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Review

The diversity of the orthoreoviruses: Molecular taxonomy and phylogentic divides

J. Michael Day *

Southeast Poultry Research Laboratory, Agricultural Research Service, United States Department of Agriculture, 934 College Station Road, Athens, GA 30605, United States

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ABSTRACT

The family *Reoviridae* is a diverse group of viruses with double-stranded RNA genomes contained within icosahedral, non-enveloped, double-layered protein capsids. Within the *Reoviridae*, the *Orthoreovirus* genus includes viruses that infect reptiles, birds and mammals (including humans). Recent sequencing efforts have produced a great deal of new molecular data for the fusogenic orthoreoviruses, a group of reoviruses that induce cell–cell fusion during an infection. This new data has allowed a fresh look at the phylogenetic relationships among the members of the *Orthoreovirus* genus, and has provided insight into the evolution of orthoreovirus species and species groups. This review mainly focuses on the molecular taxonomy of the fusogenic orthoreoviruses, and aims to provide insight into their relationships with the non-fusogenic orthoreoviruses and other selected *Reoviridae* genera.

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Contents

1. Introduction

The family *Reoviridae* is the largest of the eight recognized double-stranded RNA (dsRNA) virus families (Mertens, 2004). The non-enveloped, icosahedral members of the *Reoviridae* comprise numerous genera, with host ranges extending from fungi, plants, and insects, to mollusks, fish, reptiles, birds and mammals (including humans). These genera can be placed into one of two groups based upon the presence or absence of a turret protein structure located at each of the viral icosahedral fivefold axes (Schiff et al., 2007). The "non-turreted" group contains the much-studied and globally important *Rotavirus* genus, a widespread group infecting many mammalian and avian species. Within the "turreted" group, the genus *Orthoreovirus* includes the prototypical members of the *Reoviridae*: the mammalian reoviruses (MRVs), first described as "respiratory and enteric orphans" in the United States and Mexico in the 1950s. The orphan status of many of these early reovirus isolates referred to their presence in healthy humans with no symptoms of enteric or respiratory disease (Sabin, 1959). The *Orthoreovirus* genus can be further divided into the fusogenic and the non-fusogenic orthoreoviruses. This grouping is based upon the ability of the fusogenic orthoreoviruses to cause fusion of infected cells, resulting in multinucleated cellular syncytia that are easily discerned in cell culture and that may be important in virus dissemination *in vivo* during an infection (Benavente and Martinez-Costas, 2007; Salsman et al., 2005). These fusogenic viruses encode a fusion-associated small transmembrane (FAST) protein specifically involved in this characteristic ability (Duncan

* Tel.: +1 706 546 3975; fax: +1 706 546 3161.
E-mail address: Michael.Day@ars.usda.gov.

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and Sullivan, 1998; Salsman et al., 2005; Shmulevitz and Duncan, 2000; Shmulevitz et al., 2003). The prototypical MRVs are non-fusogenic and represent a distinct phylogenetic clade within the orthoreoviruses; the fusogenic reoviruses infect mammals, birds and reptiles, and analyses suggest that they are indeed phylogenetically distinct from the fusogenic mammalian clade (Duncan, 1999). This review will use the realization that the fusogenic and non-fusogenic orthoreovirus subgroups are phylogenetically distinct as a starting point, and will seek to analyze the increasing genomic sequence data available for the members of the *Reoviridae* that infect fish, reptiles, birds, and mammals.

2. The orthoreoviruses viruses and their distinguishing characteristics

Like the rest of the reoviruses, the orthoreoviruses contain a segmented genome enclosed in a 70–80 nm double layered protein capsid consisting of inner and outer layers (Fig. 1). The orthoreovirus inner capsid layer plus its enclosed viral genome is commonly referred to as the viral core. The orthoreovirus dsRNA genome contains 10 segments divided into three size classes based upon their characteristic mobility during gel electrophoresis: there are three large (L1, L2 and L3) segments, three medium segments (M1, M2 and M3), and four small segments (S1, S2, S3 and S4). The total genome size is approximately 23,500 base pairs. Other member of the *Reoviridae* may contain 11 (i.e., the rotaviruses) or 12 (i.e., the coltiviruses) dsRNA genome segments (Mertens, 2004), and a proposed new member of the *Reoviridae*—the genus *Dinovernavirus*—contains nine dsRNA genome segments (Attoui et al., 2005). These dsRNA genome segments each generally contain a single gene encoding a single protein, with several notable exceptions in the orthoreoviruses (see Table 1). The S1 genome segment of the non-fusogenic mammalian reoviruses (MRVs) encodes the σ1 cell attachment protein, and contained entirely within the σ1 gene is a second, smaller open reading frame encoding the non-structural protein σ1s (Ernst and Shatkin, 1985). The S1 genome segments of the avian reoviruses (ARVs) and the bat-origin reoviruses Nelson Bay virus (NBV), Pulau virus (PulV), and Melaka virus (MelV) are tricistronic and contain three sequential overlapping reading frames, the longest of which encodes the cell attachment protein σC in each virus (Chua et al., 2007; Day et al., 2007; Pritchard et al., 2006; Shmulevitz et al., 2002). The S1 genome segment of reptilian reovirus (RRV) also encodes a σC cell attachment protein but is bicistronic, with two overlapping reading frames (Duncan et al., 2004). Muscovy duck reovirus (ARV-Md) encodes its cell attachment protein uncharacteristically on the S4 genome segment, which is also bicistronic (Kuntz-Simon et al., 2002), and baboon reovirus (BRV) has a bicistronic S4 genome segment as well, which does not encode the putative BRV cell attachment protein (Dawe et al., 2002; Dawe and Duncan, 2002).

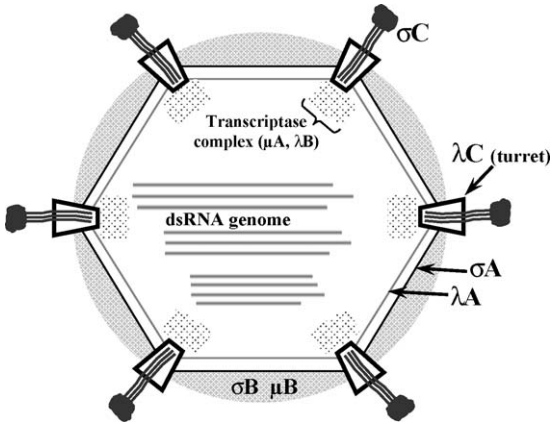

Fig. 1. Diagram of the orthoreovirus virion. The nomenclature of proteins is representative of the fusogenic orthoreoviruses. Diagram adapted from Fauquet et al. (2005) and Benavente and Martinez-Costas (2007).

Table 1
Orthoreovirus S-class genome segments, gene coding assignments.

