Structure of avian orthoreovirus virion by electron cryomicroscopy and image reconstruction

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Abstract

Among members of the genus Orthoreovirus, family Reoviridae, a group of non-enveloped viruses with genomes comprising ten segments of double-stranded RNA, only the “non-fusogenic” mammalian orthoreoviruses (MRVs) have been studied to date by electron cryomicroscopy and three-dimensional image reconstruction. In addition to MRVs, this genus comprises other species that induce syncytium formation in cultured cells, a property shared with members of the related genus Aquareovirus. To augment studies of these “fusogenic” orthoreoviruses, we used electron cryomicroscopy and image reconstruction to analyze the virions of a fusogenic avian orthoreovirus (ARV).

The structure of the ARV virion, determined from data at an effective resolution of 14.6 Å, showed strong similarities to that of MRVs. Of particular note, the ARV virion has its pentameric $\kappa$-class core turret protein in a closed conformation as in MRVs, not in a more open conformation as reported for aquareovirus. Similarly, the ARV virion contains 150 copies of its monomeric $\j$-class core-nodule protein as in MRVs, not 120 copies as reported for aquareovirus. On the other hand, unlike that of MRVs, the ARV virion lacks “hub-and-spokes” complexes within the solvent channels at sites of local sixfold symmetry in the incomplete $T = 13$ outer capsid. In MRVs, these complexes are formed by C-terminal sequences in the trimeric $\mu$-class outer-capsid protein, sequences that are genetically missing from the homologous protein of ARVs. The channel structures and C-terminal sequences of the homologous outer-capsid protein are also genetically missing from aquareoviruses. Overall, the results place ARVs between MRVs and aquareoviruses with respect to the highlighted features.

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Introduction

Electron cryomicroscopy (cryoEM) and three-dimensional image reconstruction (3DR) have been effectively used to determine the arrangements of proteins in the virions and other particle forms of “non-fusogenic” mammalian orthoreoviruses (MRVs), including those of prototype strains type 1 Lang, type 2 Jones, and type 3 Dearing (T3D) (Metcalf et al., 1991; Dryden et al., 1993, 1998). These methods have proven additionally powerful when combined with fitting of atomic models for the viral proteins derived from recent X-ray crystallographic determinations (Reimisch et al., 2000; Nason et al., 2001; Olland et al., 2001; Liemann et al., 2002; Luongo et al., 2002; Tao et al., 2002; Zhang et al., 2003, in press). This combination of approaches has contributed a number of new conclusions regarding the assembly, disassembly and functions of MRV structural proteins (Liemann et al., 2002; Odegard et al., 2003; Zhang et al., 2003, in press).

MRVs constitute one of at least five species in the genus Orthoreovirus, family Reoviridae, a group of non-enveloped
viruses with genomes comprising ten segments of double-stranded (ds) RNA (Duncan, 1999; Duncan et al., 2004). In addition to MRVs, this genus comprises at least four species that induce syncytium formation in cultures of infected cells. These “fusogenic” orthoreoviruses include two isolates from mammals (Nelson Bay virus, from a flying fox, and baboon reovirus) (Gard and Marshall, 1973; Leland et al., 2000) and numerous isolates from different birds and reptiles (Fahey and Crawley, 1954; Gershowitz and Wooley, 1973; MeFerran et al., 1976; Ahne et al., 1987; Lamirande et al., 1999; Drury et al., 2002). Sequence comparisons have revealed considerable diversity among the viral species, with levels of amino acid identities between homologous pairs of proteins that range from 16 to 36% (Duncan et al., 2004). As pathogens of poultry (van der Heide, 2000), avian orthoreoviruses (ARVs) hold particular interest for more in-depth studies of the viral particles and proteins.

Although complete genomic sequences have so far been reported only for three MRV strains (Wiener and Joklik, 1989; Breun et al., 2001; Yin et al., 2004), all Orthoreovirus species are thought to share a set of seven homologous structural proteins (Table 1), each encoded by a single long open reading frame (ORF) in each of the seven cognate genome segments. This supposition is supported by analysis of the protein composition of virions and subviral particles of ARV (Schnitzer et al., 1982; Martinez-Costas et al., 1997) as well as by the protein-coding potential of individual genome segments (Varela and Benavente, 1994). Two other monocistronic genome segments in MRVs (segments M3 and S3) and ARVs (segments M3 and S4) encode non-structural proteins (μNS and σNS, respectively) that are conserved between these species (Duncan, 1999; Touris-Otero et al., 2004) and probably conserved in the other species as well. The coding capacity of the remaining segment (S1 in MRVs and ARVs) is more variable, comprising two or three distinct ORFs (Nagata et al., 1984; Shapouri et al., 1995; Shmulevitz et al., 2002; Dawe and Duncan, 2002; Shmulevitz et al., 2002; Corcoran and Duncan, 2004).

