34 kDa were separated. Of the major structural proteins identified, two proteins with molecular weights of approximately 70 and 34 kDa were consistently present in the highest amount among the four isolates [67]. Minor virion proteins were detected but not characterized. Indeed, due to the limited copy numbers of minor virion proteins in particles, weak bands can be observed sometimes on the stained gel with the naked eye, but are hardly imaged at most times. In addition, SDS-PAGE analysis of isolated landlocked salmon aquareovirus polypeptides revealed the presence of five structural polypeptides with molecular weights ranging from 139 to 32 kDa. The polypeptide separation pattern is similar, but unique when compared with that of GSRV, CSRV, CCRV, and 13p2 [32].

In 1990, five polypeptides with molecular weights ranging from 130 to 35 kDa were identified from purified SBRV [52]. Further, the viral proteins of SBRV have been compared with those of the Atlantic salmon reovirus HBR, Smelt reovirus, Atlantic salmon reovirus (ASV), and turbot reovirus (TRV) by SDS-PAGE [32]. Similar to the identified structural proteins in AqRVs, all five viruses have been shown to contain five major structural proteins (two large, one medium, and two small), with molecular weights ranging from 137 to 34 kDa. The SBRV protein separation pattern is very different from that of SA11 and reovirus type 1 (T1L) [52].

In 1992, the polypeptides of GCRV with molecular weights ranging from 120 to 27 kDa were reported, which were analyzed by SDS-PAGE of purified particles, with separation of 11 polypeptide segments on the gel [28]. In 2002, a comparative analysis of structural proteins between TFV and GCRV revealed that structural proteins with similar molecular weights were present in both the AqRV species [14, 54]. SDS-PAGE of the virion proteins revealed five major structural proteins, approximately 136, 132, 70, 41, and 33 kDa in size. Similarities in the 136-, 132-, and 41-kDa protein bands are observed. The only major difference in the separation pattern between the two species is that with respect to the approximately 70-kDa band in TFV and the approximately 65-kDa band present in GCRV (Fig. 2.3). However, the smallest major protein band was not observed in GCRV in this study [54], which might be because of protein degradation due to the purified GCRV specimen being shipped to Singapore.

**Fig. 2.3** Structural protein analysis of GCRV and TFV. The two aquareoviruses were purified, and their virion polypeptides are analyzed on a 12% SDS-PAGE gel. The gel was stained with 0.25% Coomassie Brilliant Blue R-250



In addition, patterns of seven structural proteins have been determined for GCRV-991 with five major and two minor structural proteins [14]. The seven identified structural protein components (VP1–VP7) are approximately 138, 137, 136, 79, 67, 43, and 34 kDa in size, respectively. Three of the seven proteins, VP1, VP2, and VP3, have similar large molecular masses. VP6 and VP7 have relatively small molecular masses. Furthermore, VP5 is the most abundant protein, while VP4 is the least abundant. Similar to the structural protein profiles of GSRV, 13p2, CCRV, and CSRV, minor virion proteins can often be detected but hardly imaged based on the purified virion concentration. The GCRV protein components identified by SDS-PAGE analysis match perfectly with the GCRV structural proteins and their localization in the particle as resolved by three-dimensional image reconstruction of a single particle [7, 15, 16]. Moreover, these results are also consistent with the protein properties of AqRVs and MRVs [34, 67] and predictions deduced from amino acid sequences of GCRV [1, 13].

### 2.3.2 Various Particle Forms and Related Protein Profiles

It is known as difficult to obtain highly pure virus particles and exactly distinguish the viral particle components from infected cell lysate components upon careful virus purification, unless the virus is labeled with radioactive material during proliferation in infected cells or using western blot analysis with antibodies specific to each viral protein. In addition, avoiding degradation of intact virions during the whole purification process is also a preexisting condition for obtaining highly purified virus particles and their complete particle components. Indeed, the viral

particles need to be purified very cautiously because the outermost protein VP7 of the AqRV is readily uncoated in native surroundings or purification processes. Although each step in virion purification is performed carefully at 4 °C, it is very common to observe various particle forms by TEM. Intact AqRV protein components and other viral-type particle components (including the top component, SVP, and inner core) have been identified in GCRV, SBRV, or other AqRV species [16, 32, 35, 40, 67]. For the protein components of GCRV cores (both intact and empty), two major bands are visualized by SDS-PAGE [16]. One large band represents VP1–VP3, while the other represents the VP6 protein. Furthermore, the VP6 band appears very weak in the top empty core sample, suggesting that some of the VP6 molecules in the empty core shell may have been lost during the uncoating of VP5 and VP7 by protease treatment or automatically detached during virus purification. For intact virions and double-shell empty particles, the separated one or two large, one or two medium, and two small protein bands with molecular sizes of 139–132, 70, 43–34, respectively, can be visualized in gel by staining with Coomassie brilliant blue. Of note, in most cases, two bands of the VP5 protein (68 kDa, full-length VP5) and its cleavage segment VP5C (64 kDa) can be detected by SDS-PAGE, which is consistent with the observations for  $\mu$ 1 and cleaved  $\mu$ 1C of MRV [42, 44]. The other cleavage segment VP5N is hardly detectable because it weighs 4 kDa. Consistently,  $\mu$ 1 protein is also mostly found in virions as fragments,  $\mu$ 1N/VP5N (4 kDa) and  $\mu$ 1C/VP5C (72 kDa), which are generated by autocleavage at the site N42-P43, as confirmed by biochemical analysis and three-dimensional imaging at atomic resolution [44, 66]. This suggests that the VP5 protein in the GCRV particle can exist either in two conformations or mainly in the VP5C conformation, as observed in most cases [16, 70]. In addition, since the molecular weights of the identified structural proteins of GCRV are between 34 kDa (VP7) and 136–138 kDa (VP1–VP3), it is not possible to resolve the higher-molecular-weight proteins VP1–VP3 and lower-molecular-weight protein VP7 using the same electrophoretic conditions. Because of the instability of AqRV particles and particle related nature of autocleavage or protease induced cleavage as well as the remaining minor protein components or purification issues with experimental condition limitations, it is not surprising that more than seven structural proteins have been identified in earlier studies [28, 65].

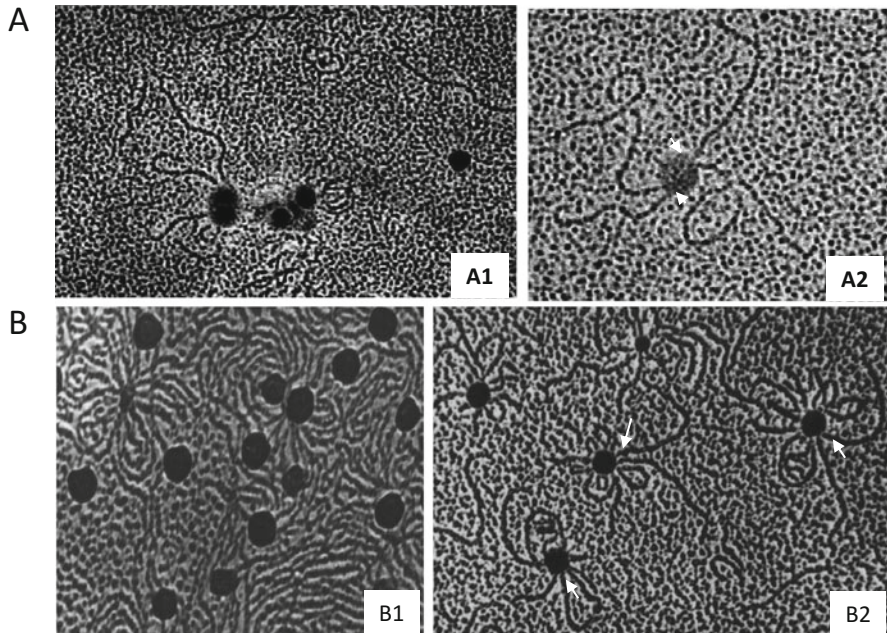
## 2.4 In Vitro Endogenous Transcription Assays of GCRV Particles

Similar to other reoviruses, the dsRNA genome segments of AqRVs are enclosed by the inner capsid shell. Enzymes that catalyze RNA transcription, capping, and replication are contained within the inner capsid, which serves as the site for the self-RNA synthetic activities. The RNA-dependent RNA polymerase

(RdRP)-related endogenous transcription activities of GCRV have been confirmed in earlier studies.

