Sequences of avian reovirus M1, M2 and M3 genes and predicted structure/function of the encoded μ proteins

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Abstract

We report the first sequence analysis of the entire complement of M-class genome segments of an avian reovirus (ARV). We analyzed the M1, M2 and M3 genome segment sequences, and sequences of the corresponding μ A, μ B and μ NS proteins, of two virus strains, ARV138 and ARV176. The ARV M1 genes were 2283 nucleotides in length and predicted to encode μ A proteins of 732 residues. Alignment of the homologous mammalian reovirus (MRV) μ 2 and ARV μ A proteins revealed a relatively low overall amino acid identity (~30%), although several highly conserved regions were identified that may contribute to conserved structural and/or functional properties of this minor core protein (i.e. the MRV μ 2 protein is an NTPase and a putative RNA-dependent RNA polymerase cofactor). The ARV M2 genes were 2158 nucleotides in length, encoding predicted μ B major outer capsid proteins of 676 amino acids, more than 30 amino acids shorter than the homologous MRV μ 1 proteins. In spite of the difference in size, the ARV/MRV μ B/ μ 1 proteins were more conserved than any of the homologous proteins encoded by other M- or S-class genome segments, exhibiting percent amino acid identities of ~45%. The conserved regions included the residues involved in the maturation- and entry- specific proteolytic cleavages that occur in the MRV μ 1 protein. Notably missing was a region recently implicated in MRV μ 1 stabilization and in forming “hub and spokes” complexes in the MRV outer capsid. The ARV M3 genes were 1996 nucleotides in length and predicted to encode a μ NS non-structural protein of 635 amino acids, significantly shorter than the homologous MRV μ NS protein, which is attributed to several substantial deletions in the aligned ARV μ NS proteins. Alignments of the ARV and MRV μ NS proteins revealed a low overall amino acid identity (~25%), although several regions were relatively conserved.

Keywords

RNA viruses; RNA sequences; Avian reovirus; Protein structure/function

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1. Introduction

Avian orthoreovirus (ARV) is one of five recognized species in the genus *Orthoreovirus*, family *Reoviridae*. ARV infects a wide variety of avian species worldwide (Jordan and Pattison, 1996) and is responsible for significant economic loss in the commercial poultry industry (Herenda and Franco, 1996). Infected birds tend to develop tenosynovitis, leading to diminished weight gain and poor feed conversion rates. Despite the agricultural and economic importance of ARV, little is known regarding its genetic and biological functioning in the host (Barnes et al., 1997).

ARV possess characteristic orthoreovirus features, including a genome of ten dsRNA segments surrounded by a nonenveloped, icosahedral double capsid of 70–80 nm in diameter. The genome segments are classified into three groups based on their electrophoretic mobilities: three large segments (L1, L2, L3), three medium segments (M1, M2, M3) and four small segments (S1, S2, S3, S4) which code for the proteins λ A, λ B, λ C, μ A, μ B, μ NS, σ C, σ A, σ B and σ NS, respectively (Varela and Benavente, 1994). The ARV S1 gene also encodes proteins p10 and p17. Although ARVs share many features with the prototypic mammalian reoviruses (MRV), several notable differences exist including host range, pathogenicity, hemagglutination properties, and syncytium formation (Ni and Ramig, 1993; Theophilos et al., 1995; Martinez-Costas et al., 1997a; Jones, 2000; van der, 2000). Genomic coding differences also exist between MRV and ARV. For example, although the ARV and MRV S1 genome segments encode homologous receptor-binding proteins (Weiner et al., 1980; Martinez-Costas et al., 1997b; Grande et al., 2002), the ARV S1 genome segment encodes two additional ARV-specific gene products, one of which is responsible for ARV's unusual cell–cell fusion ability (Shmulevitz and Duncan, 2000; Bodelon et al., 2001; Shmulevitz et al., 2002). Certain differences in the functional properties of homologous ARV and MRV proteins have also been reported. For example, two non-homologous dsRNA-binding proteins (the ARV σ A core protein and the MRV σ 3 major outer capsid protein) are predicted to regulate PKR activation (Schiff, 1998; Gonzalez-Lopez et al., 2003) while the ARV σ A core protein displays nucleoside triphosphate phosphohydrolase (NTPase) activity (Yin et al., 2002) ascribed to the non-homologous MRV μ 2 (Noble and Nibert, 1997) and λ 1 (Bisaillon et al., 1997) core proteins. Based on these early comparative studies, it seems likely that additional analysis of ARV will continue to broaden our understanding of the *Reoviridae* family, possibly leading to the identification of novel features that impact on the distinct biological and pathogenic properties of ARV.

