Baculovirus expression of the 11 mycoreovirus-1 genome segments and identification of the guanylyltransferase-encoding segment

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The type member Mycoreovirus 1 (MyRV-1) of a newly described genus, Mycoreovirus, isolated from a hypovirulent strain 9B21 of the chestnut blight fungus, has a genome composed of 11 dsRNA segments (S1–S11). All of the segments have single ORFs on their capped, positive-sense strands. Infection of insect cells by baculovirus recombinants carrying full-length cDNAs of S1–S11 resulted in overexpression of protein products of the expected sizes, based on their deduced amino acid sequences. This expression system was utilized to identify the S3-encoded protein (VP3) as a guanylyltransferase by an autoguanylylation assay, in which only VP3 was radiolabelled with \(^{32}\text{P}\)GTP. A series of progressive N-terminal and C-terminal deletion mutants was made to localize the autoguanylylation-active site of VP3 to aa residues 133–667. Within this region, a sequence stretch (aa 170–250) with relatively high sequence similarity to homologues of two other mycoreoviruses and two coltiviruses was identified. Site-directed mutagenesis of conserved aa residues revealed that H233, H242, Y243, F244 and F246, but not K172 or K202, play critical roles in guanylyltransferase activities. Together with broader sequence alignments of ‘turreted’ reoviruses, these results supported the a/vxxHx 8 Hyf/lvf motif, originally noted for orthoreovirus and aquareovirus genera, as well as for the proposed Dinovernavirus genus.

INTRODUCTION

The family Reoviridae is one of the largest and most diverse virus families, composed of 12 established genera and two other proposed genera that are important pathogens of fungi, plants, insects, fish and mammals (Mertens et al., 2005a). The family is subdivided into two groups, on the basis of a critical structural feature (‘turreted’ and ‘nonturreted’), which share common properties: they have 9–12 dsRNA genomic segments, most of which are monocistronic; virus particles are multi-shelled; and virus (core) particles serve as viral mRNA synthesis factories. Despite these common features in genome structure, RNA replication and morphology, divergence among viral sequences and lack of information on protein function within the most closely related genera makes it difficult to identify functional gene homologues among family members.

Details of deoxyoligonucleotides used for cDNA synthesis of MyRV-1 S1–S11, in construction of deletion mutants of MyRV-1 VP3, and in construction of site-directed mutants of MyRV-1 VP3, are available as supplementary material in JGV Online.
Combined results of motif searches and intensive functional studies of the capping enzymes of orthonoroviruses and AQVs have identified two putative active domains located at their N termini, Kx[V/L/I]S and Hx₈H. The Kx[V/L/I]S motif, the K residue of which was believed to be the GMP-binding site, has been found in members of the genera Orthoreovirus, Seadornavirus, Rotavirus, Orbiviruses and Phytoreovirus (Luongo, 2002; Mohd Jaafar et al., 2005), while the Hx₈H sequence has been detected in two species of the genus Orthoreovirus [Mammalian orthoreovirus (MRV) and Avian orthoreovirus (ARV)] and in grass carp reovirus (GCRV) of the genus Aquareovirus (Qiu & Luongo, 2002). Because the two motifs are not shared by all reoviral guanylyltransferases, it remains unclear whether there is a consensus sequence for a reoviral guanylyltransferase, and what it may be.

Mycoreovirus 1/Cp9B21 (MyRV-1/Cp9B21) is a member of the newly described genus Mycoreovirus of the family Reoviridae, and was isolated from hypovirulent strain 9B21 of the chestnut blight fungus (Cryphonectria parasitica) (Enebak, 1992; Hillman et al., 2004; Suzuki et al., 2004; Mertens et al., 2005b). An isogenic virus-free fungal strain, 9B21ss1, displays much greater virulence to chestnut trees and production of aerial hyphae than the virus-containing strain. Unlike many virus-infected C. parasitica strains, however (e.g. Hillman et al., 1990), no significant difference in sporulation or pigmentation is found between the virus-containing and virus-free strains. Stable transfection of fungal mycelium with virus particles has provided evidence that MyRV-1 is responsible for the phenotypic alterations (Hillman et al., 2004).