The family Reoviridae currently includes at least eleven additional genera (Mertens, 2004), the members of one of which, Aquareovirus (eleven dsRNA genome segments), show clear homologies to MRVs. In fact, the same homologous set of seven structural and two non-structural proteins putatively shared among all orthoreoviruses is also shared among aquareoviruses (Attoui et al., 2002). The virion of striped bass reovirus (SBR), the only aquareovirus yet analyzed by cryoEM and 3DR (at 23 Å resolution), also shows many similarities with that of MRVs (Nason et al., 2000). Some authors have in fact argued that it might be proper to place orthoreoviruses and aquareoviruses within a shared taxonomic group below the family level (Attoui et al., 2002; Kim et al., 2004b), such as in a distinct subfamily or even within the same genus. Interestingly, aquareoviruses share the syncytium-inducing capacity of fusogenic orthoreoviruses (Lupiani et al., 1995), an uncommon property for non-enveloped viruses. In addition to differences in host range (orthoreoviruses have not been isolated from fish), aquareoviruses, or at least SBR, differ from MRVs in a number of structural features, including partially open core turrets in virions (closed in MRVs), 120 core nodules (150 in MRVs), and no fibrous cell-adhesion protein homologous to that of MRVs (Nason et al., 2000).

To date, no cryoEM and 3DR results have been reported for any fusogenic orthoreoviruses. We recognized that such data may be useful to investigate possible divergence of structural features within the genus Orthoreovirus, as well as between orthoreoviruses and aquareoviruses. Given increasing attention to molecular and cellular studies of

### Table 1

<table>
<thead>
<tr>
<th>Protein homologs</th>
<th>Protein sizes (aa)*</th>
<th>Copy no.</th>
<th>Location in virion</th>
</tr>
</thead>
<tbody>
<tr>
<td>ARV</td>
<td>MRV</td>
<td>AqRV</td>
<td>ARVd</td>
</tr>
<tr>
<td>λA</td>
<td>λ1</td>
<td>VP3</td>
<td>1293±1275 1214 140</td>
</tr>
<tr>
<td>λB</td>
<td>λ3</td>
<td>VP2</td>
<td>1267 1274 ±  12</td>
</tr>
<tr>
<td>λC</td>
<td>λ2</td>
<td>VP1</td>
<td>1285±1289 1299 60</td>
</tr>
<tr>
<td>μA</td>
<td>μ2</td>
<td>VP5</td>
<td>732±736 728 24</td>
</tr>
<tr>
<td>μB</td>
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<td>VP4</td>
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<td>367 365 276 600</td>
</tr>
<tr>
<td>αC</td>
<td>α1</td>
<td>–</td>
<td>326 455 –  36</td>
</tr>
</tbody>
</table>

* Total number of amino acids (aa).

b Values for MRV virions, probably also correct for ARV virions. The values with approximate signs are estimates from best available data for MRV (Coombs, 1998). These values are probably also correct for aquareovirus virions, except that aquareoviruses contain only 120 core nodules formed by VP6 (Nason et al., 2000) and both genetically and structurally lack a σ1 homolog (Nason et al., 2000; Attoui et al., 2002).

c AqRV, aquareovirus.

d Values for ARV strain 138 unless otherwise indicated. GenBank accession numbers in order of listing are AAT27445, AAM46173, AAT52024, AAC18126, AAW78485, AAF45156, and AAC18122; NA, not available.

e Values for MRV strain T3D. GenBank accession numbers in order of listing are AAD42306, P17378, P11079, AAL99937, M2XR4D, AAA47261, P03527, and P03528.

f Values for golden shiner reovirus. GenBank accession numbers in order of listing are AAM92746, AAM92745, AAM92744, AAM92748, AAM92749, AAM92752 and AAM92754.

g Values for ARV-S1133.

h Assuming T = 1 shell contains two subunits in each icosahedral asymmetric unit, it is sometimes referred to as “T = 2” (Grimes et al., 1998).

i From Noad et al. (in press).
ARV in the past several years (Hsiao et al., 2002; Shmulevitz et al., 2004; Touris-Otero et al., 2004; Xu et al., 2004), we focused our efforts on one of these fusogenic isolates, ARV-138, a mildly pathogenic strain that grows to high yields in cultured cells and has been the focus of several recent analyses (Duncan and Sullivan, 1998; O’Hara et al., 2001; Patrick et al., 2001).