The complete genomic sequences of three prototype strains of MRV have recently been completed (Breun et al., 2001; Yin et al., 2004). In contrast, sequence information from several ARV isolates is limited and only available for the S-class genome segments (Chiu and Lee, 1997; Duncan, 1999; Liu and Huang, 2001; Kapczynski et al., 2002; Sellers et al., 2004). The only other ARV sequence information available is for a single ARV L-class genome segment from one virus strain (Hsiao et al., 2002). In addition, while this manuscript was in preparation two M3 genome segment sequences were reported (Touris-Otero et al., 2004a), and a variety of M and L sequences were submitted to GenBank. In order to expand the available ARV sequence database, we determined the genomic sequences of the entire complement of M-class genome segments from two different strains

of ARV. These comparative sequence studies, in combination with ongoing genomic and proteomic studies, are intended to better delineate the role(s) of individual ARV proteins in the viral replication cycle.

2. Materials and methods

2.1. Cells and viruses

Avian reovirus strain 138 (ARV138) and strain 176 (ARV176) are laboratory stocks. Viral clones were amplified to second passage in continuous QM5 quail cells. Cells were grown as monolayers in 1× Medium 199 containing 10% fetal calf serum (Gibco BRL), 10% tryptose phosphate broth (2% tryptone, 0.2% dextrose, 8.6 mM NaCl, 1.8 mM Na₂HPO₄), 0.05% NaHCO₃, 2 mM l-glutamine, 100U penicillin/ml, 100 µg streptomycin sulfate/ml and 1 µg amphotericin-B/ml in the presence of 5% CO₂ at 37 °C (Patrick et al., 2001).

2.2. Sequencing the M1, M2, and M3 genes

Genomic dsRNA was extracted from virus P2 stock with phenol/chloroform (Sambrook et al., 1989). The identity of each strain was confirmed by resolving aliquots of each virus in 10% SDS-PAGE and comparing band mobilities (Duncan, 1999). Oligonucleotide primers corresponding to the 5′ forward sense end and 5′ reverse sense end of the ARV138 genes, and additional oligonucleotides for sequencing, were designed as needed and obtained from Invitrogen. cDNA copies of the ARV M1, M2 and M3 genes of each virus were constructed by using the 5′ oligonucleotide primers and reverse transcriptase (Roche). The cDNAs were amplified by polymerase chain reaction (Saiki et al., 1985), resolved in 1% agarose gels (Sambrook et al., 1989), and the bands corresponding to the gene were excised, purified and eluted with Qiagen® columns, following the manufacturer's instructions (Qiagen Inc., Mississauga, Ontario). Sequences of the respective cDNAs were determined in both directions by dideoxynucleotide cycle sequencing (Sanger et al., 1977; Murray, 1989; Craxton, 1991), using fluorescent dideoxynucleotides.

The sequences at the ends of all genes were determined in both directions by a modification of the 3′ ligation method described by Lambden et al., 1992. Viral genes from phenol–chloroform extracted RNA were resolved in a 1% agarose gel and the M genes were excised and eluted as described above with Qiagen® columns, using the manufacturer's instructions. Oligonucleotide 3′L1 (5′-CCCCAACCCACTTTTCCATTACGCCCTTTCCCCC-3′ phosphorylated at the 5′ end and blocked with a biotin group at the 3′ end) was ligated to the 3′ ends of the M genes, according to the manufacturer's directions (Boehringer Mannheim), at 37 °C overnight. The ligated genes were then repurified by agarose gel and Qiagen® columns to remove unincorporated 3′L1 oligonucleotide and precipitated overnight with ice-cold ethanol. The precipitated genes were dissolved in 6 µl of 90% DMSO. cDNA copies of the ligated M genes were constructed by using oligonucleotide 3′L2 (5′-GGGGGAAAGGGGCGTAATGGAAAAAGTGGGTGGGG-3′) and gene-specific internal oligonucleotide primers designed to generate a product of approximately 0.5 kb in length. These constructs were amplified by PCR, purified in 1% agarose gels, excised, and eluted as described above. Sequences of these cDNAs were determined with

gene-specific internal oligonucleotides and with oligonucleotide 3' L3 (5' - GGGGGAAAGGGGCGTAAT-3') by dideoxy-fluorescence methods.

2.3. Sequence analysis

Each gene was sequenced in both directions and initially analyzed with the DNASTAR® suite (Madison, WI). Nucleotide and amino acid sequences were then analyzed with ClustalW 1.82 (European Bioinformatics Institute, United Kingdom), ExPasy (Geneva, Switzerland), Network Protein Service (Lyon, France), PredictProtein (Heidelberg, Germany), GeneBee (Moscow, Russia), Lalign (EMBLnet, Switzerland), T-Coffee (Swiss Institute for Bioinformatics), Multi-Coil (Massachusetts Institute of Technology), and BLAST (Bethesda, MD). DNA alignments were also analyzed by DNAPARS (with Jumble option) to derive phylogenetic relationships, and visualized with Treeview, and protein alignments were visualized with BOXshade v3.2 (Lausanne, Switzerland).