Virus	S1	S2	S3	S4
ARV	p10 + p17 + σC	σA	σB	σNS
ARV-Md	σA	σB[a]	σB or σNS	p10 + σC
NBV	p10 + p17 + σC	σA	σNS	σB
RRV	p14 + σC	ND	σB	ND
BRV	σA	σB	σNS	p15 + p16
MRV	σ1 + σ1s	σ2	σNS	σ3

ND = not determined.
[a] Strain 89026 only.

The reovirus dsRNA cannot serve as a template for protein translation in the host cell—i.e., it cannot serve as an mRNA—therefore, the dsRNA viruses must carry the necessary enzymes (the transcriptase complex, see Fig. 1) within their virions in order to transcribe their sequestered genomes and deliver infectious mRNA directly into the cytoplasm. The elegant molecular machinery and protein superstructure necessary for the transcription of the tightly packaged genome are located within the viral core of the dsRNA viruses, and have been described in detail (Bamford, 2000, 2002; Diprose et al., 2001; Gouet et al., 1999; Grimes et al., 1998; Reinisch et al., 2000). In general, the dsRNA viral core, with its important role in genome packaging and mRNA transcription and maturation, contains proteins that are conserved—at least at the structural and/or functional level—even among distantly related members of the dsRNA virus group, including the *Reoviridae*. The proteins of the outer capsid layer, with their roles in environmental stability and cell attachment in numerous evolving hosts, are much more variable, even within members of the same genus (Bamford et al., 2002; Cheng et al., 2008; Grimes et al., 1998; Mertens, 2004; Reinisch et al., 2000), and can diverge at the nucleotide level to the point of randomness when compared to reoviruses from other serotypes, while still retaining an overall protein configuration that retains proper function (Duncan et al., 1990; Wiener and Joklik, 1989). The growth in the amount of available sequence data for the orthoreoviruses, particularly among the members of the fusogenic reovirus group, has prompted a recent taxonomic organization of the orthoreoviruses into five species groupings (I–V), these are (I) the prototypical mammalian orthoreoviruses, including Ndelle virus; (II) the avian orthoreoviruses; (III) Nelson Bay virus and related orthoreoviruses; (IV) baboon orthoreovirus; (V) the reptilian orthoreoviruses (Duncan et al., 2004; Fauquet et al., 2005). This nomenclature and general organization will be used in the present review.

3. The fusogenic and non-fusogenic orthoreoviruses: their diversity and evidence for their phylogenetic divide

The MRVs are a ubiquitous presence in mammals, including humans. Early serum surveys in the 1950s revealed neutralizing antibodies to orthoreovirus in humans, monkeys, rabbits, and guinea pigs, and more recent surveys suggest this is still the case in humans (Sabin, 1959; Selb and Weber, 1994). The MRVs are rarely associated with disease, but they are very well-understood at the biochemical and structural levels, and their pathogenesis in newborn mice serves as an excellent model system for studying the pathogenesis of the reoviruses in general (Guglielmi et al., 2006). As a group, the MRVs have been extensively studied at the antigenic and sequence level, and three recognized serotypes exist

within a clearly defined genus (Chapell et al., 1994; Duncan et al., 1990; Goral et al., 1996; Seliger et al., 1992; Wiener and Joklik, 1988, 1989). Serotypes 1, 2, and 3 were initially described and recognized based upon virus neutralization and hemagglutination-inhibition profiles (Schiff et al., 2007), and a putative fourth serotype, Ndelle virus, has been described at the antigenic and molecular levels (Attoui et al., 2001; El Mekki et al., 1981; Zeller et al., 1989). More recent sequence-based analyses support the placement of most of the MRVs into the three classically recognized serotypes, where type 1 Lang, type 2 Jones and type 3 Dearing are the prototypical human representatives from each serogroup (Guglielmi et al., 2006).

Duncan (1999), using an analysis of three of the reovirus S-class genome segments, closely examined the phylogenetic relationship that exists between the fusogenic and non-fusogenic reoviruses. This analysis revealed a clear divide between the non-fusogenic reoviruses and the fusogenic reoviruses based upon the primary amino acid sequences of the S-class major inner capsid protein, the S-class non-structural protein, and the S-class major outer capsid protein (Fig. 2). This division extended to a separation between the classical MRVs and the fusogenic reoviruses such as NBV and BRV. Further, clear divisions between the remaining members of the fusogenic reoviruses were revealed by Duncan (1999) and subsequent analyses, which will be discussed in detail in the following sections. Available sequence data for the fusogenic orthoreoviruses has increased substantially in the past decade, allowing a fresh look at the phylogeny of the group as a whole.

4. The avian orthoreoviruses

Avian reovirus (ARV) was first implicated as the etiologic agent of viral arthritis (synovitis) in broiler chickens in 1972 (Olson and Kerr, 1966; Walker et al., 1972). ARV had been known since 1954 as Fahey–Crawley virus, and it had initially been associated with chronic respiratory disease in chickens (Fahey and Crawley, 1954; Olson and Weiss, 1972). The ARVs have since been implicated in numerous avian diseases—especially in commercial poultry—including enteric disease syndromes, myocarditis, and hepatitis, and account for considerable economic loss for the poultry industry (Jones, 2000). The pathogenicity of isolated ARV strains differs considerably, and disease states attributed to the ARVs are often difficult to recreate experimentally (Clark et al., 1990; Pantin-Jackwood et al., 2007a; Rosenberger et al., 1989; Spackman et al., 2005b). Describing the etiology of the ARVs is complicated considerably due to the fact that many ARV infections in poultry do not cause clinical signs or recognizable disease (Jones, 2008; Pantin-Jackwood et al., 2007b, 2008). Early investigations of the ARVs revealed their fusogenic nature and their inability to agglutinate red blood cells, which distinguished them from the non-fusogenic MRVs (Deshmukh et al., 1969; Felluga et al., 1974; Kawamura et al., 1965).

