Nucleoside and RNA Triphosphatase Activities of Orthoreovirus Transcriptase Cofactor μ2

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The mammalian Orthoreovirus (mORV) core particle is an icosahedral multienzyme complex for viral mRNA synthesis and provides a delimited system for mechanistic studies of that process. Previous genetic results have identified the mORV μ2 protein as a determinant of viral strain differences in the transcriptase and nucleoside triphosphatase activities of cores. New results in this report provided biochemical and genetic evidence that purified μ2 is itself a divalent cation-dependent nucleoside triphosphatase that can remove the 5′-γ-phosphate from RNA as well. Alanine substitutions in a putative nucleotide binding region of μ2 abrogated both functions but did not affect the purification profile of the protein or its known associations with microtubules and mORV μNS protein in vivo. In vitro microtubule binding by purified μ2 was also demonstrated and not affected by the mutations. Purified μ2 was further demonstrated to interact in vitro with the mORV RNA-dependent RNA polymerase, λ3, and the presence of λ3 mildly stimulated the triphosphatase activities of μ2. These findings confirm that μ2 is an enzymatic component of the mORV core and may contribute several possible functions to viral mRNA synthesis.

The mammalian Orthoreovirus (mORV) core particle is an icosahedral multienzyme complex for viral mRNA synthesis (for review, see Ref. 1–3). The core is released from the infectious virion during cell entry and is also formed from newly synthesized gene products as an assembly intermediate within infected cells. In addition to the 10 centrally condensed viral double-stranded RNA genome segments cores contain five different viral proteins in defined copy numbers (Table I). Virions contain three additional viral proteins that play key roles in particle stability and cell entry. The viral mRNAs are full-length plus-strand copies of the genome segments 1200–3900 nucleotides in length, modified by a dimethylated cap 1 structure at their 5′ ends and not polyadenylated. The manner in which the double-stranded RNA genome segments and mRNA products interact with the transcription and capping machinery in mORV cores is a topic of interest for understanding structural and mechanistic aspects of these processes in this and other viral and cellular systems.

X-ray crystallography has recently advanced our understanding of the core. A 3.6-Å crystal structure of the 52-MDa mORV core particle has provided an atomic model for most residues of the three major core proteins: λ1 in the T = 1 shell, λ2 in the pentameric surface turrets, and α2 in the monomeric surface nodules (4). Not visualized in that structure are the two stoichiometrically minor proteins, λ3 and μ2, which are located beneath the λ1 shell near the icosahedral 5-fold axes (5). To obtain an atomic model for λ3, which is the viral RNA-dependent RNA polymerase (6–8) and, thus, a functionally key element, a 2.5-Å crystal structure of recombinant λ3 has been determined (9). In addition, the λ3 structure has recently been fit into a 7.6-Å electron cryomicroscopy reconstruction of the mORV virion, revealing how λ3 associates with the λ1 shell (10). These studies leave μ2 as the only core protein for which no crystal structure is yet available (Table I). The roles of μ2 in core functions are also poorly defined.

Cores catalyze at least five different enzymatic reactions (Table I). In addition to the polymerase activity attributable to λ3, there are the four reactions in RNA 5′-capping: RNA 5′-triphosphatase (RTPase), guanylyltransferase, N-methyltransferase, and O-methyltransferase (3, 11). The RTPase activity has been assigned to the shell protein λ1 (12). The RNA guanylyl- and methyltransferases are contained in the turret protein λ2 (4, 13–18), through which the nascent transcripts are cotranscriptionally exported (19, 20). Cores also catalyze removal of the γ-phosphate group from NTPs (21–24). This activity might represent the RTPase, as observed with some other such enzymes (25–29), but might alternatively reflect a distinct activity not encompassed by the five reactions above. In fact, published findings suggest there may be two different types of nonspecific nucleoside triphosphatases (NTPases) in cores (23, 24). One of these NTPases might represent a core-associated RNA helicase that is involved in melting the double-stranded RNA genome segments during transcription (23, 24, 30), and indeed evidence for NTP-dependent RNA/DNA helicase activity by shell protein λ1 has been reported (30).