2.4. 3-D structural analysis

The $\sigma 3$ $\mu 1$ heterohexameric X-ray crystallographic structure (Accession number PDB ID 1JMU), originally determined by Liemann et al., 2002 was manipulated and modified with the graphics visualization computer program PyMol (DeLano, 2004).

3. Results and discussion

3.1. M1 gene and μA protein sequences of ARV138 and ARV176

The nucleotide sequences of the M1 genome segments of avian reovirus ARV138 (Genbank Number AY557188) and ARV176 (Genbank Number AY557189) were determined. Both ARV M1 genes were 2283 base pairs long. This is shorter than the M1 segments of the prototype members of the reovirus family, the MRV reoviruses T1L, T2J and T3D that have 2304 nucleotides, 2303 nucleotides and 2304 nucleotides, respectively (Wiener et al., 1989; Zou and Brown, 1992; Yin et al., 2004). Both ARV M1 genes contain a 5' non-translated region of 12 bases, a 3' non-translated region of 72 bases, and encode a μA protein of 732 amino acids, only 4 amino acids shorter than the homologous MRV $\mu 2$ protein (Fig. 1). Alignments of the ARV138 and ARV176 5' and 3' non-translated regions reveal complete sequence identity between them, including the previously reported tetranucleotide GCUUU at the 5' end and the UCAUC tetranucleotide at the 3' end (Duncan, 1999) (data not shown). The ARV138 M1 gene shares 91.8% identity (188 mismatches) with the ARV176 M1 gene (Table 1).

While this manuscript was in preparation, M1, M2, and M3 sequences from a number of ARV vaccine and field isolate strains were deposited in GenBank. We took this opportunity to compare our ARV138 and ARV176 sequences to the other new ARV M sequences, and to sequences representing homologous genes from various mammalian and aquareoviruses. For these representative comparisons we chose two to four clones from each group. Preliminary comparisons of the approximately 10 other new ARV clones' M sequences showed that, for most, vaccine strain S1133 and field isolate clone 918 were the most divergent; thus these 2 clones were chosen for subsequent more detailed comparative analyses. The ARV S1133 M1 gene shares 90.3% identity with the ARV138 gene and 98.4% identity with the ARV176 M1

gene (Table 1). Each of these 3 ARV clones shows approximately 51% identity with the corresponding MRV M1 genes and less than 50% identity with homologous aquareovirus M genes. The ARV918 M1 gene has <87% identity with each of the other chosen ARV M1 genes and ~50% identity with MRV and aquareovirus homologs (Table 1). Phylogenetic analysis of avian orthoreovirus, mammalian orthoreovirus and aquareovirus M1 genes (or homologs) demonstrate each generates a distinct lineage and no one group appears more closely related to either of the other two groups (Fig. 2A), reflecting the ~50% identity between any two groups (Table 1).

3.2. Comparisons of the μ A protein sequences

The ARV μ A proteins are predicted to have molecular weights of 82.0 kDa (ARV138) and 82.2 kDa (ARV176) with isoelectric points (pI) of 8.40 (ARV138) and 8.42 (ARV176). These pI are much higher than the corresponding MRV μ 2 pI , which are 6.9, 7.44 and 7.0 for T1L, T2J and T3D, respectively (Yin et al., 2004). This dramatic difference between the MRV and ARV proteins suggests a high level of variation. The ARV138 and ARV176 μ A proteins share 97.7% identity (17 substitutions out of 732 amino acids) (Table 1A). The majority of amino acid substitutions result in a conservative change to an amino acid of similar size and charge (data not shown). Alignment of the ARV μ A and MRV μ 2 proteins showed that the 4 chosen ARV clones' μ A proteins show 97.3–99.3% amino acid identity with each other. However, the ARV and MRV μ -class minor core proteins share approximately 30% amino acid identity, and the homologous aquareovirus minor core proteins share less than 25% identity with ARV and MRV μ -class minor core proteins (Table 1A). Although various alignment identity values could be adjusted upwards (up to 2%) by altering gap penalty and alignment parameters in each of the programs used (data not shown), the overall pattern remained the same; the ARV and MRV μ -class proteins appeared more similar to each other than either did to the homologous aquareovirus protein. Thus, more detailed protein comparisons (below) used mostly the ARV and MRV data. The 183 amino acid residues that are identical between ARV μ A and MRV μ 2 generate several regions in which average identity is more than twice that of the average protein, suggesting these regions are important for the structure and/or function of this minor core protein (Figs. 1 and 3A). For example, conserved regions between ARV amino acid residues 402–412 and 438–441 contain sequences bearing strong resemblance to the A and B motifs of known ATPases (Noble and Nibert, 1997). In addition, many of the amino acid substitutions in these relatively conserved regions result in amino acids of the same type and size (e.g. the replacement of ARV Met₄₀₅ with MRV Leu₄₁₂).