The fusogenic nature of the ARVs is unusual for non-enveloped viruses, and is unique in that the formation of cellular syncytia is not related to viral cell entry or exit, as is the case in enveloped viruses (Duncan et al., 1996; Jahn et al., 2003; Martens and

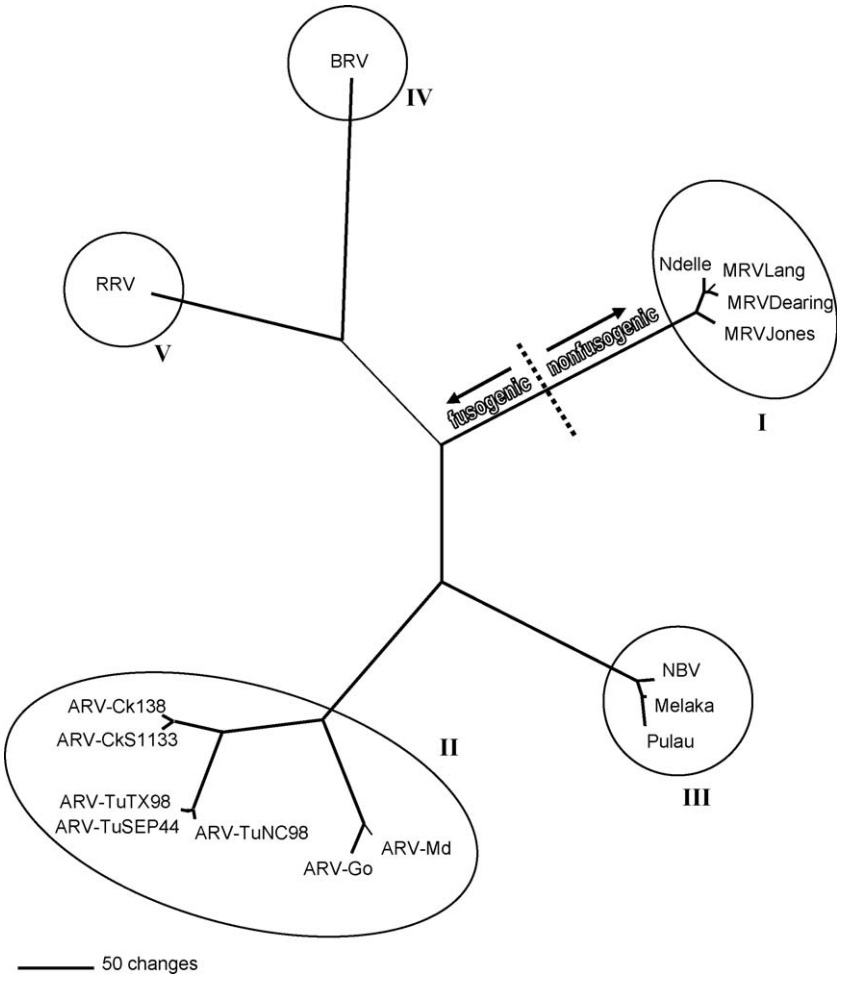

Fig. 2. Phylogentic tree based upon sequence alignment of the major outer capsid protein from selected fusogenic and non-fusogenic orthoreoviruses. Tree was generated with PAUP* (4.0b10) (Swofford, 2002) using maximum parsimony. Orthoreovirus species groups are indicated. Abbreviations as in text.

McMahon, 2008). In fact, syncytium formation by ARV is directly caused by the small (~10 kDa) p10 protein, a non-structural protein that is not part of the infectious viral capsid and that is only produced and inserted into the cell membrane following virus entry and replication (Shmulevitz and Duncan, 2000). The p10 protein is a member of the FAST protein family. The p10 open reading frame (ORF) is one of three partially overlapping ORFs located on the ARV S1 genome segment, along with the p17 and σC ORFs (Bodelon et al., 2001; Shmulevitz and Duncan, 2000). SigmaC is the ARV cell attachment protein, which exists as a homotrimer poised at the 12 icosahedral vertices of the intact virion, while p17 is a non-structural protein about which little is known, but which possesses a nuclear localization signal (NLS) and shuttles between the nucleus and the cytoplasm during an infection, and that may be associated with cell cycle arrest in infected cells (Costas et al., 2005; Grande et al., 2002; Liu et al., 2005; Martinez-Costas et al., 1997).

The σC protein induces the production of neutralizing antibodies during an infection, and can be markedly divergent at the amino acid level even among closely related strains (Day et al., 2007; Kant et al., 2003; Liu and Giambrone, 1997; Liu et al., 2003; Wickramasinghe et al., 1993). This marked divergence among circulating field strains has affected the efficacy of ARV vaccines routinely administered by the poultry industry to control viral arthritis, which highlights the importance of the use of molecular diagnostics to determine the prevalent ARVs circulating in the field and to inform subsequent vaccination strategy (Vasserman et al., 2004). SigmaC is a useful gene and protein to analyze during phylogenetic studies involving host range and vaccine efficiency due to its divergence and relatively rapid evolutionary rate compared to the other S-class genes. The ARV σC gene has a higher rate of nonsynonymous base substitutions than synonymous base substitutions, in contrast to the remaining S-class genes, where nucleotide changes do not tend to contribute to protein modifications (Liu et al., 2003). Liu et al. (2003) further identified six distinct phylogenetic lineages (I–VI) of ARV from the United States, Japan, Taiwan, and Australia based upon the σC gene sequence. Interestingly, the lineages generated from the other S-class genes (σA, σB and σNS; no more than three lineages in each case) did not necessarily correspond with the σC lineage assignments, reflecting the role of genome reassortment as a driving evolutionary force in the ARVs. Also in contrast to σC, yet consistent with the observations that the ARV/orthoreovirus core proteins are generally more conserved than the outer capsid constituents, the ARV L-class genes λA and λC and encoded proteins are very similar (mean amino acid identities of 95% and 98% for λC and λA, respectively), even among the three distinct phylogenetic lineages from 12 ARV isolates from different continents (Shen et al., 2007). The lineages described by Shen et al., 2007 based upon these L-class genes have evolved independent of any previously described serotype. The ARV λA and λC proteins share only 5–6% identity with the homologous proteins in MRV, and only 6–7% identity with homologues in Grass carp reovirus, an unusual aquareovirus described in more detail below. The ARV λA protein is a major core protein that serves as a scaffold during the early stages of viral morphogenesis, while λC is the ARV guanylyltransferase, which as a pentamer forms the ARV turrets and is involved in the enzymatic steps that place a 5′ cap on extruded viral mRNAs (Benavente and Martinez-Costas, 2007; Hsiao et al., 2002; Touris-Otero et al., 2004; Zhang et al., 2005).

A recent detailed look at the ARV RNA-dependent RNA polymerase (RdRp), encoded on the L2 genome segment, revealed an amino acid identity of about 55% when compared to the MRV RdRp. This is the highest identity value observed among any MRV and ARV proteins for which sequence is available, and suggests considerable evolutionary constraint on this core molecule (Xu and Coombs, 2008). This investigation included structure/function mapping of the ARV RdRp onto the previously determined MRV RdRp crystal structure (Tao et al., 2002), revealing that most conserved amino acids resided in the RdRp catalytic regions, while non-conserved amino acids were on the protein surface that interacts with the reovirus core.