**Results and discussion**

**CryoEM and image reconstruction of ARV virion**

Gradient-purified virions of ARV-138 were visualized by cryoEM as previously described (Baker et al., 1999). Micrographs showed the particles to have a layered capsid morphology typical of the viruses in this genus (Fig. 1A). A 3DR was generated from digitized images of 1086 individual virions obtained from fourteen such micrographs, using established procedures (Baker and Cheng, 1996; Bowman et al., 2002). The data resulting from these procedures showed an effective resolution of 14.6 Å (Fourier shell correlation = 0.5), and the final density map was computed to a resolution limit of 12.1 Å (Fourier shell correlation = 0.143) (Fig. 1B).

**Structural comparisons of ARV and MRV virions**

Different views of the 3DR of the ARV-138 virion led to an overall conclusion that it shares major structural similarities with that of MRVs, including MRV-T3D (Fig. 2). The overall diameter of the ARV virion, to the top of the major surface protein oB (Schnitzer et al., 1982; Martinez-Costas et al., 1997), is 857 Å. The previously reported diameter of the MRV virion, to the top of the homologous surface protein o3, is ~850 Å (Dryden et al., 1993), or more precisely 854 Å. Neither of these values includes the additional length of the fibrous cell-adhesion protein, o1 in MRV or oC in ARV (Schnitzer et al., 1982; Martinez-Costas et al., 1997), which is poorly visualized by cryoEM and 3DR (Dryden et al., 1993; Chandran et al., 2001; Zhang et al., 2003; this study, see below), but which is known from images of negatively stained samples of MRVs to be capable of extending 400 Å or more from the particle surface (Furlong et al., 1988).

Radially color-coded surface views of the 3DRs of ARV and MRV virions (Figs. 2A, B) revealed several of their similarities. The twelve pentameric core turrets formed by ARV protein $\lambda$C (Martinez-Costas et al., 1997), one around each icosahedral fivefold axis, are present in a closed conformation, like the turrets formed by homologous protein $\lambda$2 in MRVs (Metcalf et al., 1991; Dryden et al., 1993). These shared features are also well visualized in central cross-sections of the virions (see Figs. 2C, D). The closed turrets in the ARV and MRV virions contrast with the partially open ones formed by protein VP1 in the virion of aquareovirus SBR (Nason et al., 2000). In MRVs, closure of the turret creates a binding site for the base of cell-adhesion protein o1 (Dryden et al., 1993; Chandran et al., 2001), and the same appears true for oC in ARV. Small “knobs” of density seated above and below $\lambda$C directly at each icosahedral fivefold axis (poorly visualized in Fig. 2A, but clearer in Fig. 2C) likely represent small portions of oC. Interestingly, aquareoviruses lack a genomic o1/oC homolog, and the SBR virion, for which a 3DR has been determined, lacks a structural o1/oC analog as well, reflecting its more open turrets. The radial color cueing in Figs. 2A and B makes clear that the tops of the closed turrets (green) are recessed by ~70 Å relative to the rest of the virion surface in both ARV and MRV. Like MRV $\lambda$2, ARV $\lambda$C mediates the guanylyltransferase activity and probably also the two methyltransferase activities involved in adding 5′ caps to the viral mRNAs as they exit the viral particle following transcription (Martinez-Costas et al., 1995; Hsiao et al., 2002).