### 2.4.1 GCRV Nucleic Acid Release

To directly observe the genome of GCRV, the viral RNA component is released using the nucleic acid shadowing method with viral cores being prepared by digesting purified virus with  $\alpha$ -chymotrypsin as described previously [28]. The released RNA strand of GCRV is attached to the core turrets (Fig. 2.4a), indicating that the hollow-like spike at the fivefold axes is the channel for RNA release from the inner core into the cytoplasm, which is the same as that in MRVs [3].



**Fig. 2.4** Electron micrographs of stained and shadowed preparations of GCRV core and reaction cores. (a, b) GCRV nucleic acid release image of non-transcribing (a) and transcribing GCRV reaction core (b). Few GCRV genome RNA strands released and attached to cores from non-transcribing GCRV core in A2 as arrow indicated, and the non-transcribing core looks loosen after nucleic acid released. Many nascent nucleic acid released from transcribing cores in B. Arrowheads in B2 indicate where strands appear to emerge from spikes, and the transcribing core looks solid after some nascent nucleic acid released. The GCRV nucleic acid release from non-transcribing and transcribing GCRV reaction core preparations is followed as references [22, 28]

### 2.4.2 *GCRV Reaction Core*

RNA transcript products in GCRV reaction cores synthesized *in vitro* have been observed using a combined staining and shadowing method [22]. Up to 12 strands of nascent mRNA have been exhibited in reaction cores by TEM. The reaction cores that are intact and contain attached single-stranded RNA strands have a buoyant density of 1.48 g/cm<sup>3</sup> and an A260 to A280 ratio of 1.52. The transcribed genome products of the GCRV reaction cores with [ $\alpha$ -<sup>32</sup>P]-ATP labeling have been detected by analyzing in SDS-PAGE, which are consistent with the full-length copies of their dsRNA templates. An obvious feature of the reaction cores is that the sites of extrusion are probably the 12 hollow projections located on the surface of the core, and many strands are extruded in the form of loops (Fig. 2.4b). This evidence clearly indicates the endogenous transcription activity of GCRV [22].

### 2.4.3 *In Vitro Transcription Assays*

The endogenous RdRP activity of various GCRV particles (intact virion, top component, and core) has been assayed by *in vitro* transcription using [ $\alpha$ -<sup>32</sup>P]-ATP as a marker in earlier studies [23]. Transcriptase activation can be affected by different salt concentrations, temperatures, and reaction times. It has been shown that the cores can be activated for transcription, but the intact GCRV virions are incompetent for producing mRNA. Moreover, the intact GCRV virion can only possess transcriptase activity when the virus sample is resolved in a low-salt buffer, thereby showing effective transcriptase activity. Transcriptase activity assays performed for intermediate SVPs showed SVPs with activity higher than that of the purified viral core particles, suggesting that transcriptase can be activated by uncoating intact GCRV outer capsid shell because the outer capsid shell of intact GCRV can be loosened in a low-salt reaction buffer. Indeed, purified cores possess RNA transcriptase activity, but under the presence of the protective outermost shell, the enzyme activity can be affected. In addition, RdRP is more effective at 28 °C than at a temperature lower or higher than 28 °C, indicating that the optimum temperature for AqRV transcription is 28 °C, as determined by *in vitro* transcription assays. This is consistent with the optimal replication temperature for GCRV, as demonstrated by *in vivo* infection and *in vitro* expression studies of RNA polymerase (VP2) activities [12, 68]. The GCRV RdRP-related transcriptase complex has been verified by recent three-dimensional structural imaging analyses [66].

## 2.5 The AqRV Genome

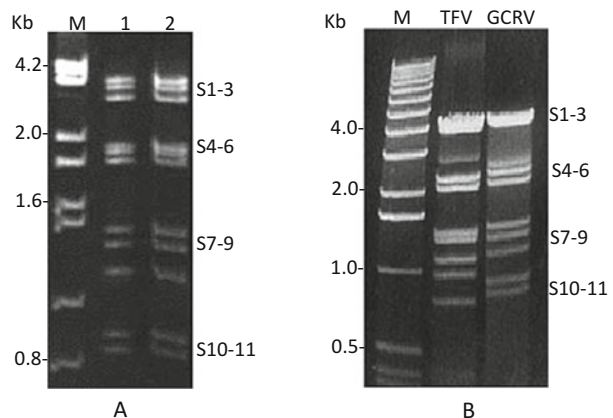
The AqRV genome, which is encased by a double-layered protein capsid shell (designated as the inner core and outer capsid), has been shown to be a dsRNA genome due to its resistance to treatment with RNase I and green orthochromatic appearance with acridine orange staining [27, 32, 54, 67]. The genome of AqRVs is composed of 11 dsRNA segments that are packaged in equimolar ratios within a concentrated core. Seven distinct species (*Aquareovirus A* to *Aquareovirus G*) have been proposed by the International Committee on Taxonomy of Viruses based on reciprocal RNA–RNA hybridization and nucleotide sequence analyses.

### 2.5.1 Nomenclature and Electrophoresis Profile of the AqRV dsRNA Genome

The electrophoretic patterns of almost all isolated AqRVs are very similar to each other with three large (segments 1–3), three medium (segments 4–6), and five small segments (segments 7–11). Therefore, the dsRNA genome of AqRVs is generally grouped into three size classes, commonly referred to as the large (S1–S3, about 3.9–3.8 kb), medium (S4–S6, approximately 2.3–2.0 kb), and small (S7–S11, about 1.6–0.9 or 1.6–0.7 kb) classes.

Viral dsRNA of AqRVs has been analyzed by SDS-PAGE using vertical slab gels (10% polyacrylamide gel) immersed in Laemmli's buffer or 1% agarose gel electrophoresis [2]. Almost all AqRV species show unique electropherotypes in SDS-PAGE. Despite the differences in gel mobility, some similar electrophoretic patterns have been observed among AqRV isolates, e.g., dsRNA genome electrophoretic patterns of GCRV and TFV (Fig. 2.5). However, the RNA profiles of some AqRVs (e.g., SBRV, Atlantic salmon reovirus HBR, Smelt reovirus, and Atlantic

**Fig. 2.5** The segmented genome profiles of GCRVs and TFV. **(a)** The genomes of GCRV-873 and GCRV-991 were resolved by 7% polyacrylamide gel; M:  $\lambda$ -DNA/EcoRI+HindIII. **(b)** The genomes of GCRV-873 and TFV were resolved by 1% agarose gel electrophoresis; M: 1 kb DNA ladder. Viral dsRNA genome segments in **a** and **b** were visualized following ethidium bromide staining





salmon reovirus (ASV)) in agarose gels have been observed to be very similar [32]. The differences in the mobility of dsRNA segments in SDS-PAGE or agarose gel electrophoresis depend on the gel properties. In addition, the mobility of dsRNA segments in SDS-PAGE gels depends on both their size and secondary structure, while in agarose gels it depends mostly on their size. In fact, the dsRNA genome of AqRVs is more expediently detected on agarose gels than on SDS-PAGE gels when adequate amounts of dsRNA from virus samples are extracted. In contrast, SDS-PAGE is recommended in cases of lower amounts of AqRV dsRNA genomic material, by staining with silver. Of note, despite the fact that most reoviruses isolated from aquatic animals have 11 dsRNA genomic segments, the genomes of some AqRVs contain 10 and/or 12 segments, as noted for piscine orthoreovirus (PRV) and reoviruses having crab hosts [29, 30], respectively, suggesting a complex and diverse molecular evolutionary course of the AqRV genome.