Two previous genetic studies have implicated μ2 in the enzymatic activities of cores. Yin et al. (31) show that the M1 genome segment, which encodes μ2, determines in vitro differences between cores of mORV strains type 1 Lang (T1L) and type 3 Dearing (T3D) in both the temperature optimum of transcription and the amounts of transcripts produced. Because λ3 is the viral polymerase (6–9), these genetic findings suggest an auxiliary role for μ2 in λ3 function. Noble and Nibert (24) show that M1/μ2 determines in vitro differences...
between T1L and T3D cores in both the response of the ATPase activities to temperature and the rate of GTP hydrolysis at certain conditions. The latter genetic associations are consistent with μ2 functioning as an NTPase or playing a regulatory role in the NTPase function of another core protein, most likely λ1 (23, 24, 30). Both μ2 and λ1 in vitro have also been shown to bind RNA (32–34). Thus, questions remain about the relative roles of μ2 and λ1 in the NTPase activities of cores and the specific roles each plays in viral mRNA synthesis.

Recent studies have used immunofluorescence microscopy to identify in vivo associations between μ2 and other proteins. Initially, μ2 was shown to associate with and stabilize microtubules in both infected and transfected cells (35). Subsequently, μ2 was shown to associate also with the major mORV nonstructural protein μNS (36). These findings suggest important roles for μ2 and μNS in building the cytoplasmic “factories” in which viral genome replication and assembly are thought to occur (35–38).

To learn more about the activities of μ2, we devised a purification protocol and undertook functional studies of the purified protein. Results in this report provide biochemical and genetic evidence that μ2 is itself a divalent cation-dependent NTPase and RTPase. Alanine substitutions in a putative nucleotide binding region of μ2 abrogated both functions but did not substantially affect the purification profile of the protein or its known associations with microtubules and μNS protein in vivo. In vitro microtubule binding by purified μ2 was also demonstrated and not affected by the mutations. Purified μ2 was further demonstrated to interact in vitro with the viral polymerase, λ3, and the presence of λ3 mildly stimulated the triphosphatase activities of μ2. These findings suggest that μ2 is an enzymatic component of the mORV core and may contribute several possible functions to viral mRNA synthesis.

**EXPERIMENTAL PROCEDURES**

**Generation of a Recombinant Baculovirus for Expression of Wild-type (wt) μ2**—Generation of plasmid pBS-M1(T1L), encoding wt T1L μ2 protein, was previously described (35). The complete μ2-encoding region was excised from this plasmid by digestion with BamHI and HindIII and ligated into the pFastBac vector (Invitrogen) under transcriptional control of the baculovirus (Autographa californica nuclear polyhedrosis virus) polyhedrin promoter. The resulting plasmid pFB-M1(T1L) was used to generate a recombinant baculovirus with the M1 gene from pBS-M1(T1L)K415A/K419A was excised by digestion with SpeI and XhoI and ligated into the pCI-neo vector (Promega) that had been digested with NheI and SalI.

**Generation of a Recombinant Baculovirus for Expression of Mutant K415A/K419A μ2**—To obtain an M1 gene encoding alanine substitutions for lysines 415 and 419 in μ2, we first removed the BamHI site from the multiple-cloning region of pBS-M1(T1L) by cutting with BamHI, filling the overhangs with Klenow polymerase, and religating. The resulting plasmid pBS-M1(T1L) was used as template in an inverse polymerase chain reaction with forward primer 5'-GGGATCC-GAGATGATGATGATC-3' and reverse primer 5'-GGGGATCCGGCCAGGCAGCAACGGC-3' (bold italics indicate nucleotide changes for Ala-419 and Ala-415, respectively). In each primer one silent nucleotide change was also introduced to generate a BamHI site (underlined) without affecting the μ2 amino acid sequence. The amplification conditions were the same as those described in Kim et al. (39). The resulting plasmid was subjected to nucleotide sequencing to confirm that between the BsmI and SpfI sites in M1, and the BsmI-SpfI fragment was then swapped for the same region of pFB-M1(T1L). The resulting plasmid pFB-M1(T1L)K415A/K419A was used to generate a recombinant baculovirus as indicated in the preceding section.

**Cloning M1 Genes into the pCI-neo Vector**—Generation of plasmid pCI-M1(T1L), encoding wt T1L μ2 protein, was previously described (35). The plasmid pCI-M1(T1L)K415A/K419A was generated in the same manner in that the M1 gene from pBS-M1(T1L)K415A/K419A was excised by digestion with SpeI and XhoI and ligated into the pCI-neo vector (Promega) that had been digested with NheI and SalI.