Recent work in our laboratory with several field isolates of turkey-origin avian reovirus (ARV-Tu) from the United States has revealed that the ARV-Tu σC protein has diverged significantly from the σC of the classical chicken ARV vaccine strain ARV-S1133 (see Fig. 3). Among the seven ARV-Tu field isolates examined, six had σC amino acid identities ranging from 53 to 56% compared to ARV-S1133 σC, while a seventh isolate, ARV-Tu NC/SEP-R44/03, shared only 35.5% identity. In fact, ARV-Tu NC/SEP-R44/03 shared only 35 to 37% identity with the σC proteins from the other ARV-Tu isolates examined in the study (Day et al., 2007). The NC/SEP-R44/03 isolate actually grouped more closely with σC from Nelson Bay reovirus (NBV) during a phylogenetic analysis, an observation supported by a recent comparison of the L-class protein RNA-dependent RNA polymerase (RdRp) from African grey parrot and NBV (Wellehan et al., 2009) Interestingly, ARV-Tu NC/SEP-R44/03 was the most pathogenic strain compared to the other ARV-Tu isolates when examined in experimental turkeys, causing a marked atrophy of the bursa of Fabricius, an organ important for immune system development in birds (Day et al., 2008; Pantin-Jackwood et al., 2007a; Spackman et al., 2005b). It is not known if the ARV-Tu σC is specifically involved in tissue tropism or the severity of clinical signs in turkeys. In general, no correlation has been found between σC sequences and the type of disease a particular ARV may cause, but the homologous MRV protein σ1 does appear to affect tissue tropism and pathogenesis in mice (Haller et al., 1995; Kant et al., 2003; Kaye et al., 1986; Spriggs et al., 1983). An ARV of enteric origin has recently been described that causes central nervous system signs in chickens; it would be interesting to see if this ARV strain's unique tissue tropism is influenced by its σC protein (Van de Zande and Kuhn, 2007). Despite the sequence divergence between the ARV-Tu isolates and other members of the ARVs, the ARV-Tu σC protein retains important conserved amino acid residues in its carboxy- and amino-terminal portions. Specifically, the ARV-Tu σC contains an amino-terminal heptad repeat motif that is found in the cell attachment proteins of all reoviruses, both fusogenic and non-fusogenic. This heptad repeat contains apolar amino acids at the "a" and "d" positions of the motif, and is important for the proper incorporation of a stable σC homotrimer into the viral capsid (Bassel-Duby et al., 1985; Day et al., 2007; Duncan et al., 1990; Leone et al., 1991a,b; Shmulevitz et al., 2002). The carboxy-terminal portion of the ARV-Tu σC contains several aromatic amino acids that are conserved in the S1133 isolate plus the mammalian fusogenic orthoreovirus NBV (Day et al., 2007). The importance of these conserved residues is not known, although they are situated in the σC globular head, the portion of σC responsible for cell receptor binding (Guardado Calvo et al., 2005).

The other overlapping ORFs located on the ARV S1 genome segment, p10 and p17, and their respective proteins, have evolved independently of σC. Specifically, the p10 protein from the ARV-Tu NC/SEP-R44/03 shared 54.5% amino acid identity with ARV S1133 (contrast this with the σC identity of 35.5%) while the p10 identity for six additional ARV-Tu isolates ranged from 62 to 72% compared to the ARV-S1133 p10. The amino acid identities of the ARV-Tu p17 proteins, when compared to the ARV-S1133 p17, are similar to the identities noted with the σC protein. It is the 3' end of the p17 ORF that overlaps the 5' portion of the σC ORF by approximately 100 bases in the ARV-Tu S1 genome segment. The ARV p10 and p17 remain more closely related when isolates that infect the same species are considered, even if the isolates are separated geogra-

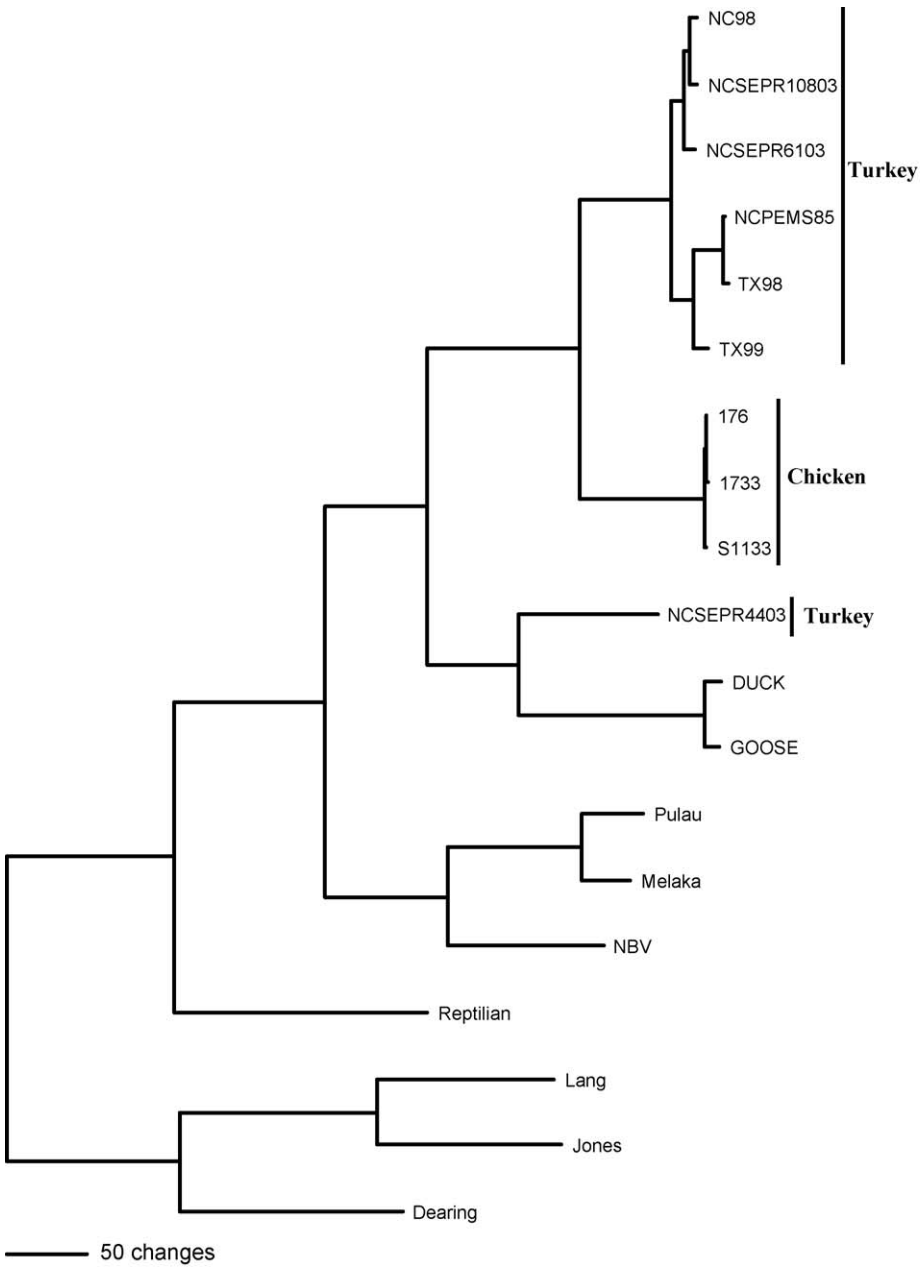

Fig. 3. Phylogenetic tree based upon sequence alignment of the cell attachment protein from selected fusogenic and non-fusogenic orthoreoviruses. Tree was generated with PAUP* (4.0b10) using maximum parsimony with midpoint rooting.

phically, and as is the case with σC, there is no correlation between p10 and p17 sequence and disease state in birds (Hsu et al., 2005). However, a study involving reassortant ARVs did implicate the S1 genome segment—specifically its ability to transfer the syncytium-inducing phenotype—in the severity of ARV pathogenesis in embryonating chicken eggs (Duncan and Sullivan, 1998).