### 2.5.2 Genome Sequence-Based Features of AqRVs

The complete genomic sequences of the prototype AqRV strains GSRV and GCRV-873 classified in the species group *Aquareovirus-C* (AqRV-C) and other AqRV species isolates, such as CSRV and AHRV, have been characterized using the single-primer amplification method-cloned cDNA libraries or an optimized strategy for full-length amplification of cDNA [1, 11, 39, 45, 64, 71]. According to the GenBank data, at least 17 full-length AqRV genome sequences and many partial genome sequences have been deposited thus far. The total length of the AqRV genome sequence is in the range of approximately 23,500–24,500 bp. The lengths of individual gene segments vary from 728 bp (the shortest; S11 gene segment) to 3949 bp (the longest; S1 gene segment), according to the National Center for Biotechnology Information (NCBI). Generally, the GC content of the AqRV genome ranges from 52% to 60%; however, the recently reported AHRV genome consists of 11 segments (with a total size of 24,171 bp) and has a relatively low GC content (ranging from 47.8% to 52.7%) [60]. Most homologous gene segments of different isolates display little or no variation in segment length, such as those of GSRV or GCRV, CSRV, Turbot *Scophthalmus maximus* reovirus (SMReV), *Micropterus salmoides* reovirus (MSReV), and SBRV. However, gene segments in the small class vary greatly in length and are more divergent at nucleic acid and protein levels, compared with the large and medium class segments. Generally, each segment encodes one protein, but the S7 segment in the different species groups appears to be bicistronic or tricistronic [2, 30, 73]. Notably, AqRVs are recognized to be fusogenic with syncytia formation as a typical CPE in their permissive cell cultures, which is consistent with the predicted protein functional features of the S7 segment homologous to that of fusogenic avian reovirus (ARV) [8, 24]. However, few AqRV species, such as GCRV-ZH08, GCRV-GD108, and GCRV-104/109, and PRV, show non-fusogenic characteristics [11, 29, 45, 64, 71], which is consistent

with that observed for MRVs [43]. The fusogenic and non-fusogenic properties of AqRVs are related to the nature of the S7 segment-encoded proteins.

### 2.5.3 Terminal Non-coding Regions

The partial and complete genomic sequences of a number of AqRV strains have been determined [1, 2, 11, 39, 45, 47, 48, 55, 56, 64, 71]. In AqRVs, genomic dsRNA segments contain six and five conserved nucleotides at the 5' terminus and 3' terminal basic group, respectively (Table 2.2). Particularly, the conserved nucleotide terminal region, with five nucleotides “-UCAUC,” at the 3' end in AqRVs is same as that in MRV [43], and the extreme 5' end with six-nucleotides is conserved within a particular virus species. For example, the conserved 5'- and 3'-terminal sequences of isolates in AqRV-C are 5'-GUUAUU.....UCAUC-3', compared to 5'-GUUUUA.....UCAUC-3' in AqRV-A,-B, and -G, and 5'-GUAAUU.....UCAUC-3' or 5'-GUAACU.....UCAUC-3' in unclassified GCRV-ZG08/108 and GCRV-109 species [1, 25, 45, 55, 56, 64, 71]. Similar to other reoviruses, each AqRV gene segment also contains terminal non-coding regions (NCRs) of a particular length. The lengths of the NCRs of genome segments vary across different AqRV species. Generally, the NCRs at the 5' ends are shorter than that at the 3' ends in AqRVs. The length ranges from 12 to 35 bp at the 5' ends, while the 3' ends contain NCRs of length ranging from 20 to more than 50 or 100 bp depending on the isolated virus

**Table 2.2** Conserved terminal sequences (positive strand) of aquareovirus genome segments

AqRV species	Terminal sequence 5'NCR.....3'NCR
<i>AqRV-A</i> (AHRV, CSRV, EFRV, SBRV, TFV, TSRV, AtSRV, SMReV, MsReV)	5'-GUUUUA... ...UCAUC-3'
<i>AqRV-B</i> (FCRV, CoSRV)	5'-GUUUUA... ...UCAUC-3'
<i>AqRV-C</i> (GSRV, GCRV873)	5'-GUUAUU... ...UCAUC-3'
<i>AqRV-D,E,F</i>	–
<i>AqRV-G</i> (AGCRV)	5'-GUUUUA... ...UCAUC-3'
<i>Unassigned strains</i>	
GCRV-ZH08	5'-GUAAUU... ...UCAUC-3'
GCRV-GD108	5'-GUAAUU... ...UCAUC-3'
GCRV-104	5'-GAAUU... ...UCAUC-3'
GCRV-109	5'-GUAACU... ...UCAUC-3'

strains [25, 45]. Moreover, the first and last nucleotides in the NCRs of all segments are complementary (G-C) and are known to be highly conserved within all AqRV species. Similar to other reoviruses, the NCRs of AqRVs are likely to include sequences important for RNA packaging, recognition by their RdRP for initiating positive- and negative-sense RNA strand synthesis, and translational efficiency [43].

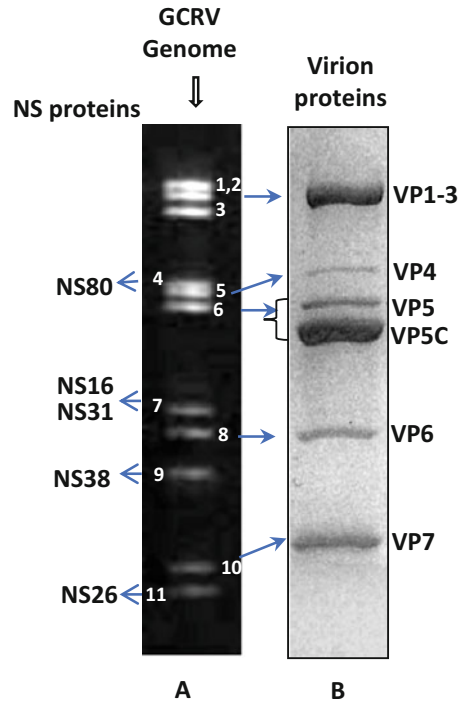
### ***2.5.4 Protein-Coding Assignments and Nomenclature***

The coding assignments and properties of the AqRV proteins encoded by each of the 11 genomic segments are now fairly well characterized. The final assignments were determined by *in vitro* translation using mRNA or genome sequencing using denatured dsRNA combined with three-dimensional structural reconstruction-based methods. It has been confirmed that the 11 genomic segments of AqRVs encode seven structural and five nonstructural (NS) proteins. According to the nomenclature of the viral structural proteins (that are contained in the virion), structural proteins are designated as VP followed by a number, with VP1 being the largest and VP7 being the smallest structural protein. Moreover, the genome segments encode proteins that are not contained in the viral particle, termed as NS proteins. For the NS proteins, two sets of nomenclature are currently used based on either the molecular weight or a number, for example, GSRV NS80 or NS1. The initial assignment of individual proteins to specific gene segments of AqRVs has been performed by *in vitro* translation of viral mRNAs in early studies on SBRV and GCRV [28, 52, 62, 65]. GCRV polypeptides encoded by each of the gene segments are designated by the molecular weights corresponding to the respective genome segments S1–S11 (Fig. 2.6).

#### **2.5.4.1 SBRV Gene-Protein-Coding Assignment**

The gene-protein-coding assignments of SBRV have been well characterized by *in vitro* translation of individual genome segments [52, 62]. [<sup>35</sup>S]methionine-labeled SBRV-infected cell lysates were used to analyze the polypeptides and gene-protein-coding assignments of SBRV. Twelve proteins with apparent molecular weights of 130, 127, 126, 97, 73, 71, 46, 39, 35, 29, 28, and 15 kDa were detected in the infected cell lysate, but not in the uninfected cells [32, 52], suggesting that these proteins are encoded by the SBRV genome. Analyzing radiolabeled cell lysates and radiolabeled purified virus proteins using SDS-PAGE and autoradiography for self-exposed X-ray film, seven structural (VP1–VP7) and five NS proteins (NS97, NS39, NS29, NS28, and NS15) have been further classified. There are two major structural proteins, whereas the other five structural proteins are present in smaller amounts. In addition, segments 1–10 of the SBRV genome encode one protein each, while segment 11 encodes two proteins.