On the surface of the ARV virion, in the regions between the turrets, are 600 knobby projections (Fig. 2A) attributable to monomers of the oB protein. These projections are similar to those formed by the o3 protein in MRVs (Metcalf et al., 1991; Dryden et al., 1993; Jané-Valbuena et al., 1999) and are also visualized in the central cross-sections (see Figs. 2C, D). Proteolytic removal of these surface-exposed
subunits is a required step in cell entry by both ARVs and MRVs (Sturzenbecker et al., 1987; Duncan, 1996). The oB (or o3) monomers are arrayed in groups of either four or six, depending on their positions on the surface (Figs. 2A, B). These arrays surround large solvent conduits, which traverse the full radial width of the outer capsid, are centered at axes of local sixfold symmetry and are comparable to the so-called P2 and P3 channels of MRVs (Metcalf et al., 1991). Arrays of six oB monomers surround the P3 channels. Arrays of four oB monomers partially surround the P2 channels, but the other two positions are occupied by parts of the λC pentamer, similar to the λ2 pentamer in MRVs (Metcalf et al., 1991; Dryden et al., 1993). In total, 60 P3 and 60 P2 channels are found in the whole outer capsid. The number and arrangement of these channels and the encircling subunits identify the outer capsid of ARV as having incomplete $T = 13$ symmetry, the same as that of MRVs (Metcalf, 1982; Metcalf et al., 1991). Notably, densities observed in the P3 channels in several previous 3DRs of MRVs (Dryden et al., 1993, 1998; Nason et al., 2001; Zhang et al., in press) are not seen in the current 3DR of ARV, as discussed in detail below.

Central cross-sections of the 3DRs of the ARV and MRV virions (Figs. 2C, D) revealed more of their similarities. Centered at a radius of 268 Å is a largely continuous inner capsid, comparable to that formed by the λ1 protein in MRVs and thus attributable to the homologous protein λA in ARV (Martinez-Costas et al., 1997). Inside this layer (i.e., at lower radii) are concentric rings of density, spaced ~23 Å apart and attributable to the closely packed genomic dsRNA as in MRVs (Dryden et al., 1993, 1998; Luongo et al., 2002; Zhang et al., 2003). Also inside the λA shell, along or near each fivefold axis, is some stronger density probably attributable to a portion of the dsRNA. This density is not seen in the current 3DR of ARV.
viral transcriptase complex as in MRVs (Dryden et al., 1998; Zhang et al., 2003). Because of their specific placements and make-ups, the transcriptase complexes located at ten to twelve of the twelve icosahedral fivefold axes per ARV or MRV virion are incompletely visualized in the 3DRs (Figs. 2C, D) but are likely to include the viral RNA-dependent RNA polymerase (protein $\lambda B$ in ARVs vs. $\lambda 3$ in MRVs) as well as the putative polymerase cofactor and nucleoside triphosphate phosphohydrolase (protein $\mu A$ in ARVs vs. $\mu 2$ in MRVs) (Schnitzer et al., 1982; Martinez-Costas et al., 1997; Tao et al., 2002; Kim et al., 2004a).

Features reminiscent of the monomeric core nodules and pentameric core turrets of MRVs contact the outside of the $\lambda A$ shell, at higher radii, in the central cross-sections (Figs. 2C, D). The nodule-like features are comparable to those formed by the $\alpha 2$ protein in MRVs and attributable to the homologous protein $\alpha A$ in ARV (Schnitzer et al., 1982; Martinez-Costas et al., 1997). These features are seen in the cross-sections as isolated “bumps”, separated by solvent density, that project upwards from the $\lambda A$ or $\lambda 1$ shell at three distinct positions: surrounding the core turrets (icosahedral fivefold axes), surrounding the icosahedral threefold axes, and across the icosahedral twofold axes (Figs. 2C, D). The different positions of these $\alpha A$ or $\alpha 2$ nodules are better discerned in radial sections of the 3DRs (see Figs. 3E, F). At their tops, the $\alpha A$ nodules of ARV contact the major $\mu$-class outer-capsid protein, as do the $\alpha 2$ nodules in MRVs (Figs. 2C, D; also see next paragraph). The turret-like features, comparable to those formed by the $\lambda 2$ protein in MRVs and attributable to the homologous protein $\lambda C$ in ARV, are seen in the cross-sections as features that project upwards from the $\lambda A$ or $\lambda 1$ shell and surround a heart-shaped cavity centered along each icosahedral fivefold axis (Figs. 2C, D). This cavity encompasses the path that the nascent viral plus-strand RNAs are thought to travel as they undergo 5'-capping and release from the transcribing viral particle, as in MRVs (Dryden et al., 1993; Reinisch et al., 2000). Also as in MRVs, the turrets are exposed on the virion surface in ARV and thus contribute to the outer capsid. The uppermost portions of each turret (i.e., those at highest radii) run approximately parallel to the particle surface and approach the icosahedral fivefold axis, closing off the top of the turret. The $\lambda C$ turrets contact all three additional outer-capsid proteins and also contact one of the three types of $\alpha A$ nodules, as do the $\lambda 2$ turrets in MRVs (Figs. 2C, D; also see next paragraph and below). The components described to this point from the cross-sections –