To date, there is no high-resolution structural information for any ARV protein. Most MRV proteins have been crystallized (for examples, see: (Reinisch et al., 2000; Liemann et al., 2002; Chappell et al., 2002; Tao et al., 2002)). However, MRV μ 2 is one of the few MRV proteins for which there is not yet any high-resolution data. Predictions of μ A secondary structure (using the Kyte-Doolittle regional hydropathy, ExPasy ProtScale surface probability and Network Protein Sequence Analysis) suggested the μ A protein can be arbitrarily divided into 4 regions (an amino terminal region from amino acid residues 1 to 149, a variable region from 150 to 462, an α -helix-rich region from 463 to 615, and a carboxyl-terminal region from 616 to 732) (Fig. 3A), similar to those predicted for the MRV

$\mu 2$ proteins (Yin et al., 2004). Overall, the ARV μA protein is approximately 38% α -helix, 20% β -sheet and 35% random coil, making it an “alpha-beta” protein according to the CATH designation (Martin et al., 1998).

In the MRV system, the M1 gene encodes the $\mu 2$ protein, which is hypothesized to be an RNA-dependent RNA polymerase cofactor. The biochemical properties of the $\mu 2$ protein have been well characterized and the complete protein sequence has been determined from a wide variety of viral isolates (Wiener et al., 1989; Zou and Brown, 1992; Parker et al., 2002; Yin et al., 2004). The $\mu 2$ protein is a minor component of the inner capsid, present in 20–24 copies per virus particle (Coombs, 1998). In vitro studies have shown that the protein interacts with the $\lambda 3$ protein, the RNA-dependent RNA polymerase, as well as functioning as an NTPase and an RNA 5' triphosphatase (Kim et al., 2004). Homology searches against protein sequence data-banks show little to no homology between the reovirus μ -class minor core protein and other known protein sequences. Other approaches will be required to determine the functional regions of this putative RdRp cofactor protein.

3.3. M2 gene and μB protein sequences of ARV138 and ARV176

The nucleotide sequences of the M2 genome segments of avian reovirus ARV138 (Genbank Number AY750052) and ARV176 (Genbank Number AY750053) were determined. Both ARV M2 genes were 2158 base pairs long, shorter than the MRV T1L, T2J and T3D M2 segments that are 2203, 2203, and 2207 nucleotides, respectively (Tarlow et al., 1988; Wiener and Joklik, 1988; Chandran et al., 1999). Both ARV M2 genes have a 5' non-translated region of 29 bases, a 3' non-translated region of 98 bases, and encode a μB protein of 676 amino acids, 32 amino acids shorter than the homologous MRV $\mu 1$ protein. Alignments of the ARV138 and ARV176 5' and 3' non-translated regions reveal complete sequence identity between them, including the previously reported tetranucleotide GCUUU at the 5' end and the UCAUC tetranucleotide at the 3' end (Duncan, 1999) (data not shown). The ARV138 M2 gene shares 90.4% identity (208 mismatches) with the ARV176 M2 gene. As with the M1 gene, ARV176 and ARV S1133 show the greatest identity (95.8% for M2) and ARV918 shows the lowest identity compared to other ARV strains (<76%). The ARV M2 genes show about 55% identity with the corresponding MRV M2 genes and 50% identity with corresponding aquareovirus genes (Table 1B). Phylogenetic analyses suggest the ARV and MRV M2 genes are more closely related to each other than either is to the corresponding aquareovirus genes (Fig. 2B).

3.4. Comparisons of the μB protein sequences

The ARV μB proteins are predicted to have molecular weights of 73.1 kDa (ARV138) and 73.3 kDa (ARV176) and pI of 5.24 (ARV138) and 5.53 (ARV176). The ARV138 and ARV176 μB proteins share approximately 95% identity (34 substitutions out of 676 amino acids). Most amino acid substitutions result in a conservative change to an amino acid of similar size and charge (data not shown). Comparisons of the ARV138, ARV176, and ARV S1133 μB proteins show 95.0–97.5% identity whereas the ARV918 μB protein shows <86% identity to the other ARV μB proteins. Alignments of the ARV μB , MRV $\mu 1$, and homologous aquareovirus outer capsid proteins showed that ARV and MRV proteins share approximately 44% amino acid identity and aquareovirus proteins showed only ~25%

identity with either the ARV and MRV proteins. The value of 44% identity between ARV μ B and MRV μ 1 proteins is significantly higher than has been found for any other ARV/MRV protein comparison, which range from about 25–30% ((Duncan, 1999); this study). To corroborate this unusually high sequence identity, we resolved gradient-purified ARV virions by SDS-PAGE, and subjected the μ B protein band to in-gel tryptic digestion and mass spectrometry (MS). Database searches and confirmatory tandem MS/MS identified several different regions scattered throughout the μ B sequence (from amino acids 65–675), and provided 54.6% protein coverage of the μ B sequence, which strongly suggests the nucleotide-deduced μ B sequences are correct (data not shown).