MyRV-1 has a genome composed of 11 dsRNA segments (S1–S11). All of the segments have recently been sequenced and shown to have single ORFs on their capped, positive-sense strands, with the conserved terminal sequences 5’-GAUCA...GCAGUC-3’ (Suzuki et al., 2004). Phylogenetic analysis has revealed a close relationship to other mycoreoviruses recently characterized, Mycoreovirus 2 (MyRV-2) and Mycoreovirus 3 (MyRV-3), and to members of the genus Coltivirus of the family Reoviridae, which includes the tick-transmitted human pathogens Colorado tick fever virus (CTFV) and Eyach virus (EyaV) (Hillman & Suzuki, 2004). Sequence analyses have led to the tentative identification of VP1 as the viral RNA-dependent RNA polymerase (RdRp), VP6 as a nucleoside triphosphate (NTP)-binding protein, and VP4 as a myristoylated structural protein. Functional roles of proteins encoded by other segments are unknown.

We now report expression in insect cells of the 11 MyRV-1 genomic segments and identification of the S3-encoded protein as the capping enzyme. A VP3 domain required for guanylyltransferase activity, as mapped by deletion and site-directed mutational analyses, contained a consensus sequence, Hx₈Hx₈Hy[I/V][F] (residues 233–246), that was conserved in counterparts of the other two mycoreoviruses MyRV-2/CpCl8 and MyRV-3/RnW370, and in the two sequenced coltiviruses. Interestingly, the a/vx₈Hx₈Hy[I/V][F] motif is found in all members of the ‘turreted’ viruses in the Reoviridae family, supporting an association between particle structure and evolution, as proposed by Hill et al. (1999) and Nibert & Kim (2004).

**METHODS**

**Fungal strains and culturing.** C. parasitica strain 9B21, which was naturally infected with the virus ultimately characterized as MyRV-1/Cp9B21, and its isogenic virus-free counterpart, 9B21ss1, have been described previously (Hillman et al., 2004). EP155, the standard virus-free strain of C. parasitica, was stably transfected with virus particles of MyRV-1/Cp9B21 purified from strain 9B21 (Hillman et al., 2004). Fungal colonies were grown under bench-top conditions at 23–26°C on potato dextrose agar (PDA, Difco) solid plates, or in potato dextrose broth (PDB, Difco) liquid media for RNA extraction. For maintenance, strains were cultured on regeneration plates (Churchill et al., 1990) and stored at 4°C until use.

**Full-length cDNA cloning.** Genomic dsRNA of MyRV-1/Cp9B21 was extracted and purified from mycelia of MyRV-1/Cp9B21-infected EP155 cultured in PDB (Difco), as described by Hillman et al. (2004). For synthesis of full-length cDNA of each segment, a pair of primers was designed on the basis of the terminal RNA sequence of each segment. For segments 1–7, forward primers contained BanHI sites and reverse primers contained NotI sites. The forward and reverse primers for S8 cDNA synthesis contained EcoRI and NotI sites, and no restriction enzyme recognition sites were added to primers used for S9–S11. The primers used in these reactions are listed in Supplementary Table S1. Total genomic dsRNA was denatured in DMSO at 65°C for 15 min in the presence of the primer pairs (Asamizu et al., 1985), and cDNA representing each segment was synthesized by reverse transcription using RevertAid H Minus M-MuLV reverse transcriptase (Fermentas). The resultant cDNA was amplified by PCR using KOD polymerase with high fidelity (Toyobo) with the same sets of primers as in cDNA synthesis. The PCR products derived from S1–S8 were digested with BanHI or EcoRI and NotI, and ligated into the BanHI–NotI site or EcoRI–NotI site of pbBlueScript II (SK+) (Strategene), while full-length cDNA of S9–S11 was cloned into pGEMT-easy (Promega). These ligates were transformed into Escherichia coli DH5α (Takara). At least three clones of each segment were obtained and used for sequence analysis. All full-length cDNA clones used in this study were identical in nucleotide sequence to those reported by us previously (Hillman et al., 2004; Suzuki et al., 2004).