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**Fig. 3.** Radial sections of the 3DRs of ARV and MRV virions. The row of numbers across the middle of the figure indicates the radii, in Å, at which the sectional views above and below each number were generated. Fivefold-symmetrical densities attributable to pentamers of the $\lambda C$ protein in ARV-138 (A, C, E) or the $\lambda 2$ protein in MRV-T3D (B, D, F) are surrounded by a black pentagon in each panel. Threefold-symmetrical densities attributable to trimers of the $\mu B$ protein in ARV-138 or the $\mu 1$ protein in MRV-T3D are labeled with white letters in panels A to D; the different letters (Q, R, S, and T) denote the four symmetrically distinct positions that the $\mu B$ or $\mu 1$ trimers occupy within the $T = 13$ outer capsid. A region centered on one P3 channel of each particle, and shown in magnified view in Fig. 4, is surrounded by a white circle in panels C and D; arrowheads denote densities in the channel that are absent in ARV-138, but present in MRV-T3D. Ovoid densities attributable to monomers of the $\alpha A$ protein in ARV-138 or the $\alpha 2$ protein in MRV-T3D are labeled with white letters in panels E and F; the different letters (2f, 3f, and 5f) denote the three symmetrically distinct positions that the $\alpha A$ or $\alpha 2$ monomers occupy between the $\lambda 1$ (inner) and $\mu 1$ (outer) layers, at or surrounding the icosahedral twofold (2f), threefold, (3f), or fivefold (5f) axes. Scale bar in panel F applies to all panels, 20 nm.
dsRNA genome segments and proteins \( \lambda A, \lambda B, \lambda C, \mu A, \) and \( \omega A \) – constitute the ARV core particle (Schnitzer et al., 1982; Martinez-Costas et al., 1997).

The remaining features apparent in the central cross-sections of the ARV and MRV virions (Figs. 2C, D) represent the major parts of the outer capsid. Contacting the sides of the \( \lambda C \) turrets as well as the tops of the \( \omega A \) nodules are features comparable to those formed by the \( \mu 1 \) protein in MRVs and thus attributable to the homologous protein \( \mu B \) in ARV (Schnitzer et al., 1982; Martinez-Costas et al., 1997). Like MRV \( \mu 1 \), ARV \( \mu B \) is N-terminally myristoylated and is proteolytically cleaved at several discrete sites during assembly or cell entry (Duncan, 1996; Martinez-Costas et al., 1997). Atop the \( \mu B \) densities are additional features comparable to those formed by the \( \omega 3 \) protein in MRVs and thus attributable to the homologous protein \( \omega B \) in ARV. The reduced electron density attributable to the \( \omega B \) subunits in this 3DR of ARV virions (Fig. 2C) probably reflects that \( \omega B \) was partially degraded during purification and/or storage prior to microscopy in this study. Lastly, outside the \( \lambda C \) turrets along the icosahedral fivefold axes are beads of weak density (poorly visualized in Fig. 2C) comparable to those formed by cell-adhesion protein \( \omega 1 \) in MRV and thus likely attributable to the homologous protein, \( \omega C \), in ARV.

Sectional views at specific radii within viral particles are another effective tool for discerning elements of structure and making comparisons between the particles (Dryden et al., 1993). For example, a comparison of features at a radius of 343 Å in the ARV and MRV virions (Figs. 3A, B) shows remarkable similarities in the densities attributable to proteins \( \lambda C \) and \( \mu B \) of ARV and proteins \( \lambda 2 \) and \( \mu 1 \) of MRV. Similarly, a comparison of features at a radius of 287 Å in these virions (Figs. 3E, F) shows remarkable similarities in the densities attributable to proteins \( \lambda C \) and \( \omega A \) of ARV and proteins \( \lambda 2 \) and \( \omega 2 \) of MRV. Such views strongly support the conclusion that ARV and MRV virions share homologous components and very similar organizations in both inner and outer capsids. On the other hand, some interesting differences are seen near radius 329 Å in the ARV and MRV virions (Figs. 3C, D), as discussed below.