The MRV μ 1 protein is the major outer capsid protein, accounting itself for approximately one half the total protein mass of the virion. Protein μ 1 plays major roles in cell entry and assembly, and during these processes undergoes a number of maturation events. Residues Asn₄₂-Pro₄₃, between which μ 1 is cut to generate the aminoterminal μ 1N and carboxyl-terminal μ 1C molecules, is completely conserved in all mammalian, avian, and aquareovirus μ -class outer capsid proteins sequenced to date (Fig. 4), suggesting all viruses have similar μ -class autolytic activity. The Arg residue recognized by trypsin to generate ISVP-associated peptides δ and ϕ during cell entry and uncoating (Arg₅₈₄ in MRV) is conserved in our ARV μ B sequences (Arg₅₈₃) but not found in the available aquareovirus sequences. However, MRV μ 1 residue Tyr₅₈₁, which is recognized by α -chymotrypsin to generate ISVPs, is not present in either the avian or aquareoviruses (Fig. 4). These data suggest that while ARV, aquareovirus, and MRV virions can be digested to produce ISVPs and cores, there may be subtle differences in the way their outer capsid proteins are processed during uncoating. Finally, the MRV μ 1 carboxyl terminus is extended 33-amino acids compared to the homologous avian reovirus and aquareovirus proteins (Fig. 4). This region contains a variety of molecular signals and the presence or absence of these signals could represent virus species- and genus-specific structural/functional differences. For example, the cysteine residue that is involved in MRV μ 1 intramolecular disulfide bond cross-linking (Cys₆₇₉) (Nibert et al., 2005), is either lacking or located elsewhere in the ARV μ B protein, suggesting some differences in μ 1 multimerization and supramolecular organization. In addition, analyses of peptides released from virion particles during MRV protease digestions also indicate the C-terminal portions of this region are released from virions prior to σ 3 peptide release (Mendez et al., 2003), suggesting this region may serve as a “trigger” for entry-dependent σ 3 proteolysis. Despite these differences, the unusually high overall μ 1: μ B amino acid identity values may relate to the roles this major outer capsid protein plays in interacting with other capsid proteins within the virion (Dryden et al., 1993), and/or evolutionary constraints placed upon a protein that is involved in so many protein–protein interactions.

The MRV μ 1 protein is the only orthoreovirus μ -class protein for which a high-resolution structure is available (Liemann et al., 2002). We took advantage of the unusually high identity between the ARV and MRV μ -class major outer capsid protein sequences to attempt to fit the ARV μ B sequence into the MRV μ 1 crystal structure. The MRV μ 1 crystal structure (PDB # 1JMU (Liemann et al., 2002)) was manipulated with the PyMOL graphics program (DeLano, 2004) to highlight identical amino acid residues (Fig. 5). In addition to identities highlighted in the previous paragraph (for example, the completely conserved Asn₄₂-Pro₄₃

peptide at which $\mu 1 \rightarrow \mu 1N/\mu 1C$ autolysis occurs), it is clear that ARV μB and MRV $\mu 1$ amino acid identities are relatively evenly distributed throughout the molecule's 3-dimensional structure, rather than localized, suggesting very similar overall morphology. However, as indicated above, there are differences with respect to the extra 33 amino acids at the C-terminus of MRV $\mu 1$. Although these extra MRV residues are disordered and not visible in available crystal structures (Liemann et al., 2002), more recent high-resolution cryoelectron microscopy and image reconstructions indicate these residues form "hub and spokes" complexes within the outer capsid P2 and P3 solvent channels (Zhang et al., 2003, 2005). These hub and spokes complexes are lacking in cryoelectron microscopic reconstructions of aquareovirus (Nason et al., 2000) and ARV (Zhang et al., in press), completely consistent with lack of these residues in the ARV μB and aquareovirus VP5 proteins. These comparative differences suggest there will be differences in structure/function of the ARV and MRV μ -class outer capsid proteins. Apparent lack of disulfide bridges that hold adjacent outer capsid heterohexameric aggregates together in ARV, as well as presence or absence of extra peptides within the solvent channels, may account for slightly lower long-term stability of ARV virions during storage.

3.5. M3 gene sequences and μNS sequences of ARV138 and ARV176

The M3 genome segment nucleotide sequences of ARV strains 138 (Genbank Number AY557190) and 176 (Genbank Number AY557191) were determined. Both genes were 1996 nucleotides long, shorter than the MRV M3 genes that are 2241 base pairs (T1L), 2240 base pairs (T2J) and 2241 base pairs (T3D) long (McCutcheon et al., 1999). Both ARV M3 genes possess a 5' non-translated region of 24 bases, a 3' non-translated region of 64 bases, and encode a μNS protein of 635 amino acids, significantly shorter than the 719-amino acid long homologous MRV μNS protein (Table 1). Alignment of the two ARV non-translated regions show them to be identical and to contain the consensus 5'-GCUUU and UCAUC-3' tetranucleotides (data not shown). The ARV138 M3 gene shares 93.6% identity (110 mismatches) with the ARV176 M3 gene, and each shows >90% identity to ARV S1133 and <82% identity to ARV918 (Table 1C). As a group, the ARV M3 genes share <50% identity with homologous MRV and aquareovirus genes. Phylogenetic analysis demonstrates the ARV, MRV, and aquareovirus genes generate distinct lineages with no one group more closely related to either of the other two groups (Fig. 2C).