**Fig. 2.6** Gene-protein-coding assignment of GCRV. A SDS-PAGE gel shows the 11 segments of dsRNA that comprise GCRV genome and the proteins encoded by each of these genes (a). The genome segments encode nonstructural (NS) and structural (virion) proteins marked at left and right, respectively. The virion proteins are analyzed on a 12% SDS-PAGE gel (b). The viral genome and structural proteins of GCRV are obtained from purified particle



To confirm the above data, further translational analysis of the individual genome segments using a nuclease-treated rabbit reticulocyte lysate *in vitro* was conducted by Subramanian et al. in 1994 [62]. The synthesis of viral proteins by *in vitro* translation was quantitatively and qualitatively very similar to that observed in SBRV-infected CHSE-214 cells. Of the 12 proteins, the genome segment 11 codes for two proteins, NS29 and NS15. According to the understanding of the proteins encoded by the 11 genome segments of SBRV and their three-dimensional image reconstruction, the proteins VP5 (S5; 71 kDa) and VP7 (S10; 34 kDa) comprise the outer shell [40]. VP1–VP3 and VP6 are the core proteins. Additionally, VP1 is the most likely candidate protein responsible for the turret structure, while the role of VP4 is unknown [40].

#### 2.5.4.2 GCRV Gene-Protein-Coding Assignment

The gene-protein-coding assignment of GCRV was reported by Ke et al. in 1992 [28]. The 11 polypeptides isolated from purified virus particles with molecular weights ranging from 130 kDa to 27 kDa were observed by SDS-PAGE analysis. Furthermore, the denatured viral genome segments were translated using a cell-free rabbit reticulocyte translation system labeled with L-[<sup>35</sup>S]methionine, which showed 11 protein bands in the exposed X-ray film with autoradiography. Further

gene-protein-coding assignment of GCHV was conducted with the wheat germ translation system labeled with L-[<sup>35</sup>S]methionine using each separated GCRV genome segment [65]. The translation products displayed on the X-ray film showed 12 protein bands with probable molecular weights in the range of 130–19.5 kDa. However, the study failed to distinguish the structural and NS proteins of GCRV. This might be due to poor experimental conditions and some technology-related problems in the 1990s. Indeed, the gene-protein-coding assignments of GCRV were obscure until 2002 [1]. Complete sequence determination and identification of GCRV structural proteins from purified natural virus particles indicated that the GCRV 11 dsRNA genome segments encode 12 proteins. Segments 1, 2, 3, 4, 6, 8, and 10 encode seven structural proteins that form the virus particle [1, 7, 15, 76], and the remaining segments encode five NS proteins that are involved in viral replication and transcription [18, 19, 77, 78].

Further full-length sequence analysis indicated that each dsRNA genome segment of GCRV generally contains a single gene encoding a single protein, except the S7 segment. Twelve proteins have been translated from the 11 gene segments of GCRV, such that 10 of these gene segments encode a single protein, and only the S7 gene encodes two proteins. Genome sequence-based homologous protein analysis indicated that 7 of the 12 proteins are structural components of the virion (VP1–VP7) and five are NS (NS80, NS38, NS31, NS26, and NS16) proteins involved in viral replication [1]. The GCRV particle structural protein compositions were further confirmed by SDS-PAGE, cryo-electron microscopy, and three-dimensional image reconstruction analyses [7, 15, 66]. The 11 segments of dsRNA that comprise GCRV genome and the proteins encoded by each of these genes are shown in Fig. 2.6. Recently, a putative NS12 protein has been identified to be encoded by the S7 genomic segment [73].

### 2.5.5 *In Vitro and In Vivo Protein Expression Analyses*

Based on the obtained genome sequence of AqRVs, almost all proteins (including 7 structural and 5 NS proteins) encoded by GCRV genome have been expressed *in vitro* using different expression vectors (including prokaryotic and yeast expression plasmids, recombinant baculovirus Bac-to-Bac insect expression system, and mammalian expression plasmids) [63, 69, 72]. All the expressed proteins *in vitro* have been shown to correspond to the predicted protein products from the corresponding GCRV genome, which validates the deduced protein sequences from the corresponding dsRNA genome segment open reading frame (ORF). In addition, the structural and NS protein expressions in virus-infected cells were detected and confirmed with prepared multi-clonal antibodies generated from individual genome segments [69, 76–78]. It is important to note that some additional or truncated isoforms of the GCRV proteins, such as VP5/VP5C, NS80/NS80C, which are homologous to MRV protein  $\mu$ 1 and  $\mu$ NSC, respectively, have been detected in both transfected and infected cells [5, 26, 69, 70]. Moreover, NS80 and NS38

expressions are detected prior to that of the structural proteins, indicating that the NS proteins are important for viral replication [69, 78]. As a fusogenic reovirus, the fusion-associated small transmembrane (FAST) protein NS16/NS12, which is similar in function to the ARV p10, p14, and p15 [49], has also been detected in infected and transfected cells. These findings indicate that the AqRV genome may perform translation in infected cells beyond the ATG in-frame rules [25, 49]. The structural proteins and their functions are described in Chap. 3, while the AqRV replication-related NS proteins are described further in this chapter.

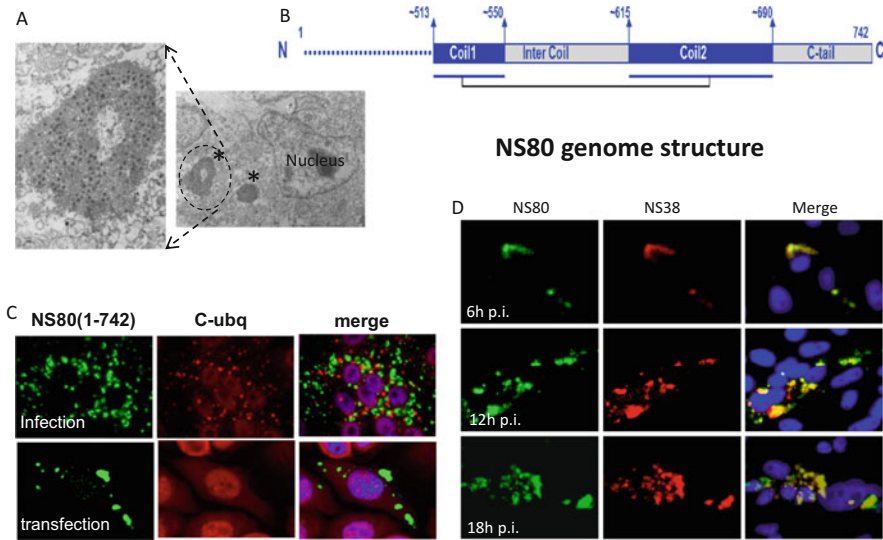
## 2.6 AqRV Morphogenesis

The most characteristic feature of reovirus replication in infected cells is the formation of cytoplasmic factories, named viral inclusion bodies (VIBs), viral factories, or viroplasm, which consist of progeny viruses, usually appearing as paracrystalline arrays of full and empty virions. VIBs are specific intracellular compartments for reovirus replication and assembly [43]. AqRV morphogenesis in host cells has been studied extensively using the ultrastructure and molecular biology methods, which are related to evaluating the mechanism of VIB formation in infected cells [5, 45, 58, 69, 77, 78]. It has been found that the NS proteins NS80 and NS38 of the AqRV as well as the viral core structural proteins play critical roles in AqRV morphogenesis.

### 2.6.1 TEM Analysis of VIB Formation in Infected Cells

The thin-section ultrastructural TEM images revealed that the AqRV replication and assembly occur within VIBs in infected fish or cultivated cells [32, 51]. Paracrystalline arrays have been observed in cell lines infected by various AqRVs, such as GSRV, 13p2, TRV, GCRV, TFV, TSRV, and AHRV [10, 32, 54, 74]. It has been found that the AqRV particles bind to the plasma membrane of permissive cells at 0 or 10 min post-infection (p.i.) and are subsequently internalized. The SVPs are found in the cytoplasm at 40 and 60 min p.i [51, 76]. It has been shown that the core particles of AqRV are observed within the dense viroplasm in the early stages of infection, and the size and number of viroplasmic inclusions increase as the infection progresses; mature virions are formed with an overall size of about 70–80 nm in the late stage of replication [10, 51]. In addition, a number of viroplasms can be detected at one time in the cytoplasm of a cell, suggesting that there is no limitation on the number of virions penetrating and replicating in a single cell [10, 51].