**Deletion and site-directed mutation.** A series of four progressive deletion mutants of MyRV-1/Cp9B21 VP3 from each terminus was constructed, eight in total (AC1–AC4 and AN1–AN4). Deletion mutants were generated by amplifying from the full-length wild-type cDNA of S3 (pbBlueScript-S3-16) with the thermostable KOD DNA polymerase. Mutants AC1–AC4 contained truncated forms of VP3, aa residues 1–833, 1–667, 1–333 and 1–167, while AN1–AN4 lacked aa 1–132, 1–362, 1–522 and 1–729. The specific primer pairs used in these amplifications are listed in Supplementary Table S2. The deleted mutants were each subcloned into the BanHI–NotI site of the pbBlueScript SK+ plasmid vector and then moved to the baculovirus transfer vector.

All single amino acid substitution mutants were generated by the overlap-extension method (Sambrook & Russell, 2001). Two partial fragments of MyRV-1 S3 cDNA overlapping at their termini were amplified from full-length cDNA of segment 3 (pbBlueScript-S3-16) using a pair of a mutant primer and forward (S3FL1) or reverse (S3FL2) primer. Site-directed mutations were introduced into the overlapping region with mutagenic primers. Taking advantage of the
overlapping sequences, full-length site-directed mutants were amplified using mixtures of two partial fragments as template by PCR reactions. Forward and reverse primers S3FL1 and S3FL2 were commonly used for all the mutants in the first and second PCR reactions. The primers used for generating each mutant are listed in Supplementary Table S3. After digestion with BamHI and NotI, the site-directed mutants were subcloned into the pBlueScript SK+ plasmid vector and subsequently to the baculovirus transfer vector. Sequence integrity was confirmed for all deletion and substitution mutants.

**Baculovirus expression in Spodoptera frugiperda cells.** S. frugiperda cells (S9) were cultured in TC100 insect medium (Gibco) supplemented with 10% fetal bovine serum, as described by Matsuura et al. (1987). The cDNAs of wild-type and mutant S1–S11 of MyRV-1/Cp9B21 were cloned into the baculovirus (Autographa californica multiple nucleopolyhedrovirus, AcMNPV) transfer vectors, pAcYM1 (Matsuura et al., 1987) or pBacDual (Invitrogen). pAcYM1-containing inserts were transfected into insect cells along with the BaculoGold baculovirus DNA (BD Biosciences Pharmingen). Baculovirus recombinants were cloned by plaque purification (Matsuura et al., 1987). MyRV-1/Cp9B21 S1–S8 cDNAs cloned downstream of the polyhedrin promoter in pFastBacDual were moved to baculovirus DNA contained in the bacmid, which was maintained in DH10Bac *E. coli* cells. Recombinant baculovirus DNA was isolated from *E. coli* cells according to the manufacturer’s protocol (Bac-to-Bac Baculovirus Expression System, Invitrogen). Resulting bacmid clones were transfected into insect cells to launch baculovirus recombinants with the aid of TransFector (B-Bridge International), SDS-PAGE of proteins in insect cells was according to Suzuki et al. (1994).

**Autoguanylation assays.** Autoguanylation was performed following the method of Hsiao et al. (2002), with several modifications. S9 cells were infected with recombinant baculovirus at 5 p.f.u. per cell, and the infected cells were harvested 3 days post-infection by centrifugation at 10,000 r.p.m. for 3 min. After being washed with PBS, pH 7.2, the resulting pellet was resuspended in cold lysis buffer (10 mM Tris/HCl, pH 7.5; 5 mM MgCl2; 200 mM NaCl; 0.5% Triton X-100) containing proteinase inhibitors, and incubated on ice for 1 h. To 20 μl aliquots of the preparation, 2.5 mM Ci ml−1 (92.5 MBq ml−1) (final concentration) of [α-32P]GTP (Amersham) and 20 μg ml−1 (final concentration) of inorganic pyrophosphatase (Sigma) were added, and incubated at 25 °C for 20 min. Samples were boiled for 3 min and subjected to SDS-PAGE (Laemmli, 1970) to remove unbound GTP and non-covalently bound radiolabel. The gel was then dried using a gel-drying processor model At-3700 (ATTO). Protein–guanylate complex formation on the dried gel was assessed by phosphorimager (Bio-imaging analyser BAS-1000, Fuji Film).