3.6. Comparisons of the μNS protein sequences

The ARV μNS proteins are composed of 635 amino acids, making them 84 amino acids shorter than the homologous MRV proteins. Alignments of the ARV, MRV, and aquareovirus μNS (and homologous) protein sequences showed considerable differences, most of which were represented by numerous gaps in the alignments, that range from a few to as many as 25 amino acid residues in length, depending upon the alignment program and gap penalties used (Fig. 6). The molecular weight of the ARV138 μNS protein is 70.9 kDa and the molecular weight of the ARV176 μNS protein is 70.8 kDa. The two ARV proteins sequenced here have approximately 97% identity, with 20 amino acid differences between them. The majority of these amino acid substitutions result in conservative changes to amino acids of similar charges and size (data not shown). The predicted isoelectric points of the ARV μNS proteins are 5.92 (ARV138) and 5.88 (ARV176). Alignment of the ARV μNS

proteins with that of Muscovy duck reovirus (MDRV) suggested only 71% identity, despite the M3 genes sharing 72–73% identity, and suggested the MDRV protein was truncated by 83 residues. Closer inspection of the nucleotide sequence alignments revealed that the predicted MDRV M3 sequence contains an extra nucleotide between residues 1668–1675 relative to each of the other avian M3 sequences. Some alignment programs identified the extra nucleotide as A₁₆₆₈, whereas others identified the extra nucleotide as C₁₆₇₅. Removal of either nucleotide altered the open reading frame, extended the predicted MDRV μ NS protein by 83 residues, and raised the percent identities between MDRV and each of the other avian reovirus μ NS proteins to 80.2–80.6% (data not shown). Alignment of the MRV μ NS proteins and the ARV μ NS proteins showed that the two sets of proteins share approximately 25% amino acid identity, and that homologous aquareovirus proteins share even less identity (Table 1C), even when alignment parameters were altered to optimize alignments (data not shown).

Predictions of μ NS secondary structure (determined using ExPasy ProtScale surface predictions, Kyte & Doolittle regional hydropathy scale and Network Protein Sequence Analysis Programs) suggest the ARV μ NS protein can be arbitrarily divided into 5 regions (an amino terminal region from residues 1 to 68, a α -helix-rich region from 69 to 209, a variable region from 210 to 349, a second α -helix-rich region from 350 to 603, and the carboxyl-terminal region from 604 to 635 (Fig. 3C). The variable region (a.a. 210–349) and the carboxyl-terminal region (a.a. 604–635) are predicted to be the most structurally divergent among the ARV serotypes. Overall, the ARV μ NS protein is approximately 52% α -helix, 13% β -sheet and 30% random coil, making it a “mixed” protein according to the CATH designation (Martin et al., 1998). The high helical content predicted in the ARV μ NS protein is similar to what is predicted for its MRV counterpart (McCutcheon et al., 1999). MOTIF and FingerPRINTScan programs were used to compare the ARV μ NS sequences with other sequences in protein data banks (ProSite, Blocks, and ProDomain). This comparison suggested that no currently recognized motifs or runs of sequence are present in either ARV μ NS protein. However, the MultiCoil program suggested that regions of the ARV μ NS protein, between amino acid residues 561–596, had a high likelihood of forming a coiled-coil structure (Fig. 6), similar to what has been reported for this portion of the MRV protein (Broering et al., 2005).

The role of the μ -class non-structural protein in reovirus infection has not yet been clarified. The MRV μ NS protein is produced in relative abundance compared with other virally produced proteins (Joklik, 1983). Protein μ NS interacts with viral plus-strand RNA transcripts, associates with virus assembly intermediates (Morgan and Zweerink, 1975) and with core particles (Broering et al., 2000), and participates in sequestering viral proteins into cellular inclusions (Broering et al., 2002, 2004; Miller et al., 2003), which suggests μ NS may play a role in the regulation of virus transcription or assembly. The sequence similarity between the predicted ARV and MRV proteins encoded by the M3 gene suggest the ARV μ NS protein may have a function similar to that of MRV μ NS, as recently demonstrated (Touris-Otero et al., 2004a,b).