The differences between the ARV-Tu isolates and other members of the ARVs (i.e. chicken-origin ARVs) extend to other genome segments and genes as well. The σB protein of ARV-Tu, the major outer capsid structural protein, has also diverged from the chicken σB protein, although not to the extent that σC has. Interestingly, ARV-Tu NC/SEP-R44/03 σB is very similar to the σB from other ARV-Tu isolates (Fig. 2). The σB and σC/S1 data taken together lend support to the recognition of ARV-Tu as a separate species within species group II of the *Orthoreoviruses* (Kapczynski et al., 2002; Sellers et al., 2004). This suggestion is further supported by pathogenesis studies that determined the ARV-Tu isolates cannot produce disease signs in chickens, although they may replicate at low levels in the chicken gut. Even more striking is the fact that a severely pathogenic strain of chicken-origin ARV, ARV-1733, which causes mortality in chickens, produces no disease signs in commercial turkeys and only mild clinical signs in specific pathogen-free (SPF) turkeys (Spackman et al., 2005b). Indeed, chicken- and turkey-origin ARVs group separately in a phylogenetic analysis of diagnostic data collected during an enteric virus survey of commercial poultry in the United States (Fig. 4). The diagnostic test targets the σNS gene of genome segment S4, and takes advantage of the nucleotide conservation observed in this gene among circulating chicken- and turkey-origin ARV strains. Earlier diagnostic tests were based on more divergent genes that code for the outer capsid protein σB and the σC cell attachment protein (Pantin-Jackwood et al., 2008; Spackman et al., 2005a).

Recent studies focusing on the ARV M-class proteins has revealed, interestingly, that the ARV protein μB and the MRV

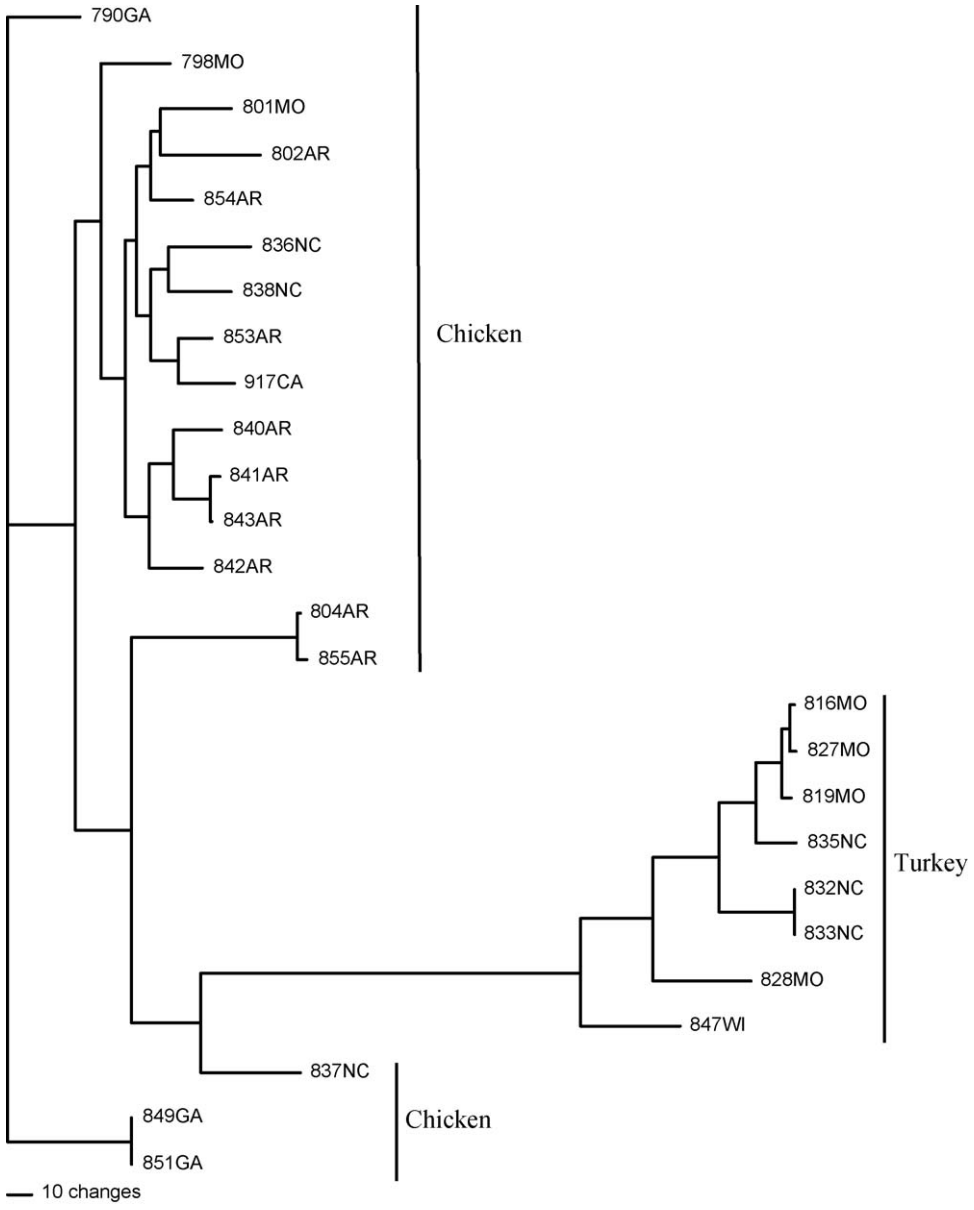

Fig. 4. Phylogenetic tree based upon nucleotide alignment of cDNA generated from the σNS (S4 genome segment) of Turkey- and chicken-origin avian reoviruses collected in a nationwide survey of enteric viruses in poultry in the United States. Tree was generated with PAUP* (4.0b10) using maximum parsimony. Chicken and turkey origin isolates are indicated, and the virus labels contain a laboratory-generated number followed by the state abbreviation.

protein μ1—the homologous M-class major outer capsid proteins—were surprisingly similar, with a 45% amino acid identity and conservation of functional regions. The other two proteins of the ARV M-class, μA (a putative RdRp cofactor) and μNS (which may play a role in virion assembly or transcription), showed only 25–30% identity to their MRV counterparts, similar to the identities observed in the S-class proteins (Noad et al., 2006). For all of the M-class genes analyzed, phylogenetic analysis revealed the expected groupings based upon the recognized orthoreovirus species groups, with the ARVs and MRVs segregating into distinct groups in each case, and with aquareovirus isolates forming distinct groups for each M-class gene (Noad et al., 2006). Of the M-class proteins, μB displayed the highest degree of sequence divergence among 12 ARVs investigated, which reflects its position in the virion outer capsid. As is the case with other investigated gene classes from the ARVs, the M-class proteins, which group phylogenetically into multiple co-circulating lineages, do not correlate with described serotypes or pathotypes noted in the field (Su et al., 2006).