Once an AqRV species enters the cell, electron-dense particles condense in the periphery of the viroplasms, and the cellular ultrastructural changes can be observed in ultra-thin section images during early infection [10, 32]. The AqRV infection forms neorganelles in infected cells. The dense globular inclusion bodies/



**Fig. 2.7** NS80 and NS38 are involved in viral inclusion formation. (a, right) VIBs in cytoplasm of infected cells (left is the zoomed-in view of the VIB shown in right). (b) Schematic representation of aquareovirus NS80 C-terminal regions with four potential inclusion formation related domains. (c) NS80 induced VIBs were detected in infected and transfected cells. (d) The subcellular localizations of NS80 and NS38 were detected in GCRV-infected CIK cells by IF. The subcellular localizations of NS80 and NS38 immunostained with rabbit anti-NS80 or/and mouse anti-NS38 polyclonal antibodies followed by FITC-conjugated goat anti-rabbit IgG (green) and Texas Red-conjugated goat anti-mouse IgG (red), respectively. Nuclei were counterstained with DAPI (blue). Images in c and d are modified from reference [58]

viroplasm appear to be the site for initiating assembly of the viral progeny in the AqRV replication cycle (Fig. 2.7a). The presence of viral polypeptides inside the viroplasm at the medium and late replication stages has been observed by immune electron microscopy and immunofluorescence assays [51, 69, 77, 78]. During replication and assembly of AqRVs, these VIB-like structures have a peculiarly dense consistency that distinguishes them from the adjacent cytoplasm and causes them to appear highly refractile when viewed by phase-contrast microscopy, and they contain fully or partially assembled viral particles, viral proteins, dsRNA, and microtubules [58, 77]. VIBs are formed throughout the cytoplasm of infected cells as the infection progresses. Studies clearly indicate that the AqRV replication is typical to the cytoplasm of infected cells, which is consistent with the reovirus replication features in general.

### 2.6.2 NS80: A Scaffold of VIBs

The AqRV NS protein NS80, also termed NS1, has been identified as the major constituent for forming globular VIBs [58]. GCRV NS80 consists of 742 amino

acids and is encoded by a gene on S4 (2320 nt), with a molecular weight of approximately 80 kDa. Phylogenetic analyses indicated that the NS protein NS80 of GCRV or the homologous protein in other AqRV isolates is related to the formation of VIBs. BLAST analysis comparing the GCRV NS80 analogue with that of CSRV in the genus *Aquareovirus* and MRV and ARV in the genus *Orthoreovirus* showed identities mostly within the C-terminal region of the protein. In particular, the NS80 homologous proteins across different AqRV species share two typical coiled-coil regions (513–548 and 615–700 amino acids) in their carboxyl-proximal region (Fig. 2.7b), which have been shown to be important in VIB formation in MRVs [10, 26, 58]. Immunofluorescence assay showed that NS80 can form VIBs when expressed alone in transfected cells or during viral infection (Fig. 2.7c). It has been also identified that the C-terminus of NS80 is responsible for VIB formation, and the N-terminal NS80 interacts with viral proteins [58, 77]. In addition, a shorter specific fragment of NS80, an approximately 58-kDa product of NS80, has been detected in infected and transfected cells [5]. As NS80 is a multi-functional protein, the different isoforms of NS80 proteins present in infected cells may be functional at various replication stages, suggesting that the isoforms may play different functions during viral replication.

### 2.6.3 Interaction of NS80 with Viral Proteins

It has been observed that NS80 retains five ISPs (VP1–VP4 and VP6) and NS38 within VIBs in co-transfected or infected cells (Fig. 2.7d). NS80 interacts with each core protein and newly synthesized viral RNAs colocalized with VIBs [26, 69, 77]. Furthermore, time-course analysis of the viral structural protein expression showed that the expression of NS80 is first detected, followed by that of the ISP VP3 and other ISPs, suggesting that VIBs are essential for the formation of progeny virions. Further experiments indicated that knockdown of NS80 by shRNA not only inhibits the expression of the AqRV structural proteins, but also reduces viral infection. These results indicate that NS80-based VIBs are formed at an earlier stage of viral infection, and the protein NS80 is able to coordinate the expression of viral structural proteins and viral replication [69, 77].

### 2.6.4 Role of NS38 in GCRV Morphogenesis

AqRV NS38, encoded by GCRV S9 (1130 nt), contains 352 amino acids with a molecular mass of approximately 38 kDa. BLAST analyses suggested that there is approximately 23% similarity between the AqRV NS38 and MRV  $\sigma$ NS at the protein level. It has been found that protein  $\sigma$ NS of MRVs, together with another NS protein  $\mu$ NS, and protein  $\sigma$ 3 can associate with mRNA molecules to form single-stranded RNA-containing complexes [43, 78]. Some studies have implicated  $\sigma$ NS as



a minimally essential viral component for forming VIBs together with  $\mu$ NS, which then recruits other reovirus proteins and RNA to initiate viral genome replication. Studies have indicated that the AqRV NS38, a virus genome-encoded putative single-stranded RNA-binding protein, interacts with not only NS80 in VIBs, but also ISPs (VP1–VP4 and VP6). Interactions between NS38 and NS80-RNA complexes in both transfected and infected cells have also been detected. Knockdown of NS38 by siRNAs-115/219 clearly reduces viral infection, with decreased mRNA and protein yields. Moreover, NS38 can interact with the host cellular eukaryotic translation initiation factor 3 subunit A (eIF3A) in transfected cells. Furthermore, it has been identified that NS38 directly interacts with outer shell protein VP5 and VP7 (unpublished data). These findings indicate that NS38 may function as a mediator by interacting with the viral and host cellular components in VIBs during viral replication and particle assembly [78].

## **2.7 GCRV S7 Genome Segment-Encoded FAST Protein and Associated Syncytium Formation**

Similar to fusogenic orthoreoviruses that can promote the formation of multinucleated syncytia in infected cells [8, 49], AqRVs are able to induce typical CPEs and finally lead to multinucleated syncytium formation in permissive cell lines during cultivation. AqRVs and fusogenic orthoreoviruses are the only known examples of nonenveloped viruses that lead to cell–cell fusion and syncytium formation in virus-infected cells [24, 49]. Studies have indicated that the FAST protein encoded by the AqRV S7 genome segment is responsible for cell–cell fusion during the viral replication cycle.