In conclusion, we report the first sequence analysis of the entire complement of M-class genome segments from two distinct avian reovirus clones. All ARV M genes are more

closely related to homologous MRV M genes than they are to homologous aquareovirus M genes. Comparisons of the ARV and MRV sequences showed nucleotide identity values of approximately 50%. Comparisons of ARV and MRV M1- and M3-encoded protein sequences showed amino acid identity values of about 25–30%, as previously reported for ARV and MRV S gene-encoded proteins. Despite these relatively low global identities, alignments of the proteins demonstrated localized regions of significantly higher amino acid identity that likely are involved in the proteins' functions, including the putative NTPase motifs in the μ -class minor core protein. Comparison of the ARV and MRV μ -class major outer capsid proteins (MRV μ 1 and ARV μ B) showed dramatically higher amino acid identities (about 45%), and important regions involved in protein–protein interactions, maturation and entry-related proteolysis were especially highly conserved. Finally, analysis of the M3 genes suggested an error in the reported Muscovy duck M3 gene sequence which could be re-examined by analysis of the amino acid sequence near the carboxyl terminus of the MDRV μ NS protein.

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Fig. 1.

Alignment of the deduced μ A amino acid sequences of ARV138 and ARV176. Clones are: ARV–ARV138 (GenBank No. AY557188), ARV176 (GenBank No. AY557189), ARV S1133 (GenBank No. AY639610), and ARV918 (GenBank No. AY639617); MRV–Type 1 Lang (T1L) (GenBank No. X59945), Type 2 Jones (T2J) (GenBank No. NC4254), and Type 3 Dearing (T3D) (GenBank No. NC4280); aquareoviruses–grass carp reovirus (GCRV) (GenBank No. NC5159), grass carp hemorrhagic virus (GCHV) (GenBank No. AF251262), golden ide reovirus (GIRV) (GenBank No. AF450324), and golden shiner reovirus (GSRV) (GenBank No. AF403402). Alignments were performed by the T-Coffee program (Notredame et al., 2000) and visualized with BOXShade. Amino acid residues that are identical in at least six of the sequences are indicated by black background shading. Grey background shading indicates conservative amino acid substitutions in at least six of the sequences. The single letter amino acid code is used. Regions of relatively high sequence identity between the ARV and MRV proteins (see Fig. 3) are indicated by horizontal lines above the sequences. The amino acids that represent the putative ATPase A and B motifs are indicated by (●).

Fig. 2.

Phylogenetic tree analyses of the prototype ARV M1 (A), M2 (B), and M3 (C) genes and homologous genes in other reoviruses. Abbreviations are as defined in the legends to Figs. 1, 4 and 6. Lines are proportional in length to nucleotide substitution. Alignments were performed by ClustalW and visualized by Treeview (Page, 1996).

Fig. 3.

Window-averaged scores for sequence identity among the ARV and MRV μ -class minor core protein (A), μ -class major outer capsid protein (B) and μ -class non-structural proteins (C). Identity scores averaged over running windows of 21 amino acids and centered at consecutive amino acid positions are shown. The global identity score for the five sequences is indicated by the horizontal line. Regions of greater-than-average sequence conservation are marked with lines above the plot. The two putative NTP-binding motifs in the μ -class minor core protein are marked with filled circles in A. Secondary structure predictions: N–amino terminal region; V–variable region; H– α -helical region; and C–carboxyl terminal region are indicated in boxes above the plots in A and C.

Fig. 4. Alignment of the deduced μ B amino acid sequences of ARV138 and ARV176. Clones are: ARV–ARV138 (GenBank No. AY750052), ARV176 (GenBank No. AY750053), ARV S1133 (GenBank No. AY635934), and ARV918 (Genbank No. AY635945); MRV–Type 1 Lang (T1L) (GenBank No. NC4262), Type 2 Jones (T2J) (GenBank No. NC4270), Type 3 Dearing (T3D) (GenBank No. NC4278), and Ndelle virus (GenBank No. AF368034); aquareoviruses–grass carp reovirus (GCRV) (GenBank No. NC5169), grass carp hemorrhagic virus (GCHV) (GenBank No. AF239175), and goldenshiner reovirus (GSRV) (GenBank No. AF403403). The shading scheme is the same as in Fig. 1. The single letter amino acid code is used. The μ 1 to μ 1N/ μ 1C cleavage site is indicated by ($\blacktriangledown$); the MRV α -chymotrypsin-specified μ 1C to δ/ϕ cleavage site is indicated by ($\ddagger$); the MRV trypsin-specified μ 1C to δ/ϕ cleavage site is indicated by ($\dagger$); and the MRV Cysteine residue involved in ϕ : ϕ cross-linking is indicated by (=).

Fig. 5.

Crystal structure of the MRV outer capsid proteins. (A) Heterohexameric ($\mu_1\sigma_3$) crystal structure (as determined by (Liemann et al., 2002)) and manipulated with PyMol (DeLano, 2004)). σ_3 molecules are shown in green, lime, and olive; μ_1 molecules depicted in white, wheat, and grey. (B) “Exploded” view of white μ_1 molecule shown above in (A). (C) And (D) represent same μ_1 molecule but rotated 120° (C) and 240° (D) around vertical axis. Identical ARV μ_B residues are shown in blue, the completely conserved Asn₄₂-Pro₄₃ dipeptide that is cut to generate μ_1N and μ_1C is shown near bottom of μ_1 molecule in orange, and the trypsin cleavage site (Arg₅₈₄ in MRV) that is cut to generate ISVP peptides δ and ϕ is shown near middle of μ_1 molecule in red. MRV residues Lys₆₇₆-Arg₇₀₈, missing in the ARV μ_B protein, are disordered and not visible in these structures.