5. The special cases of the goose and the Muscovy duck

Like the other ARVs, Muscovy duck reovirus (ARV-Md) causes syncytium formation in cell culture and does not hemagglutinate red blood cells. Goose reovirus (ARV-Go) causes a disease in young goslings that is similar to the disease state attributed to ARV-Md, but ARV-Go fails to produce multinucleated syncytia in cell culture (Malkinson et al., 1981; Palya et al., 2003). ARV-Go and ARV-Md possess electropherotypes that are markedly different than the prototypical chicken ARV S1133, particularly in the case of the S1 genome segment (Kuntz-Simon et al., 2002; Palya et al., 2003). As the genomes of different ARV-Md strains are analyzed, an unpredictable genomic organization is being revealed. The S2 genome segment of ARV-Md strain 89026 encodes the major outer capsid protein σB and contains the same 5′ and 3′ untranslated terminal nucleotide motifs as described for chicken ARVs (5′-GCTTTTT···TATTCATC-3′). At the nucleotide level, the ARV-Md σB gene has nucleotide identities ranging from 62.1 to 64.1% when

compared to the well-studied chicken ARVs; amino acid identities are similar for the σB protein (approximately 61%) (Kuntz-Simon et al., 2002; Le Gall-Recule et al., 1999; Zhang et al., 2007b). In ARV-Md strain 89330, the S3 genome segment encodes the σB gene, while the S3 genome segment of strain 89026 encodes the σNS gene, reflecting an interesting shift in electrophoretic mobilities of genome segments between these two related strains. Further genomic analysis has revealed that the polycistronic genome segment of both ARV-Md and ARV-Go is the S4 genome segment, which encodes the overlapping ORFs for p10 and σC (Banyai et al., 2005). The ~10.8 kDa ARV-Md p10 has no homology to other described proteins (including ARV p10), while the ARV-Md σC only shares about 25% amino acid identity with the prototypical chicken-origin ARVs and encodes a truncated cell attachment protein (Kuntz-Simon et al., 2002; Zhang et al., 2006a). Phylogenetic analysis further reveals that, based upon a comparison of outer capsid proteins, the ARV-Md is distinct from all other described reovirus species groups, but is still contained within the orthoreovirus species group II along with the other ARVs (Fauquet et al., 2005; Kuntz-Simon et al., 2002; Zhang et al., 2006a). ARV-Go is also a member of species group II, and initial analysis of the σA gene and encoded protein shows some geographical differences in amino acid identities in isolates from China and Europe (Zhang et al., 2006b). The ARV-Md M-class genes and encoded proteins retain the familiar gene organization of the ARVs, with genome segments M1, M2, and M3 coding for the μA, μB and μNS proteins, respectively. The M-class proteins of ARV-Md are more similar to their ARV counterparts, with amino acid identities of 85.3–86.2% for μA, 75–76.5% for μB, and 78.4–79.8% for μNS, supporting their inclusion as a separate ARV species within orthoreovirus species group II (Zhang et al., 2007a).

6. Mammalian fusogenic orthoreoviruses

In 1970 a virus with typical reovirus morphology was isolated from the heart blood of a grey-headed flying fox (*Pteropus poliocephalus*) (a fruit bat) in the Nelson Bay area of New South Wales, Australia. This putative reovirus did not behave similarly to previously described reoviruses of mammalian origin, in that it produced multinucleated syncytia in mammalian cell culture, nor did it kill chick embryos when inoculated onto the chorioallantoic membrane (CAM) of embryonated chicken eggs. It did, however, produce pocks on the CAM (Gard and Compans, 1970; Gard and Marshall, 1973). This behavior in cell culture and in eggs suggested a virus with characteristics intermediate between ARV and MRV. It was further noted that the cell fusion induced by Nelson Bay virus (NBV) was distinct from the glycoprotein-mediated cell fusion caused by certain mammalian enveloped viruses, and that the inability to prevent syncytium formation in cell culture using anti-NBV antibodies suggested the membrane fusion was not due to the direct action of a cell surface protein (Wilcox and Compans, 1982). NBV was subsequently shown to have a tricistronic S1 genome segment encoding the genes p10, p17 and σC in a sequential, overlapping manner (Shmulevitz and Duncan, 2000). This genome organization is essentially identical to that observed in the avian reoviruses. The NBV and ARV p10 proteins are quite divergent, with 33% amino acid identity, while the NBV and ARV p17 proteins share 29% amino acid identity. This is similar to the amino acid identities observed between the NBV and ARV σC proteins, which range from 24 to 27% (see Fig. 3 for phylogeny). Despite this degree of divergence, the NBV and ARV σC proteins do share conserved N-terminal amino acid residues which may be important for the formation of σC trimers *in vivo*, and conserved C-terminal aromatic residues located in the σC globular head region (Day et al., 2007; Shmulevitz et al., 2002). Further, NBV and ARV share conserved amino acids within the transmembrane domain of p10 and within the putative nuclear localization signal (NLS) of p17 (Day et al., 2007). NBV has only been isolated from the field a single time, so little is known about pathogenesis in its natural host or about its possible host range.

A similar fusogenic reovirus has recently been isolated from the urine of another fruit bat species (*P. hypomelanus*) in Malaysia (Chua, 2003; Pritchard et al., 2006). This reovirus, named Pulau virus (PulV), has an electropherotype that differs in significant ways from NBV, particularly in the S-class genome segments. Sequence analysis of the S-class genome segments of PulV and comparison to NBV revealed nucleotide identities that ranged from 60% for the S1 genome segment to 88% for the S3 genome segment (see Figs. 2 and 3 for phylogenies). As in ARV and NBV, the S1 genome segment is tricistronic and encodes the p10, p17 and σC genes. At the amino acid level, the p17 and σC proteins (Fig. 2) are quite divergent from the NBV proteins, with 53 and 43% identity respectively. In contrast, the PulV p10 protein retained 100% identity with the NBV p10, suggesting identical mechanisms of cell–cell fusion. The PulV and NBV σA and σNS proteins were very similar, with 97% amino acid identity. The amino acid identity of the NBV and PulV major outer capsid protein σB (Fig. 3) was 89%, intermediate between the minor outer capsid protein σC and the more conserved core protein σA. PulV and NBV are also related on the antigenic level, with PulV being neutralized by anti-NBV serum and vice versa (Pritchard et al., 2006).