### **2.7.1 Detection of FAST Protein**

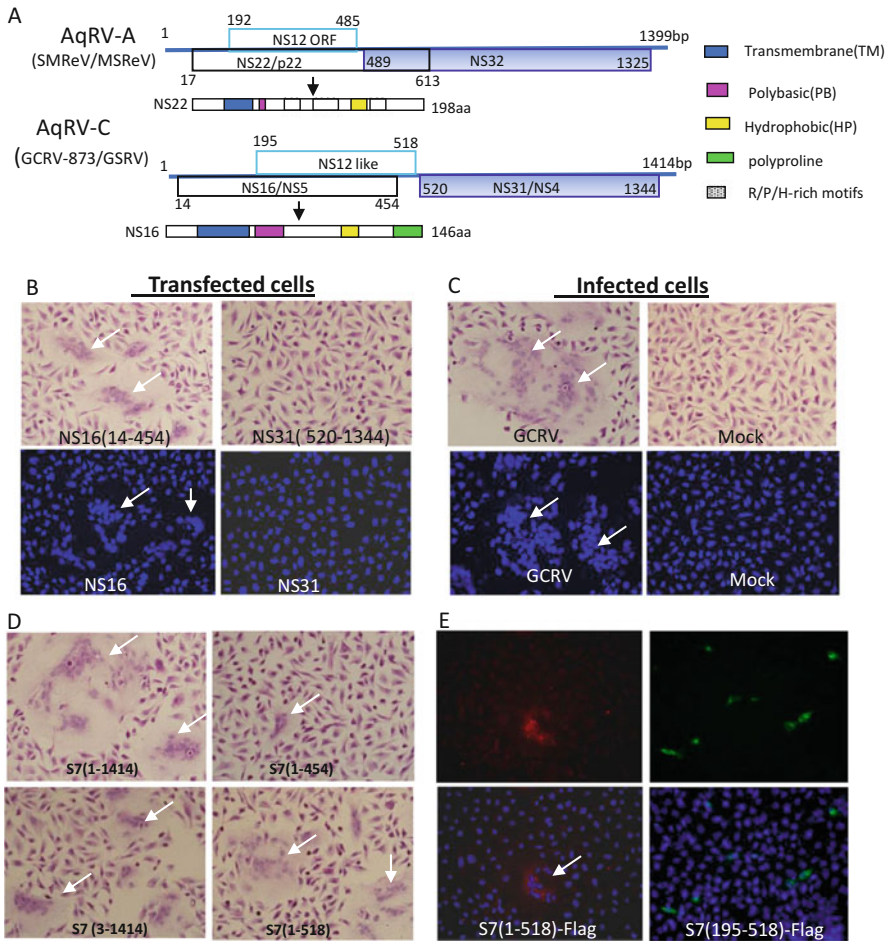
Early investigations have revealed that ARVs can induce multinucleated syncytia formation in infected cell cultures, which distinguishes them from non-fusogenic MRVs [49]. It has also been found that the fusogenic nature of ARVs is such that the formation of cellular syncytia is not related to viral cellular entry or exit, as is the case for enveloped viruses, such as the human immunodeficiency virus (HIV) gp41 (HIV-1), which mediates fusion between target cells through a complex interaction of viral glycoproteins with cell receptors [24]. FAST proteins are small (95–198 amino acids) and expressed as NS proteins during the viral replication cycle [49]. Studies on the reovirus fusion mechanism suggest that the reovirus FAST proteins may function as virus genome-encoded fusogens and utilize many accessory host factors to drive cell–cell fusion, and therefore, may benefit the rapid spread of infection after being expressed in infected cells [8, 49]. A recent study on ARVs

demonstrated that cell–cell fusion induced by the reovirus FAST proteins enhances replication and pathogenicity of nonenveloped dsRNA viruses [24].

### 2.7.2 Sequence-Based Features of FAST Protein

Fusogenic reovirus-induced cell–cell fusion and syncytium formation are dependent on a distinct family of FAST proteins encoded by polycistronic genomic segments. Some FAST proteins of the ARV, such as p10, p14, and p15, have been well characterized in the genus *Orthoreovirus* [8, 24]. A few FAST proteins, such as the p22 protein from AtSRV, SMReV, and MSReV (AqRV-A) and NS16 from GCRV and GSRV (AqRV-C), have been predicted in the genus *Aquareovirus* based on bioinformatics analyses [18, 25, 49]. Subsequently, the proteins NS16, NS22, or p22 encoded by the polycistronic genome segment S7 of AtSRV, MSReV, SMReV, and GCRV have been identified as FAST proteins in the genus *Aquareovirus* [6, 18, 25]. Generally, the basic structure of the FAST protein (Fig. 2.8a), including the transmembrane (TM) domain, polybasic (PB) region, hydrophobic region, and polyproline and proline-rich motif, has been determined in the AtSRV p22, GCRV NS16, and MSReV/SMReV NS22 [6, 18, 25, 49]. In particular, sequence analysis has also suggested an additional dileucine motif in the cytoplasmic region at amino acid positions 113–114, which may regulate protein sorting and other cellular processes.

Bioinformatics analysis has shown that NS16 and NS22 of the AqRV share basic structural motifs with the ARV FAST proteins p10, p14, and p15. NS16 and NS22 in AqRV-C and AqRV-A species, respectively, have been predicted to be single-pass membrane proteins comprising 146 and 198 amino acids, respectively, which display essential features similar to those of the identified FAST protein motifs and are assumed to possess an N-terminus-outside/C-terminus-inside asymmetric topology on the plasma membrane. In the N-terminal region, there is a strong TM domain (covering 37–60 amino acids for NS16 and 35–57 amino acids for NS22). Following the TM domain, there is a PB region that contains a stretch of basic residues (covering 63–78 amino acids for NS16 and amino acid positions 61–68 and 82–95 for NS22). The PB region is thought to support the translocation of the N-terminal domain (34 amino acids for NS22 and 36 amino acids for NS16) into the extracellular environment [18], and it has been implicated as essential for FAST protein activity. In addition to the TM domain, a hydrophobic region called the hydrophobic patch has been predicted to exist in the C-terminal fragment of both proteins (amino acid positions 113–121 for NS16 and 140–150 for NS22). Moreover, two regions rich in arginine, proline, and histidine have been found in NS22 [25]. In contrast, there are three regions rich in arginine, proline, and histidine in the S7 encoded FAST protein of the AtSRV [49], despite its high sequence similarity with the SMReV NS22. Furthermore, some differences have been found in the motifs in NS22 and NS16. A myristoylation consensus sequence (MGXXXS) has been identified at the N-terminus of NS22 [25], but no such myristoylation site exists



**Fig. 2.8** Identification of GCRV genome S7 encoded FAST protein. (a) Diagram of AqRVs genome segment S7 encoded proteins NS16/NS22, NS12, and NS31/NS32. Virus strains and accession numbers for segment 7 of the genomes are as follows: AqRV-A: MsReV (KJ740731.1), SMReV (HM989936.1); AqRV-C: GCRV-873 (AF403393.1), GSRV (AF403404.1). (b) Expression of GCRV S7 encoded NS16 and NS31 protein. NS16 induced cell-cell fusion and multinucleated syncytium formation in transfected CIK cells (left), no syncytium formation is observed with NS31 expression (right). Cells were fixed at 48 hpt and stained with HE or Hoechst. (c) Cytopathic effects are observed in GCRV-infected CIK cells at 12 hpi. Mock-infected cells served as a negative control. (d) Syncytia formation in transfected CIK cells with recombinant plasmids (pCI-Neo) containing different truncation regions of GCRV S7 sequence. (e) Immunofluorescence assays of truncated S7 fragment expressions in transfected cells. The expressed proteins were detected by IF assay at 24 hpt with an anti-FLAG MAb followed by Alexa 488 (green) or 568 (red) labeled secondary antibody, respectively. Arrows indicate the syncytia and nucleus aggregation

in NS16 of GCRV or GSRV. It has been reported that N-terminal myristoylation is necessary for the fusion activity of the reptilian reovirus protein p14 [8, 41]. Notably, the viruses utilize a noncanonical CUG codon for initiating translation to produce the fusion protein p22 responsible for syncytiogenesis [25, 49, 73].

Interestingly, GCRV-104 initially appeared to lack an NS FAST protein. However, further careful analysis indicated that the potential N-terminal region of NS15 (ORF2) in the S11 segment may contain a TM domain with its size consistent with that of the FAST protein ectodomains; additionally, a cluster of basic residues contained at the C-terminus that is enriched in arginine and proline residues, and several cysteine residues that may be palmitoylated [41]. Similarly, a bioinformatics assay predicted that the GCRV-HZ08 NS41 and/or GCRV-GD108 NS11/9 (ORF2) potentially encode membrane-interacting NS proteins having one or more TM domains [41]. These may be additional examples of non-fusogenic integral membrane proteins encoded by AqRVs that are similar to the PRV protein p13 [29]. However, these predicted transmembrane proteins with relative biological functions are not clearly defined in AqRVs and orthoreoviruses.