**RESULTS**

**High-level expression in insect cells of MyRV-1/Cp9B21 S1–S11**

Toward functional analyses of the MyRV-1 proteins, we utilized the baculovirus expression system, which has been demonstrated to be useful for proteins of other reoviruses. SDS-PAGE analysis of lysates from insect cells infected with baculovirus recombinants carrying the full-length cDNAs of MyRV-1 S1–S11 (AcMyRV-1S1 to AcMyRV-S11) is shown in Fig. 1. Infection of the baculovirus recombinants resulted in overproduction of polypeptides of approximately 135 kDa (AcMyRV-1S1, lane 1), 125 kDa (AcMyRV-1S2, lane 2), 115 kDa (AcMyRV-1S3, lane 3), 65 kDa (AcMyRV-1S4, lane 4), 63 kDa (AcMyRV-1S5, lane 5), 60 kDa (AcMyRV-1S6, lane 6), 58 kDa (AcMyRV-1S7, lane 7), 65 and 60 kDa (AcMyRV-1S8, lane 8), 30 kDa (AcMyRV-1S9, lane 9), 24 kDa (AcMyRV-1S1, lane 10) and 10 kDa (AcMyRV-1S11, lane 11) that were consistent with the molecular sizes expected from the nucleotide sequences. The nature of the two protein bands from AcMyRV-1S8-infected cells is not known. S11-encoded VP11 was not detectable by Coomassie brilliant blue staining in a lower percentage gel;
however, a higher concentration polyacrylamide gel resolved trace amounts of a unique product consistent with the size predicted for S11 (Fig. 1b, lane 11) and more clearly differentiated between the 24 kDa virus-specific product of S10 and a similar-sized protein common to all samples (Fig. 1b, lane 10).

**GTP specifically binds MyRV-1/Cp9B21 VP3**

Three methods are commonly used for identification of a guanylyltransferase capping enzyme (Mizumoto & Kaziro, 1987): (1) a GTP–enzyme binding assay (autoguanylylation assay); (2) a GTP–PPi exchange assay in the absence of acceptor RNA; and (3) a transguanylylation (cap formation) assay using nucleoside diphosphate or diphosphorylated RNA as cap acceptors. A GTP–enzyme binding assay was employed here, in which an intermediate form, a GMP–guanylyltransferase complex made during the capping reaction, was detected. Baculovirus-expressed proteins encoded by MyRV-1 S1–S11, shown in Fig. 1(a, b), were tested for their ability to bind GTP, the property indicative of a guanylyltransferase. Initially, soluble and insoluble fractions of cell lysates were used. Fractionation resulted in degradation of some of the proteins, however, even in the presence of protease inhibitors. This has also been observed for the C protein of an avian reovirus during its storage (Hsiao et al., 2002). Therefore, unfractionated cell lysates were used for the assay. Of the 11 lysates, only S3-expressing cell lysates contained a protein of 115 kDa labelled specifically (Fig. 1c). Minor signals corresponding to ~80 kDa were found in most of the lanes, so this is likely an insect-cell-derived protein. Thus, MyRV-1/Cp9B21 S3-encoded VP3 is the viral guanylyltransferase.

**Mapping of the guanylyltransferase catalytic domain**

To identify the guanylyltransferase catalytic domain, a series of eight progressive deletion mutants of S3 (ΔC1–ΔC4 and ΔN1–ΔN4) (Fig. 2) was cloned into the baculovirus transfer vector pFastBacDual and expressed in insect cells. All of the mutant VP3 recombinants directed the synthesis of proteins of the expected sizes and at detectable levels by SDS-PAGE analysis (Fig. 3a), while their expression levels were similar to (ΔC1, ΔN1, ΔN2) or higher than (ΔC2, ΔC3, ΔC4, ΔN3, ΔN4) those of wild-type VP3 of MyRV-1/Cp9B21, as judged from the intensity of protein bands stained by Coomassie brilliant blue. The lysates of insect cells infected with the baculovirus recombinants were examined for their GTP-binding activities as described above. Two deletion mutants of VP3 lacking the amino acids from the C terminus, ΔC1 and ΔC2, retained their binding activities, although the levels of labelling were much lower than that of the wild-type VP3 of MyRV-1/Cp9B21 (Fig. 3b). The smallest and second-smallest mutants, ΔC3 and ΔC4, lost their binding activities. In contrast, only one mutant lacking the N-terminal region, 132 aa residues (ΔN1), was still able to bind GTP, while no other ΔN2–ΔN4 mutant was labelled with [γ-32P]GTP.