Another fusogenic orthoreovirus that appears to be of bat origin was recently isolated in Malaysia from a throat swab taken from a human male patient with acute respiratory disease. This virus, called Melaka virus (MelV), caused syncytium formation in numerous mammalian cell lines and did not react to antiserum raised against known respiratory viruses, including influenza A and B (Chua et al., 2007). MelV was subsequently found to contain a segmented dsRNA genome, a tricistronic S1 genome segment encoding p10, p17 and σC in the same manner as NBV and PulV, and produced an electropherotype essentially identical to that of PulV. Phylogenetic analysis of the major outer and inner capsid proteins showed a close relationship to PulV in each case (see Fig. 2), placing MelV along with PulV and NBV in species group III of orthoreovirus. An epidemiological follow-up of the human index case revealed that the patient had been exposed to a bat in his home about a week before the onset of symptoms, but no direct contact or direct bat-to-human transmission was confirmed. This interesting case is the first report of a fusogenic orthoreovirus implicated in human disease, and raises the intriguing possibility that cell–cell fusion may be necessary for pathology to develop during human reovirus infection (Chua et al., 2007), since the non-fusogenic MRVs are common in human populations, but do not generally cause disease symptoms. Interestingly, serological evidence suggests that a small percentage of the human population on Timoan Island, Malaysia—where MelV was discovered—have been infected with both MelV and PulV (Chua et al., 2007).

In 1995 an orthoreovirus was described that was isolated from the brain homogenates of a baboon that had succumbed to meningoencephalomyelitis. Analysis of the electropherotype of this baboon reovirus (BRV) revealed a dsRNA genome that was distinct from all other fusogenic orthoreoviruses that had been described at the time, including those of mammalian origin (Duncan et al., 1995). BRV also caused syncytium formation in Vero cells and appeared to have diverged extensively from other fusogenic orthoreoviruses based upon homologous and heterologous immune precipitations of viral proteins. The animal from which the BRV was isolated was part of a baboon colony at a biomedical research facility in Texas, and although other members of the colony possessed antibodies to BRV, a natural reservoir for BRV has not been determined (Duncan et al., 1995). Subsequent sequence analysis revealed that the organization of the BRV S-class

genome segments differed in significant ways from the organization of the S-class genome segments of NBV, PulV and MelV. The BRV S1 genome segment does not encode a cell attachment protein, σC, as is the case in the ARVs and in NBV and closely related fusogenic mammalian viruses. The BRV S1 segment instead encodes the major core protein σA, which shares only about 30 to 32% amino acid identity with the ARV and NBV σA proteins (Duncan, 1999). Further, the BRV S4 genome segment is bicistronic, and neither of the small proteins it encodes, p15 and p16, share any homology with other known viral or cellular proteins. However, the p15 protein has been definitively identified as the BRV cell fusion protein, and it can induce syncytium formation on its own, like the p10 protein of ARV and NBV; this establishes the BRV p15 as a novel member of the FAST protein family, although it differs markedly from ARV/NBV p10 (Dawe et al., 2005; Dawe and Duncan, 2002). An analysis of the BRV S-class genome segments revealed another interesting fact about BRV: it does not encode a putative cell attachment protein on any of its S-class genome segments, which differentiates it from all other orthoreoviruses, both fusogenic and non-fusogenic. In fact, BRV may have evolved a cell entry mechanism that does not involve a σC/σ1 homolog or similar pathway, since convalescent sera from baboons infected with BRV do not contain neutralizing antibodies to BRV (Leland et al., 2000). Neither a function nor a homolog has been ascribed to BRV p16, although its possible role in cell attachment has been ruled out (Dawe et al., 2002).

7. Reptilian orthoreoviruses

In 1987 a virus was isolated from a moribund python (*Python regius*) with hemorrhagic kidney lesions. The virus was subsequently found to contain a segmented dsRNA genome and had characteristic reovirus morphology when examined using electron microscopy. Further, the putative reptilian reovirus (RRV) caused extensive syncytium formation in cell culture, similar to what had been reported for the ARVs at the time, yet did not hemagglutinate human erythrocytes, a trait typical of the non-fusogenic MRVs (Ahne et al., 1987). Since this time, RRVs have been described in snakes, lizards and iguanas, where they have been mainly implicated in respiratory or neurological disease (Drury et al., 2002; Lamirande et al., 1999; Vieler et al., 1994). Surveys of reptiles in the wild suggest that infections with RRV may be common and widespread, but rarely associated with a recognized disease state (Gravendyck et al., 1998; Marschang et al., 2002).

Recent genomic analysis has revealed that RRV contains a bicistronic S1 genome segment with overlapping ORFs that encode σC and an RRV-specific protein of about 14 kDa called p14 (Duncan et al., 2004). The p14 protein contains a transmembrane motif and has been identified as the RRV cell fusion protein and a member of the FAST protein family, although it has no significant sequence identity to the other members of the FAST family (Corcoran and Duncan, 2004). The RRV major outer capsid protein σB shares only 21–25% amino acid identity with the ARVs, NBV and BRV (see Fig. 2 for phylogeny). The divergence noted in σB, coupled with the unique RRV genome organization and observed host range, provided ample evidence that the RRVs constitute a new orthoreovirus species, and they have been placed in the new orthoreovirus species group V (Duncan et al., 2004; Fauquet et al., 2005). Recent analysis using degenerate primers and nested PCR targeting the L-class RNA-dependent RNA polymerase (RdRp) gene of several RRVs isolated from snakes and a tortoise revealed three distinct phylogenetic clusters within the orthoreovirus species group V. The nested PCR technique used to examine the orthoreovirus RdRP may provide a more robust tool for reovirus detection and phylogenetic analysis, since the RdRp does not diverge as quickly as the proteins of the outer capsid, particularly σC (Wellehan et al., 2009). The fact that this particular study was able to amplify and compare orthoreoviruses infecting three classes of vertebrate species (reptiles, birds and mammals) supports the focus on the viral RdRp for extended phylogenies of distantly related hosts.

8. The evolutionary past of the aquareoviruses: a special case

Beginning in the late 1970s, reovirus-like particles were isolated from moribund fish such as golden shiner (*Notemigonus crysoleucas*) and chum salmon (*Oncorhynchus keta*) and from shellfish such as the Easter oyster (*Crassostrea virginica*) (Winton et al., 1987). These aquareoviruses shared distinct morphological similarities with the MRVs, and have since been found in numerous fish and shellfish species, and several reovirus-like particles have been isolated from crustaceans (Lupiani et al., 1995; Winton et al., 1987).