Fig. 6.

Alignment of the deduced μ NS amino acid sequences of ARV138 (GenBank No. AY557190), ARV176 (GenBank No. AY557191), ARV S1133 (GenBank No. AY608700), and ARV918 (GenBank No. AY573911); Muscovy duck reovirus (MDRV) (Genbank No. AJ293969); MRV–Type 1 Lang (T1L) (GenBank No. NC4257), Type 2 Jones (T2J) (GenBank No. NC4258), and Type 3 Dearing (T3D) (GenBank No. AF174384); aquareoviruses–grass carp reovirus (GCRV) (GenBank No. NC5158), and goldenshiner reovirus (GSRV) (GenBank No. AF403401). The shading scheme is the same as in Fig. 1. The single letter amino acid code is used. Regions of relatively high sequence identity between the ARV and MRV proteins (see Fig. 3C) are indicated by horizontal lines above the sequences. Regions predicted to have a high propensity to form coiled-coil interactions (as determined by MultiCoil (Wolf et al., 1997)) are denoted by *a* and *d* above the sequences.

Table 1

Comparisons of ARV, MRV, and aquareovirus M gene and μ protein identities^a

Isolate	ARV138	ARV176	ARVS1133	ARV918	MRVT1L	MRVT2J	MRVT3D	GCRV	GSRV
(A) μ -Class minor core protein (M1 gene- μ A protein)									
ARV138	–	97.7	97.3	97.5	30.7	29.0	30.1	22.7	22.3
ARV176	91.8	–	99.3	97.8	30.9	29.7	30.1	22.7	22.4
ARVS1133	90.3	98.4	–	98.0	31.1	29.6	30.3	22.6	22.3
ARV918	86.8	86.6	86.4	–	32.1	29.7	30.7	23.0	22.7
MRVT1L	50.8	51.7	51.6	49.5	–	80.8	98.6	24.1	23.5
MRVT2J	50.5	51.0	51.0	50.5	71.5	–	80.0	23.0	24.1
MRVT3D	51.2	50.9	50.7	49.2	97.8	70.9	–	23.7	23.6
GCRV	49.4	49.7	49.3	48.9	49.2	48.3	49.1	–	97.0
GSRV	49.9	49.4	48.9	49.4	48.8	49.0	48.8	91.5	–
(B) μ -Class major outer capsid protein (M2 gene- μ B protein)									
ARV138	–	95.3	95.0	85.8	43.2	43.2	42.9	25.4	25.4
ARV176	90.4	–	97.5	84.9	43.2	43.2	43.2	25.3	35.3
ARVS1133	86.7	95.8	–	85.7	43.6	43.6	43.6	25.4	25.4
ARV918	74.9	74.8	75.2	–	44.0	44.0	44.2	25.4	25.6
MRVT1L	54.4	54.3	53.6	53.6	–	97.5	97.2	25.1	25.1
MRVT2J	54.0	55.0	54.7	54.4	76.9	–	96.2	25.2	25.2
MRVT3D	54.1	54.8	54.6	54.8	85.0	76.6	–	24.6	25.0
GCRV	50.2	50.7	50.3	48.9	48.9	49.3	50.1	–	99.2
GSRV	49.6	50.6	51.1	49.7	49.3	48.9	49.3	94.2	–
(C) μ -Class non-structural protein (M3 gene- μ NS protein)									
ARV138	–	97.0	96.5	90.1	24.4	25.0	24.4	20.5	20.7
ARV176	93.6	–	98.9	90.2	24.2	25.4	24.3	20.0	20.1
ARVS1133	92.1	98.0	–	89.8	24.5	25.1	24.6	20.4	20.4
ARV918	81.2	80.9	80.9	–	24.2	24.9	24.2	20.3	20.7
MRVT1L	49.6	49.0	49.4	49.2	–	82.4	95.6	16.5	16.4
MRVT2J	48.6	48.9	48.1	49.6	72.1	–	83.5	18.6	18.6
MRVT3D	49.6	49.4	49.7	49.6	88.0	71.7	–	17.6	17.8

Isolate	ARV138	ARV176	ARVSI133	ARV918	MRVT1L	MRVT2J	MRVT3D	GCRV	GSRV
GCRV	48.6	48.6	48.0	48.8	48.0	48.6	47.2	–	98.5
GSRV	47.8	48.4	48.4	48.0	47.7	48.2	47.4	96.3	–

^aValues for M gene sequences shown in bold below diagonal; values for U proteins shown above diagonal. Values determined using LaLign (<http://www.ch.embnet.org/software/LALIGNform.html>).