**Construct**

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**Fig. 2.** Schematic representation of deletion mutants of MyRV-1 S3. MyRV-1/Cp9B21 S3 (wild-type, WT) is 3258 bp in length with an ORF encoding 1065 aa. Mutants ΔC1–ΔC4 contain deletions from the C terminus that encode truncated forms of VP3 comprising aa residues 1–833, 1–667, 1–333 and 1–167. The N-terminal regions were deleted in ΔN1–ΔN4, retaining aa residues 133–1065, 363–1065, 523–1065 and 730–1065. cDNA constructs of these mutants were cloned into the baculovirus transfer vector pFastBacDual, as described in Methods. Sizes of the deleted forms of VP3 are shown to the right of the figure.

The observation that ΔC1, ΔC2 and ΔN1 bound GTP, while the other deletion mutants failed to bind GTP (Fig. 3b), suggested that an activity domain or an aa sequence stretch important for the proper folding of VP3 resides within aa residues 133–667.

**Identification of the active residues**

Alignment of MyRV-1/Cp9B21 with homologous proteins of the other two mycoreoviruses revealed 24% overall identity to MyRV-2/CpC18 VP3 and 20% overall identity to MyRV-3/RnW370. The regions of greatest identity were within aa 119–362 (29 and 25% identities, respectively) and aa 762–1055 (28 and 24% identities, respectively). The former includes a stretch of sequences at the N-terminal region reported previously to show similarities among equivalent segments of the evolutionarily related reoviruses (Suzuki et al., 2004) (Fig. 4). It was of interest to note that only the N-terminal region included a sequence stretch with similarities to corresponding proteins of the two coltivirus, CTFV and EyaV. Assuming that the catalytic residues are strictly conserved among the viruses, the N-terminal region was targeted for further analysis. Fig. 4 shows the sequence alignment of the equivalent domains and 12 sites (numbered 1–13) at which alanine substitution mutations were introduced (K172A, K202A, P229A, G231A, H233A, S237A, H242A, Y243A, F244A, V245A, F246A and
D251A). The valine residue at position 245 (numbered 11) was mutated to serine as well as to alanine to obtain V245S and V245A. The substitution mutants were cloned into the baculovirus transfer vector for expression in insect cells. All mutants were expressed almost equally, at levels comparable to that of the wild-type VP3 (Fig. 5). No protein of 115 kDa was detected in insect cells uninfected (Fig. 5, lane C) or infected with a baculovirus recombinant (AcMyRV-1S7) that contained cDNA of MyRV-1 S7 (Fig. 5, lane S7).

When tested in the autoguanylylation assay, these mutants were variable in their abilities to form GMP complexes, and separated into four groups. The first group of MyRV-1 VP3 variants included mutants at residues 172 (K172A), 202 (K202A), 231 (G231A) and 251 (D251A) in which no effects of alanine substitutions were observed, and autoradiography revealed protein bands with similar intensities to those of wild-type VP3 (Fig. 5, lanes 1, 2, 4, 10 and 13). Only one mutant, S237A protein, belonging to the second group, was reproducibly labelled more strongly than the wild-type VP3 (Fig. 5, lane 6), consistent with observations for a valine substitution mutant of MRV 22 at the flexible L192 (Luongo, 2002). In the third group, substitutions at H233, H242, Y243, F244 and F246 caused detrimental effects to GTP-binding activities (Fig. 5, lanes 5, 7, 8, 9 and 12). Site-directed mutants in the fourth group, P229A and V245S, retained only low levels of autoguanylylation activity (Fig. 5b, lanes 3 and 11). It is of interest that alanine substitution of V245 did not change the activity, whereas replacement of the same amino acid with serine conserved in MyRV-3 VP3 in the identical position decreased the activity. Together, these results suggest pivotal roles for residues H233, H242, Y243, F244 and F246 in the autoguanylylation of MyRV-1 VP3.