The aquareoviruses, although not a member of the *Orthoreovirus* genus, are an interesting group to consider in any evolutionary analysis of the fusogenic orthoreoviruses. They are a distinct genus within the *Reoviridae*, comprising six different species (*Aquareovirus* A–F, with a proposed species group G), and differ from the orthoreoviruses in that they posses a dsRNA genome containing 11 segments (Fauquet et al., 2005; Winton et al., 1987). The aquareoviruses are generally syncytium forming in cell culture, although the mechanism of cell–cell fusion has not been elucidated as in the fusogenic orthoreoviruses. The aquareoviruses encode seven structural and five nonstructural proteins, two of which are encoded by the smallest genome segment, Seg-11 (Subramanian et al., 1994).

Analysis of the aquareovirus polymerase gene revealed an unexpected degree of amino acid identity (about 42%) with the orthoreovirus (MRV) polymerase. This degree of identity is usually only found among members of the same *Reoviridae* genus. In fact, unusual homology between several other genes has been noted between MRV and aquareovirus, indicating a common evolutionary origin for the two genera, one which infects fish and shellfish, and the other which infects reptiles, birds, and mammals (sometimes humans) (Attoui et al., 2002). A subsequent detailed look at the proteins that make up the viral core in the aquareoviruses and the orthoreoviruses revealed that the enzymatic structure and function of the mRNA synthesis machinery is also homologous between the two genera. The analysis further revealed—through mapping conserved aquareovirus residues onto the available orthoreovirus protein structures—that residues involved in protein–protein interactions had evolved more quickly than the residues found at enzymatic surfaces as the two groups diverged from their common ancestor (Kim et al., 2004). Just as functional constraints have caused the sequence and structure of the reovirus viral core proteins to remain more conserved than the sequence and structure of the outer capsid proteins, so too are the enzyme residues of the reovirus core proteins conserved to a greater extent than the residues at protein–protein interfaces. Further, recent genomic analysis of the American grass carp reovirus (AGCRV)—an unusual aquareovirus that does not induce syncytium formation in cell culture—has revealed an evolutionary link between the aquareoviruses and the coltiviruses, members of the *Reoviridae* whose prototypical member is Colorado tick fever virus (CTFV) and that have a genome consisting of 12 dsRNA segments. Specifically, homologs of the two proteins encoded by bicistronic Seg-7 of AGCRV are found on two separate genome segments in CTFV (Mohd Jaafar et al., 2008). Interestingly, the coltiviruses are a member of the nonturreted group within the *Reoviridae*, while the orthoreoviruses and aquareoviruses are in the turreted group (Schiff et al., 2007). Examining the interesting evolutionary past of

the aquareoviruses provides insight into the fundamental mechanisms involved in the evolution of the segmented, dsRNA viruses in general, and could lead to an understanding of the complex evolutionary connections of the orthoreoviruses in particular (see Table 1).

9. Conclusions and considerations

The diversity of the fusogenic orthoreoviruses along with more closely related *Reoviridae* genera such as *Aquareovirus* and *Colitivirus* is truly impressive, with a host range encompassing multiple vertebrates and invertebrates. The increase in the amount of reovirus sequence data, particularly for the members of the fusogenic orthoreoviruses, has allowed a more detailed look at the often complex phylogenetic relationships among the members of the this group and with the members of the fusogenic orthoreoviruses.

The divergence observed among coding sequences and proteins in the S-class genome segments is often remarkable, with nucleotide sequences evolving to the point of apparent randomness. This has been particularly true of the orthoreovirus cell attachment proteins and the members of the FAST protein family. Despite this divergence, the evolution of the orthoreovirus cell attachment proteins is checked by structural constraints that ensure the retention of residues crucial for the formation of a stable homotrimer and for cell surface recognition and binding. The FAST proteins, even though they are a diverse group with little to no sequence homology between the described p10, p14, and p15 proteins, each have retained structural motifs that are essential to their predicted membrane topology and therefore their syncytium producing abilities. Each has an N-terminal (amino terminal) external domain or "ectodomain", that is outside of the cell surface, one or two transmembrane domains, and a polybasic domain (Dawe et al., 2005) (Corcoran and Duncan, 2004). Recent functional studies of the diminutive FAST proteins revealed that they have evolved the ability to induce syncytium formation specifically through the recruitment of surrogate cellular factors to induce cell–cell fusion of multiple cell types (Salsman et al., 2008). As the available sequence data for the orthoreovirus L-class and M-class gene segments and proteins continues to grow—and as fully sequenced fusogenic orthoreovirus genomes become available—we can perhaps begin to correlate certain genotypes with disease signs or patterns of pathogenesis in the field, something that has generally eluded orthoreovirus investigators.

As new members of the *Orthoreovirus* genus are discovered and characterized, it is likely that the accumulated data regarding the organization of sequenced genomes will continue to increase in complexity (see Table 1). The elucidation of the taxonomic relationships between the genus *Orthoreovirus* and the *Aquareovirus* and *Coltivirus* genera serves as a lesson in the mechanisms involved in the evolution of the reoviral dsRNA genome. Understanding the evolutionary relationships among the members of the orthoreoviruses is of particular importance as viruses that infect higher mammals such as BRV and viruses with potential zoonotic importance such as MelV are discovered and characterized.

10. Accession numbers

Major outer capsid proteins: MRV-Lang, AAA47272; MRV-Dearing, AAA47283; MRV-Jones, CAA42670; MRV-Ndelle, AAL36031; NBV, AAC18127; MelV, ABM67660; PulV, AAR13236; BRV, AAC18128; RRV, AAP03133; ARV-Md, CAC44893; ARV-Go, AAM55474; ARV-Tu TX/98, AAR27797; ARV-Tu NC/98, AAM10637; ARV-S1133, AAA67065; ARV-138, AAC18126; ARV-Tu NC/SEP-R44/03, FJ211385. Cell attachment proteins: ARV-Tu NC/98, ABL96275; ARV-Tu NC/SEP-R108/03, ABM89085; ARV-Tu NC/SEP-R61/03, ABM89086; ARV-Tu NC/PEMS/85, ABM89084; ARV-Tu TX/98, ABM89082; ARV-Tu TX/99, ABM89083; ARV-176, AAF45153; ARV-1733, AAB61607; ARV-S1133, AAK18188; ARV-Tu NC/SEP-R44/03, ABG24271; ARV-Md, CAC83879; ARV-Go, CAG30722; PulV, AAR13233; MelV, ABM67657; NBV, AAF45159; RRV, AAP03135; MRV-Lang, AAA66877; MRV-Jones, AAA66879; MRV-Dearing, AAA47275. SigmaNS cDNA: EU400274 to EU400299.

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