### 2.7.3 NS16 and NS22: The AqRV FAST Proteins

Bioinformatics analysis has shown that NS16 and NS22 (p22 for the AtSRV) of the AqRV share basic structural motifs with the reovirus FAST proteins, suggesting that the AqRV NS proteins NS16/NS22 may be a fusion protein responsible for AqRV-C and AqRV-A syncytiogenesis. As expected, the expression of NS16 (ORF14-454 nt) in transfected cells could induce cell–cell fusion and multinucleated syncytium formation (left panel in Fig. 2.8b), consistent with typical CPE formed in CIK cells infected with GCRV (Fig. 2.8c). However, the expression of S7 cDNA with another ORF (GCRV NS31: 520–1344 nt; SMReV NS32: 489–1325 nt) does not cause cell–cell fusion (right panel in Fig. 2.8b). Especially, many large syncytia formed when full-length cDNA of the S7 segment expressed in transfected CIK cells at 24 hpt, hinting that one more membrane fusion relevant ORF contained in the 5' end sequence of S7 segment. In fact, detailed analyses have shown that cell–cell fusion and nucleus aggregation could be formed when truncated constructions of S7 fragment, such as S7(1-454), S7(1-518), expressed in transfected cells (Fig. 2.8d,e), indicating that the initial 5' end ORFs of the GCRV and SMReV/MSReV S7 segment encode proteins critical for cell–cell fusion [6, 25]. It may need to note that the NS16 alone is capable of inducing cell–cell fusion and syncytium formation in transfected cells; nevertheless, the fusion activity is less efficient in comparison with the viral infection [18]. Unlike NS16, which has an AUG ORF, NS22 of SMReV has been found to be translated from a non-AUG translation start site. In addition, the NS16 FAST protein has been observed to display an N-terminus-outside/C-terminus-inside orientation, with the N-terminal ectodomain critical for effective fusion [18]. Moreover, immunofluorescence assays of GCRV have revealed that NS16 co-localizes with the NS protein NS26 in co-transfected cells.

The enhanced fusion efficiency can be detected when NS16 is co-expressed with NS26, implying that NS26 may participate in cell–cell fusion through cooperation with NS16 in AqRV infection [18, 19].

### **2.7.4 NS12: A Novel Membrane-Associated Protein**

It is known that the GCRV S7 segment encodes two proteins NS16 and NS31. A neglected ORF, tentatively named NS12, residing between NS16 and NS31 in the S7 segment, has been found to have atypical fusogenic activity [73]. With an additional ORF(195-518), the nucleotide sequence of NS12 partially overlaps with the 3' expressible nucleotide sequence of NS16 (Fig. 2.8a). Furthermore, bioinformatics analysis has indicated that NS12 is a transmembrane protein, which has been confirmed by its exclusive presence in the membrane-associated fraction of the cell lysate. In particular, the NS12 product can be detected in infected cells, indicating that NS12 is expressed in cells with GCRV infection. However, the expression of NS12 alone cannot induce visible syncytium formation in transfected CIK cells (Fig. 2.8e), which distinguishes it from the approved FAST protein NS16. Subsequent bioinformatics analysis showed that NS12-like ORFs (with an AUG or non-AUG initiation codon) are also present in the S7 segment of other AqRVs [73], suggesting that NS12 homologs may be widely distributed in the genus *Aquareovirus*. Collectively, the novel membrane-associated protein NS12 is functionally distinct from the known AqRV FAST protein NS16. The identification of bicistron/tricistron mRNAs in AqRVs and their genetic variety provides a basis for further understanding the mechanism of molecular evolution of fusogenic AqRVs.

### **2.7.5 Role of NS31 in Virus Replication**

Compared with the aforementioned AqRV NS proteins, very little is known about the role of NS31 or NS32, which is encoded by the GCRV or SMReV/MsReV genomic segment S7 downstream of NS16 or NS22 (Fig. 2.8a). When NS31 or NS32 solely expressed in transfected cell, no syncytium formation can be observed [25] (right panel in Fig. 2.8b). Immunofluorescence assays showed that NS31 colocalized with NS16 in transfected cells (unpublished data), hinting that NS31 might associate with NS16 during cell–cell fusion. However, no biological relationship has been determined between the GCRV S7 segment-encoded FAST proteins NS16 and NS31. Bioinformatics analysis has predicted that the NS31 protein contains a helix-turn-helix-like domain and a C-terminal acidic  $\alpha$ -helix motif. Using a GAL4-based yeast reporter system and a grass carp cDNA library, it has been found that a fusion protein composed of the Gal4-BD domain and NS31 (BD-NS31) is able to activate the expression of reporter genes (*Gall/MEL1* promoter) without the Gal4-AD domain. In addition, NS31 homologues from other AqRVs have been shown to

possess a similar transcriptional activation in yeast, suggesting that the AqRV/GCRV NS31 protein may be a potent transcription regulator. Further analyses are found that GCRV NS31 and other NS31-like proteins in Aquareovirus genus could efficiently induce host heat-shock 70-kda protein (HSP70) expression, and GCRV protein synthesis or progeny virus yields was restrained by an inhibitor of host HSP70 in infected cells. All these results indicated NS31 cognate proteins in the Aquareovirus genus should play a regulating role during aquareovirus replication [72].

### ***2.7.6 Possible Function of GCRV NS26 in Syncytia Formation***

GCRV NS26 is encoded by the S11 genomic segment, which shares no sequence homology with the known proteins in MRVs. NS26 has been found to interact with NS80 and co-localize with NS16 in co-transfected cells. Co-expression of NS16 with NS26 has been observed to enhance cell–cell fusion efficiency [18]. These results suggest that NS26 may participate in the fusion process of viral infection. Further functional analysis of NS26 has suggested that the TLPK motif is important for NS26 to enhance the fusogenic activity of NS16, and NS26 may utilize lysosomes to benefit the fusion activity [19].

## **2.8 MRV $\sigma$ 1-like Cell Attachment Protein of GCRV-II Species**

In contrast to the majority of AqRVs, the newly identified GCRV-ZH08, GCRV-GD108, and GCRV-104/-109 have been found to have the MRV  $\sigma$ 1-like cell attachment protein (VP55 or VP56) on the particle surface [11, 41, 45, 64, 71]. The recombinant MRV  $\sigma$ 1 protein of GCRV-GD108 can bind to grass carp snout fibroblast cells, as observed by cell attachment assays. Neutralization tests have shown that the polyclonal antibody generated from the expressed fiber protein VP55 is able to prevent viral infection in both fish and grass carp snout fibroblast cells. A more recent study indicated that fiber-like protein VP55 can repress interferon production by degrading the phosphorylated cellular transcription factor interferon regulatory factor 7 (IRF7) [75]. As  $\sigma$ 1 protein is a major epitope and possesses hemagglutination activity, further studies on the GCRV-ZH08/-GD108/-104/-109 (GCRV-II) S7 segment-encoded cell attachment protein will provide a basis for understanding the mechanism of VP55 in cell entry and pathogenesis.

## 2.9 Conclusions and Future Considerations

According to classic gene-protein-coding assignment and genome sequence-based protein prediction, the 11 dsRNA genome segments of GCRV (AqRV-C) encode at least 12 proteins. Of the 12 approved proteins, seven structural proteins VP1–VP7 have currently been confirmed by high-resolution single-particle three-dimensional image reconstruction, as well as SDS-PAGE analysis of the structural proteins of the viral particle. The basic biological and molecular characteristics of the remaining proposed NS proteins NS80, NS38, NS31, NS26, and NS16 have also been investigated [5, 10, 18, 25, 26, 58, 69, 72, 78]. In addition, a novel protein NS12, encoded by the S7 genomic segment of GCRV, has recently been identified to play a partial role in syncytium formation [73]. This evidence suggests that another unknown NS protein may be expressed in virus-infected host cells and facilitate AqRV replication. Much progress has been made in the elucidation of the AqRV molecular biology in the past 30 years. However, for more in-depth knowledge, some issues need to be addressed in future studies.