**DISCUSSION**

Capping reactions in eukaryotic cells for the synthesis of m7G(5')pppNmp- require RNA triphosphatase, guanylyltransferase, RNA (guanine-7-)methyltransferase and RNA (nucleoside-O')methyltransferase. These enzymic activities may be performed by separate protein subunits or by single subunits. The key enzyme in the capping reactions, guanylyltransferase, is a member of the nucleotidyltransferase family that includes RNA ligase, DNA ligase and RNA guanylyltransferase. All cellular and DNA viral guanylyltransferases contain the motif KxDG (Furuichi & Shatkin, 2000), in which the K residue serves as the GMP-linkage site. Including this work, guanylyltransferases have now been identified biochemically in members of seven genera of the family Reoviridae. Although guanylyltransferases in two genera, Orthoreovirus and Aquareovirus, show low levels of sequence similarity, little discernible similarity is found in other combinations (Luongo, 2002; Mohd Jaafar et al., 2005), making it difficult to predict a capping enzyme across genera based on sequence comparison alone. Some of the reoviral guanylyltransferases also possess methyltransferase activity, e.g. MRV 22, Rotavirus VP3 and Bluetongue virus VP4 (Lawton et al., 2000; Ramadevi et al., 1998). Interestingly, the C-terminus of sequences of both MyRV-1/9B21 and MyRV-2/C18 contain regions that identified bacterial methyltransferases, albeit with poor e values, among relatively few hits in PSI-BLAST searches. Whether or not this is significant is unknown.

The baculovirus expression system has proven to be useful for structural and functional analyses of reoviruses, as exemplified by studies on the dissection of replicase complexes and their template specificity, structure determination of core-like particles, and functional assessment of viroplasms (e.g. Patton et al., 1997; Estes, 2001; Roy, 2001; Wei et al., 2006). In the current study, all 11 genome
segments of MyRV-1/Cp9B21, the type member of a newly established genus *Mycoreovirus* within the family *Reoviridae*, were expressed in insect cells to levels sufficient for detection by Coomassie brilliant blue staining. We used this system to show that VP3 is a guanylyltransferase, with its catalytic domain in the N-terminal region. Expression of the 11 MyRV-1 segments will be a basis for further functional analysis of the encoded proteins.

The results in this study show striking similarities to previous findings on the guanylyltransferases of turreted reoviruses. Our deletion mutational analysis revealed that a guanylyltransferase activity domain resides at the N-terminal region. This finding is consistent with the observation for MRV and ARV (orthoreoviruses), and AQV (an aquareovirus), that the catalytic regions of their guanylyltransferases are located at their N termini (Luongo, 2004).

![Amino acid sequence alignment of MyRV-1/Cp9B21 VP3 and its counterparts among evolutionarily related viruses. Amino acid sequences of VP3s of MyRV-1/Cp9B21 (Hillman et al., 2004), MyRV-2/CpC18 (this study), MyRV-3-RnW370 (Wei et al., 2004), CTFV (Attoui et al., 2002) and EyaV (Attoui et al., 2002) were aligned using the multi-alignment program in CLUSTAL W (Thompson et al., 1997). For each viral VP3, map positions of the first residues of the alignment are shown on the right, while amino acid residues changed in substitution mutation of MyRV-1/Cp9B21 VP3 are numbered above the alignment. Map positions of residues mutated on MyRV-1/Cp9B21 VP3 are shown on the top of the alignment. Amino acids strictly conserved among the five viruses are indicated by asterisks, while changes to chemically similar amino acids are shown by one or two dots.](http://vir.sgmjournals.org/)