**ARV has 150 core nodules**

Another major difference between the virions of MRVs and aquareovirus SBR – in addition to whether or not the core turrets are closed and whether or not a cell-adhesion protein is anchored to the top of the turrets (Nason et al., 2000) – concerns the number of core nodules. In SBR, there are 120 core nodules attributed to protein VP6: 60 at sites surrounding the 12 core turrets (icosahedral fivefold axes) and 60 more at sites surrounding the 20 icosahedral threefold axes (Nason et al., 2000). Insect reoviruses from the genus *Cypovirus* also contain 120 such nodules, attributed to protein LPP (Zhou et al., 2003). In MRVs, however, there are 150 core nodules, formed by protein \( \omega 2 \):

120 at similar sites as those in SBR and 30 more at sites overlying the 30 icosahedral twofold axes (Dryden et al., 1993; Reinisch et al., 2000). In the ARV virion, as indicated by the central cross-section (Fig. 2C), but more clearly shown by the radial section at 287 Å (Fig. 3E), there are 150 core nodules, attributable to protein \( \omega A \). Thus, ARV is like MRV and not like SBR in this regard. The slightly smeared density of the twofold nodules apparent in both ARV and MRV virions (Figs. 3E, F) likely reflects that the single \( \omega A \) or \( \omega 2 \) subunit at each of these sites can be bound in either of two orientations (Reinisch et al., 2000), which does not reinforce the averaged density to the same high extent as at the other nodule positions, where only one orientation of the \( \omega A \) or \( \omega 2 \) subunit is found (Reinisch et al., 2000).

**ARV outer capsid lacks hub-and-spokes complexes**

The detached spheroidal densities observed in the P3 channels of MRVs in several previous 3DRs (Dryden et al., 1993, 1998; Nason et al., 2001) have been recently visualized as more connected and elaborate “hub-and-spokes” complexes in 3DRs of MRV-T3D at resolutions near 7.0 Å or better (Zhang et al., 2003, in press). Related complexes have been visualized as well in the P2 channels of MRV (Zhang et al., in press), as well as in the P2 and P3 channels of MRV intermediate subvirion particles (X. Zhang, M.A. Agosto, M.L. Nibert, and T.S. Baker, unpublished results). In both locations, the channel structures are attributed to all or part of the C-terminal 33 residues of the major outer-capsid protein \( \mu 1 \) (Liemann et al., 2002; Odegard et al., 2003; Zhang et al., in press), residues that are disordered and not visible in the X-ray crystal structure of the \( \mu 1/\omega 3 \) heterohexamer (Liemann et al., 2002). Whether or not such complexes are present in the ARV virion is somewhat difficult to discern with confidence from the radially color-coded surface view of the whole particle (Fig. 2A), but, in other views, it is clear that such complexes are missing from ARV. For example, the radial sections at 329 Å in the ARV and MRV virions show densities within the P2 and P3 channels of MRV, but not within those of ARV (Figs. 3C, D). This difference is further highlighted by a close-up view of a P3 channel from either virion, across the particle radii at which the hub-and-spokes complex is present in MRV, but not in ARV (Fig. 4). In fact, revisiting the radially color-coded surface view of the whole virions in Figs. 2A and B, one can see that, in the ARV virion, the surfaces of \( \lambda A \) and \( \omega A \) (red) are visible through the channels, whereas, in the MRV virion, they are largely obscured by the hub-and-spokes complexes (yellow). The absence of these complexes in ARV-138 is consistent with the fact that the genetically encoded sequence (i.e., ORF) of the \( \mu 1 \) homolog of ARVs, \( \mu B \), terminates just before the C-terminal sequences that contribute to forming the hub-and-spokes complexes in MRVs; in sequence alignments, the C-terminal-most encoded residue in \( \mu B \), Ser676, aligns with \( \mu 1 \) residue Pro675 (Noad et al., in press) whereas the C-
terminal-most encoded residue in $\mu 1$ is Arg708 (Fig. 4C). Interestingly, the genetically encoded sequence of the $\mu 1/\mu B$ homolog of aquareoviruses, VP4, terminates at the same aligned residue as does $\mu B$ (Qiu et al., 2001; Liemann et al., 2002), and the SBR virion, for which a 3DR has been determined, also shows no evidence of hub-and-spokes complexes in its P2 or P3 channels (Nason et al., 2000). Thus, ARVs appear to be more similar to aquareoviruses than to MRVs with regard to these features.