**Particle Instability of AqRVs** A number of morphological studies have shown that various particle components (intact virions, SVPs, empty particles, and inner cores) can be observed in highly purified or partially purified AqRV preparations by TEM. Indeed, based on the fusogenic properties of most AqRVs inoculated in permissive host cells, mature virions can be released from infected cells by lysis. For general purification of the GCRVs, the collected virus-cell suspension can be purified directly without undergoing any physical methods, such as sonication, or chemical treatment, such as treatment with deoxycholate and extraction with freon, which are frequently used in the purification of the non-fusogenic *Orthoreovirus* [16, 43, 61]. A combination of freeze-thaw cycles before conducting differential centrifugation or ultracentrifugation might be a routine preparatory procedure used in the AqRV purification to obtain a high yield of virus from infected cell lysates. Despite the lack of protease treatment in the general purification of GCRV from lysed cellular components, some degraded viral particles can be observed in untreated viral sample preparations, suggesting that the GCRV particle is capable of degrading spontaneously without the action of exogenous proteases. The fact that there are some SVPs present in the purified viral stock leads us to speculate that the AqRV particle capsid structures are not stable or outer shell protection protein VP7 can easily become detached from penetration protein VP5 under natural conditions or during storage. Based on this phenomenon, stringent and careful handling of viral preparations is needed during AqRV purification. The unstable nature of the AqRV particles might be related to the host-dependent conditions beneficial for establishing efficient infection.

**Gene-Protein-Coding Assignment and Protein Nomenclature** Generally, each protein of AqRVs encoded by a genome segment is named by its corresponding size. The coding assignments of the SBRV genome segments were the first to be well

determined among the identified AqRVs by *in vitro* translation in 1994 [32, 62]. Comparison of [<sup>35</sup>S]methionine-radiolabeled proteins from infected cell lysates and purified virions has led to the distinction of the structural and NS proteins [52, 62]. In this way, 12 proteins (with molecular weights of 130, 127, 126, 97, 73, 71, 46, 39, 35, 29, 28, and 15 kDa) that are encoded by the 11 genomic segments have been identified. Each SBRV segment is monocistronic, with the exception of segment 11, which encodes two NS proteins. Furthermore, of the 12 proteins, seven are structural proteins: VP1 (~130 kDa), VP2 (~127 kDa), VP3 (~126 kDa), VP4 (~73 kDa), VP5 (~71 kDa), VP6 (~46 kDa), and VP7 (~35 kDa) [32, 59, 62], and the remaining five are NS proteins. Further studies on SBRV with SVP-enhanced infectivity assays and single-particle three-dimensional images have shown that segments S5 and S6 encode proteins VP4 and/or VP5, respectively, which resemble the protein product  $\mu$ 1 in MRV encoded by the M2 genome segment [40]. VP4 and VP5 may be isoforms of protein VP5, as reported by McPhillips et al. [35]. The VP5 protein appears to be generated from the products of “VP4,” which correspond to the homologous MRV proteins  $\mu$ 1 and  $\mu$ 1C [44]. In fact, comparing the native virion and 5 min trypsinized [5MT] structures of SBRV shows a noticeable structural change in the trimeric subunits [40]. This observation is highly correlated with the biochemical data of SBRV infectivity assays performed by treatment with protease and visualized by SDS-PAGE [35, 62]. It has been shown that the putative VP5 protein is cleaved after 5 min of trypsin treatment, resulting in a 52-kDa fragment that stays associated with the particle [35]. Further, three-dimensional image reconstruction has been used to determine the VP5 density in an SBRV particle [40]. Therefore, in this regard, the protein VP4 encoded by the S6 segment of the SBRV [35, 62] should be termed as VP5, although no further studies have been conducted on SBRV. Similarly, the copy number of the VP4 protein encoded by the GCRV S5 segment is low, and it is very difficult to detect the protein from purified viral preparations via SDS-PAGE. Early studies have failed to define GCRV gene-protein-coding assignments accurately [28, 64]. Nonetheless, the appropriate gene-protein-coding assignment of GCRV has been obtained and confirmed from purified GCRV proteins by combining full-length genome sequence analysis and three-dimensional image reconstruction [1, 13, 15]. In fact, in another study on MRV [31] and the GCRV S6 sequence [48], the S6 segment-encoded protein has also been defined as VP5. Therefore, based on the current understanding of the molecular virology and high-resolution structural biology of GCRV or AqRVs, it is time to modify the nomenclature of the GCRV-873 (GCRV-I) S6 genome segment-encoded VP4 protein to VP5 to avoid further confusion between VP4 and VP5, which are encoded by genome segments S5 and S6, respectively, in AqRVs.

**Proteins Involved in AqRV Morphogenesis** The GCRV NS80, a protein homologous to the  $\mu$ NS protein of MRV, has been well characterized [5, 10, 26, 58, 69, 77, 78]. Studies have shown that the NS protein NS80 of GCRV can form VIBs in singly expressed or infected cells and recruit all the ISPs (VP1–VP4 and VP6) in addition to NS38 within its VIBs [69, 78]. It has been confirmed that the coiled-coil motifs in the C-terminal regions of NS80 are crucial for forming VIBs in infected cells, whereas



the N-terminal regions of NS80 play important roles in interacting with viral proteins and supporting viral replication [58, 77]. Similar to  $\sigma$ NS in MRV, NS38 is considered to have single-stranded RNA-binding ability and is thought to be involved in viral protein synthesis. In addition to NS80, the GCRV NS38 has also been found to interact with five viral core structural proteins, RNA, and host eIF3A during viral replication for efficient viral protein synthesis. Furthermore, recent evidence showed that NS38 interacts with the OCPs VP5 and VP7 in transfected and infected cells, suggesting that NS38 plays a significant role in progeny particle assembly via interactions with viral proteins and host cell factors. The detailed roles of the viral structural and NS proteins in AqRV morphogenesis remain obscure; therefore, it is further necessary to elucidate the function of each AqRV genome-encoded protein and the interaction among all the proteins during AqRV morphogenesis.

**Fusogenic Characteristics of AqRVs** Based on the currently characterized genome sequences and deduced protein functions related to the presence or absence of cell fusion-related NS FAST or surface structural  $\sigma$ 1-like cell attachment protein, the genus *Aquareovirus* should be subdivided into two distinct subgroups relative to the fusogenic and non-fusogenic orthoreoviruses. The evidence for their phylogenetic classification based on this distinct feature is dependent on the diversity of the S7 genomic segment of the AqRVs. In the genus *Orthoreovirus*, this differentiation is determined by the S class gene, especially the S1 gene in most cases. In fact, most orthoreoviruses, except MRV, are found to encode FAST proteins responsible for inducing cell–cell fusion and syncytium formation [8, 49]. Along with the proteins encoded by fusogenic orthoreoviruses, which are well-characterized examples of transmembrane fusion proteins encoded by nonenveloped viruses, the AqRV FAST proteins are the other known examples. In contrast, the newly identified PRV with 10 genomic segments and the GCRV-II or -III (GCRV-ZH08, GCRV-GD108, and GCRV-104/-109) species have been found to possess a fiber-like cell attachment protein that is similar to  $\sigma$ 1, the cell adsorption protein of MRV [41]. Interestingly, it appears that GCRV-104 can induce a typical CPE in infected permissive cells 5 days p.i [11]. This phenomenon may be related to the potential membrane-associated protein NS15 (ORF2), which contains a FAST protein-specific TM domain in the N-terminal region that is found to be encoded by the GCRV-104 genome S11 segment. If this is the case, then GCRV-104 might not only possess  $\sigma$ 1-fiber protein in the particle, but also bear fusogenic activity in cell cultures. In addition, a novel membrane-associated protein NS12 has been found to be encoded by the GCRV-I S7 gene segment residing between the gene segments encoding NS16 and NS31. Therefore, this indicates that the fusogenic nature is too complex in AqRVs. To understand the molecular mechanism underlying the pathogenesis of GCRV and other AqRVs, it is important to conduct further extensive and in-depth investigations of the NS FAST-related proteins and their roles in AqRV replication.

To summarize, the molecular characteristics of AqRVs have been extensively studied in recent years. Our current knowledge of the AqRV particle, genome, and encoded protein functions provides a strong basis for further understanding the detailed molecular mechanism of action of AqRV pathogenesis in host cells during

infection. Studies on the interactions between NS80 and NS38 and the OCPs VP5 and VP7 as well as the five core proteins during morphogenesis and particle assembly and those on different fusogenic mechanisms used by AqRVs to enhance viral replication efficiency are warranted. In addition, understanding the role of other unknown NS proteins, such as NS26 and NS31, in regulating viral replication in cooperation with host cellular factors is also critical to reveal the detailed events involved in AqRV replication and pathogenesis.

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