![GTP-binding activities of site-directed mutants of MyRV-1/Cp9B21 VP3 expressed in insect cells. (a) Each lane of a 10% polyacrylamide gel was loaded with lysates from baculovirus recombinant-infected insect cells. Lane numbers refer to substitutions at amino acids shown in the sequence alignments in Fig. 4: K172A (lane 1); K202A (lane 2); P229A (lane 3); G231A (lane 4); H233A (lane 5); S237A (lane 6); H242A (lane 7); Y243A (lane 8); F244A (lane 9); V245A (lane 10); V245S (lane 11); F246A (lane 12); D251A (lane 13). Insect cells infected (lane S7) or uninfected (C) with AcMyRV-1S7 were harvested and subjected to SDS-PAGE analysis. The sizes of the molecular mass standards (Fermentas) are 170, 130, 100, 72, 55, 40, 33 and 24 kDa. (b) GTP-binding assay of site-directed mutants of MyRV-1/Cp9B21 VP3. Autoguanylylation reactions were performed with lysates of cells expressing the single amino acid substitution mutants. GMP-bound proteins were detected by autoradiography preceded by 17.5% polyacrylamide gel electrophoresis. Lane numbers indicated correspond to those in Figs 4 and 5(a).](http://vir.sgmjournals.org/)
2002; Hsiao et al., 2002; Qiu & Luongo, 2003). By site-directed mutagenesis of amino acids that are well conserved among VP3s of the closely related mycoreoviruses and coltiviruses, we identified several amino acid residues important for autoguanylylation activity: H233, H242, Y243, F244 and F246. The sequence stretch containing these amino acids conforms to the Hx₈H motif (aa positions 223–232 for MRV l²) in the genera Orthoreovirus (MRV, ARV) and Aquareovirus (golden shiner reovirus, GCRV; cham salmon reovirus, GCRV) (Qiu & Luongo, 2003). However, no Kx[V/L/I]S motif (aa residues 190–193 for MRV l²), proposed for the guanylyltransferases of Rotavirus, Orbivirus, Orthoreovirus, Phytorovirus and Seadornavirus (Luongo, 2002; Mohd Jaafar et al., 2005) is found in MyRV-1 or related viruses.

As previously noted by us (Suzuki et al., 2004), low levels of sequence similarity between MyRV-1 VP3 and Cypovirus 1 (CPV-1) VP3, a capsid protein (Ikeda et al., 2001), was found. Interestingly, the overall alignment showed the histidine couple of MyRV-1 VP3 and CPV-1 VP3 themselves aligning, prompting us to search available sequences of reovirus proteins for the Hx₈H motif. In a broader search, the Hx₈H-like motifs shown in Fig. 6 were detected in eight of the 12 genera described in the 8th report of the International Committee on Taxonomy of Viruses (ICTV) (Mertens et al., 2005a) and two recently proposed genera (Dinovernavirus and Mimoreovirus) (Attoui et al., 2005, 2006). These included members of the relatively well-studied genera Orzyavirus, Fijiivirus and Coltivirus, in which the guanylyltransferase had not yet been identified. The motif in the genus Seadornavirus was not included in our alignments, because of the space difference between the histidine couple (12 versus 8 aa residues) (Mohd Jaafar et al., 2005), but the proposed dinovernavirus aedes pseudoscutellaris reovirus, with a 7 aa space, was included. CPV-1, Cypovirus 14 (CPV-14) and Cypovirus 15 (CPV-15) VP3s show approximately 30 % identity, and the histidine couple is conserved in all three. Similarly, the motif was found in groups 1, 3 and 5 of the genus Fijivirus, in which only low levels of amino acid sequence identity, ranging from 18.8 to 21.4 %, were found among counterparts. Some of the proteins in Fig. 6 show inter-genus sequence similarities (e.g. cypovirus VP3 versus fijivirus counterparts; oryzavirus P2 versus fijivirus counterparts) detectable by BLAST search and the two histidines are readily alignable. Similarities in

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**Fig. 6.** Proposed motif for guanylyltransferases of some reoviruses. A sequence motif is proposed for guanylyltransferases or guanylyltransferase candidates of members of eight genera in the family Reoviridae. Segments encoding the proteins, sizes (aa) of the proteins, positions of the first amino acids of the sequence stretch and accession numbers are shown. References are shown for proteins already identified as guanylyltransferases. The two strictly conserved histidine residues are shown in red. Amino acids conserved in at least three genera are in blue, while others are in black. MyRV-1, MyRV-1/Cp9B21; MyRV-2, MyRV-2/CpC18; MyRV-3, MyRV-3/RnW370; CTFV, CTFV-Florio; EyaV, EyaV-France578; MRV-3, Mammalian orthoreovirus-type 3-Dearing; ARV, Avian orthoreovirus-1733; GCRV, grass carp reovirus (Aquareovirus C); GSRV, golden shiner reovirus (Aquareovirus C); CSRV, cham salmon reovirus CS (Aquareovirus C); CPV-1, CPV-1 (bombyx mori cypovirus 1); CPV-14, CPV-14 (lymantria dispar cypovirus 14); CPV-15, CPV-15 (trichoplusia ni cypovirus 15); APRV, aedes pseudoscutellaris reovirus; RRSV, Rice ragged stunt virus; FDV, Fiji disease virus; RBSDV, Rice black streaked dwarf virus; NLRV, Nilaparvata lugens reovirus.
the proteins in Fig. 6 include not only two strictly conserved histidines but also the size of the proteins (1056–1299 aa) and the positions of the conserved histidines relative to the N terminus (positions 186–278). Site-directed mutational analyses of MyRV-1 VP3 (Figs 4 and 5) showed that not only the two histidines but also the moderately well-conserved residues immediately downstream of H242 (e.g. Y243, F244, F246) affect the guanylyltransferase activity.