**ARV outer capsid has $T = 13$ laevo symmetry**

Preceding comments about the ARV virion structure do not address the handedness of its capsid lattices. The $T = 13$ outer capsid of MRV virions exhibits laevo handedness (Metcalf, 1982; Dryden et al., 1993). The same is presumably true for the ARV virion, but we did not obtain micrographs from tilted grids in this study that would have allowed us to determine the handedness directly. Instead, we addressed this question by fitting a refined atomic model of the MRV virion (see Materials and methods) into the 3DR of the ARV-138 virion displayed with laevo handedness in its $T = 13$ outer capsid. The extremely good fits that we obtained for nearly all regions or domains of the $\lambda 2$ (Fig. 5A) and $\mu 1$ (Fig. 5C) subunits of MRV within the 3DR of ARV indicate that the outer capsid of the ARV virion does indeed exhibit the same, incomplete $T = 13/1$ symmetry as does that of MRV. In addition, the crystal structure of the $\lambda 2$ pentamer (Reinisch et al., 2000) could be fitted only poorly into the dextro enantiomer of the 3DR of the ARV-138 virion (Fig. 5B). The refined atomic model of the MRV virion fitted into the 3DR of the ARV virion also exhibited close correspondence of features attributable to the $T = 1$ core-shell protein $\lambda 1$ and the core-nodule protein $\alpha 2$ (data not shown). These remarkably similar details of arrangement and shape for the ARV and MRV proteins observed in this experiment thus provide further strong evidence for their major structural similarities.

**ARV taxonomy and relation to other reoviruses**

In summary, the results of this first 3DR of a fusogenic orthoreovirus, ARV-138, demonstrate strong structural similarities between ARV and non-fusogenic MRV virions, from interior to surface and including many features of both inner and outer capsids. Unique distributions of protein components along different symmetry axes are also shared. Although these strong similarities may have been largely expected from reported protein homologies, the results in this study place structural and functional comparisons of these two most commonly isolated species of the genus *Orthoreovirus* on much firmer ground. Indeed, the many similar details in the ARV and MRV virions observed in this study lead us to conclude with greater confidence that many of the basic functional mechanisms in particle stability, cell entry, mRNA synthesis, and particle assembly are likely to be largely conserved between ARVs and MRVs.

In terms of the evolutionary relationships among ARVs, MRVs, and members of the genus *Aquareovirus*, conformations of the core turrets, numbers of core nodules, and presence or absence of a fibrous cell-adhesion protein homologous to MRV $\alpha 1$ identify ARVs and MRVs as the closer relatives. Other properties such as numbers of genome segments (ten in ARVs and MRVs vs. eleven in aquareoviruses) and levels of sequence identity among the conserved proteins support this conclusion. Notably, in this
study, however, we found that ARV-138 is more similar to aquareovirus SBR than to MRVs in lacking hub-and-spokes complexes in its P2 and P3 outer-capsid channels, as formed by C-terminal sequences unique to the \(A\)-class outer-capsid protein of MRVs. Since ARVs and aquareoviruses share syncytium formation in infected cultures as a property that MRVs do not share, it is tempting to speculate that the lack of the channel structures in ARVs and aquareoviruses might represent a covariation that is functionally linked to syncytium formation. However, since the syncytium-inducing p10 protein of ARV is a non-structural protein (Shmulevitz and Duncan, 2000), it is difficult to envision how or why such a linkage might operate. Another possibility is that the presence or absence of the hub-and-spokes complexes reflects a host-range difference relating to different requirements for particle stability during spread between or within avian or piscine vs. mammalian individuals in the natural world. Further experiments are needed to resolve this question. Structural analysis of the baboon reovirus virion and sequence analysis of its \(\mu\)-class outer-capsid protein may be informative in this regard since baboon reovirus infects mammals but resembles aquareoviruses in the apparent lack of a \(\alpha1/\alphaC\)-like cell-adhesion protein (Dawe and Duncan, 2002).

**Materials and methods**

**Purifying ARV virions**

ARV-138 was grown in QM5 quail fibroblasts as previously described (Duncan and Sullivan, 1998). Viral particles were isolated from the infected cell-culture supernatants by extraction with trichlorotrifluoroethane, ultracentrifugation, and purification on linear CsCl density gradients (1.25 to 1.45 g/cm\(^3\)) (Duncan, 1996). The well-defined band of virions at a buoyant density of \(\approx 1.36 \text{ g/cm}^3\) was harvested and dialyzed overnight against virion storage buffer (150 mM NaCl, 10 mM MgCl\(_2\), 10 mM Tris, pH 7.5). Gradient-purified virions were stored at 4°C before use.

**Performing cryoEM on ARV virions and determining a 3DR**

Gradient-purified ARV-138 virions were embedded in vitreous ice on holey carbon grids maintained at \(-176°C\) and viewed in a Philips CM200 FEG microscope as previously described (Baker et al., 1999). Micrographs were recorded at a nominal magnification of 38,000× following low-dose procedures (\(~20 \text{ e}^-/\text{Å}^2\) ). The actual magnification was later determined to be \(~38,740\text{ ×}\) by calibration against an atomic model of the MRV-T3D virion (see below). Micrographs were digitized at 7-Å intervals on a Zeiss PHODIS scanner, and the data were bin-averaged to obtain pixels corresponding to 3.68 Å in the specimen. Images of 1086 individual particles, well isolated and seemingly intact, were extracted from fourteen scanned micrographs that had been recorded at defocus settings of 0.98 to 4.43 μm underfocus. A 3DR was generated using established procedures for icosahedral particles (Baker and Cheng, 1996), including corrections by established procedures to compensate in part for effects of the microscope contrast transfer function (Bowman et al., 2002). Orientation angles were evenly distributed throughout the asymmetric unit as shown by all inverse eigenvalues being <0.01. The data resulting from these procedures showed an effective resolution of 14.6 Å (Fourier shell correlation = 0.5) (see Fig. 1B). The final
density map was then computed to a resolution limit of 12.1 Å (Fourier shell correlation = 0.143) (see Fig. 1B) by applying an inverse temperature factor of 1/500 Å² to enhance the high-resolution features out to 14.6 Å, after which the inverse factor was held constant, and a Gaussian function was applied to smoothly attenuate the Fourier data to zero amplitude from 14.6 to 12.1 Å.

Displaying 3DRs of the ARV and MRV virions

A previously determined 3DR of the MRV-T3D virion at 7.0-Å resolution (Zhang et al., in press) was used for the comparisons with ARV-138 in this report; however, in order to match the resolution of the ARV map, the MRV map was recomputed in an identical manner as described in the preceding paragraph for ARV. Based on reconstructions of other MRVs (Metcalf et al., 1991; Dryden et al., 1993; X. Zhang, S.B. Walker, M.L.Nibert, and T.S. Baker, unpublished results), the MRV-T3D structure is thought to be representative of all members of that species. All contour-shaded surface views and density-coded (darker, higher density; lighter, lower density) cross-sections and radial sections were generated with RobEM (http://www.bilbo.bio.purdue.edu/~workshop/help_robem/). Radially color-coded surface views were generated with the UCSF Chimera package (http://www.cgl.ucsf.edu/chimera/) (Pettersen et al., 2004) from the Computer Graphics Laboratory, University of California, San Francisco (supported by NIH P41 RR-01081). Fig. 1B was prepared by using XMGrace (http://www.plasma-gate.weizmann.sc.il/Grace/) and Adobe illustrator CS. Fig. 5 was prepared by using PMV and VISION (Sanner, 1999; Sanner et al., 2002). All panels in each figure were combined and labeled by using Adobe Photoshop CS.

Fitting crystal structures of MRV proteins into the 3DR of the ARV virion

The pixel size of the MRV-T3D virion reconstruction at 7.0-Å resolution (Zhang et al., in press) was first calibrated against the crystal structure of the reovirus core (Reinisch et al., 2000). The previously reported atomic model of the MRV-T3D virion (Zhang et al., 2003) was then refined against the calibrated MRV-T3D 3DR by use of a real-space procedure contained in the program RSRef (Chapman, 1996). The pixel size of the ARV-138 virion reconstruction at 14.6-Å resolution (this study) was then calibrated, again relative to the crystal structure of the reovirus core. The refined atomic model of the MRV-T3D virion was then fitted into the 3DR of the ARV-138 virion without any further adjustment of individual protein subunits.

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References

Dawe, S., Duncan, R., 2002. The S4 genome segment of baboon reovirus is


Duncan, R., 1996. The pH-dependent entry of avian reovirus is accompanied by two specific cleavages of the major outer capsid protein μ2C. Virology 219, 179–189.