An important remaining question is which residue of MyRV-1 VP3 is the GMP-binding site. It has been shown elsewhere that covalent intermediates are formed via a phosphaamide bond between a GMP and a basic amino acid on the enzyme, usually with the ε-amino group of a lysine (Fausnau & Shatkin, 1990; Hsiao et al., 2002). Within the region of aa 133–667 defined by our deletion analysis, only two lysine residues, K172 and K202, are found at relatively similar positions to the Kx[V/L]/I[S] of MRV J2. K202 is at an identical or similar position to those of its counterparts in both the mycoreoviruses and the coltiviruses, while K172 is found in identical positions only in the mycoreoviruses. Neither K172 nor K202, however, was shown to be necessary for the guanylyltransferase activity of MyRV-1 VP3. As reported by Shuman & Hurwitz (1980), enzyme–GMP covalent binding can also occur with the imidamino group of a histidine residue and the guanido-amino group of an arginine residue. This raises the possibility that one of the histidines within the HxH consensus sequence might serve as the GMP-binding site, although other conserved basic residues cannot be ruled out as a GMP-linking residue. Supporting this, the active regions mapped for the ARV and GCV guanylyltransferases do not contain the Kx[V/L]/I[S] motif (Hsiao et al., 2002), while the HxH motif is conserved. Furthermore, a histidine residue conserved strictly in capping enzymes of positive-stranded RNA virus members of the alpha-like superfamily is implicated in GMP binding (Huang et al., 2004). Direct determination of the GMP-binding site of MyRV-1 VP3 is needed to clarify this.

Reovirus core particles are believed to contain all enzymic activities necessary for the synthesis of capped viral mRNA, and all reovirus guanylyltransferases identified to date are structural (core) proteins. Consistent with this, our preliminary data suggest that MyRV-1 VP3 is a structural protein, although its specific location within the particle has yet to be determined. The reovirus genera Orthoreovirus, Cypovirus, Agareovirus, Fifivirus, Oryzavirus, Idenoreovirus and Mycoreovirus have been described as turreted, while the genera Phytoreovirus, Rotavirus, Orbivirus, Coltivirus and Seadornavirus are considered nonturreted (Mertens et al., 2005a). Particles of the nonturreted group contain capping enzymes as part of the transcriptase complex, which also contains helicase and RdRp inside the core (Roy, 2001; see Estes, 2001, for a review). In the turreted group, the capping enzyme complex is part of the spike structures protruding from the core through the outer capsid (Reinish et al., 2000; see Nibert & Schiff, 2001, for a review). In MRV, turrets around the fivefold axes are composed of pentamers of the capping protein λ2 (Nibert & Schiff, 2001). This structure is believed to play a role in RNA capping and the release of transcripts from the particle. The NTP-binding motifs A and B on NTPase have recently been shown to be conserved in all turreted members and the genus Coltivirus (Nibert & Kim, 2004). The HxH motif analysed here is another consensus sequence found in members of the turreted group and in coltiviruses. Thus, although coltiviruses are tentatively classified as nonturreted (Mertens et al., 2005a), similarities in transcription components and strategies, and RdRp-based phylogeny (Attoui et al., 2002; Nibert & Kim, 2004; Hillman et al., 2004), suggest that they may in fact be turreted. Clarification of the relation among the HxH motif, capping reactions and the turret structure awaits further biochemical and structural analyses.

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