**Purification of wt and K415A/K419A μ2**—SF21 cells were prepared in a 1-liter spinner at a concentration of 1 × 10^6 cells/ml, into which the recombinant baculovirus to express either wt or K415A/K419A μ2 was inoculated at 5–10 plaque-forming units/cell. At 60 h post-infection, the cells were harvested and washed 3 times with cold phosphate-buffered saline (137 mM NaCl, 3 mM KCl, 8 mM NaHPO4, 1 mM KH2PO4, pH 7.5). Nuclear extracts were then prepared from these cells as follows. The cell pellet was washed twice with 50 ml of ice-cold Nonidet P-40 buffer and then twice with 50 ml of ice-cold CaCl2 buffer. To make the Nonidet P-40 and CaCl2 buffers 100 ml of Chelsky buffer (10 mM Tris, pH 7.0, 10 mM NaCl, 3 mM MgCl2, 30 mM sucrose) was supplemented with Nonidet P-40 to 0.5% or CaCl2 to 10 mM. The nuclear pellet was resuspended in 50 ml of lysis buffer (20 mM Tris, pH 7.9, 100 mM KCl, 0.2 mM EDTA, 20% glycerol, 1% protease inhibitor mixture (Roche Diagnostics)). Nuclei were lysed on ice with a syringe, and the nucleoplasm was separated from the postnuclear pellet by centrifugation at 15,000 rpm for 30 min in an RC-5B centrifuge with an SS34 rotor (DuPont Sorvall). The nucleoplasm was dialyzed overnight at 4 °C against 50 mM Tris acetate, pH 8.5, 50 mM KCl, 5 mM MgCl2, 10% glycerol, 2 mM β-mercaptoethanol.

All subsequent column work was performed at room temperature with an AKTA-FPLC system (Amersham Biosciences). At each step the column fractions containing μ2 were identified by SDS-polyacrylamide gel electrophoresis and immunoblotting with μ2-specific polyclonal antiserum (36) and an alkaline phosphatase-based color detection method (39). The dialyzed nucleoplasm was applied to a 5-ml HiTrap DEAE-Sepharose column (Amersham Biosciences) preequilibrated with Aephosphate buffer (Aephosphate buffer supplemented with 50 mM NaCl). μ2 eluted from this column at 0.8–1.2 m NaCl in a 0–2 mM NaCl gradient in Aephosphate buffer. The fractions containing μ2 were pooled, dialyzed over-
night at 4 °C against A260 buffer, and applied to a 1-ml HiTrap heparin-Sepharose column (Amersham Biosciences) pre-equilibrated with A260 buffer. Elution was performed with a gradient of 0 to 1 M NaCl gradient in A260 buffer. The fractions containing µ2 were pooled, dialyzed against A260 buffer (A260 buffer supplemented with 100 mM NaCl), and used for all subsequent work as purified µ2. Protein concentration was estimated from absorbance at 280 nm. Yields from this procedure were routinely in the range of 300–400 µg of µ2 per 4 × 10^7 cells.

**Purification of µ2**—The T3D µ3 protein was expressed and purified as previously described (9). Immediately before use in each experiment, µ3 (or bovine serum albumin used in parallel samples) was dialyzed against A260 buffer for 2 h at 4 °C using a Slide-A-Lyzer 10K cassette (Pierce).

**RTPase Assays**—Reactions for analysis in the colorimetric NTPase assay were performed in A260 buffer lacking β-mercaptoethanol. The reactions also contained 2 mM ATP and the estimated amount(s) of protein(s) in a total reaction volume of 60 µL. For some experiments, ingredients were altered as specifically described in the figure legends. After incubation at room temperature for an appropriate time (standard, 45 min), the reaction was stopped with 60 µL of 10% trichloroacetic acid. 100 µL of the stopped reaction was then mixed with 100 µL of colorimetric reagent prepared by mixing 6 N sulfuric acid, 0.8% ammonium molybdate, and 10% ascorbic acid in a 1:3:1 ratio. The mixture was incubated at 57 °C for 30 min, and absorbance at 655 nm (A655) was measured with a microplate colorimeter (Molecular Devices). Standard curves were generated with a dilution series of [γ-32P]ATP, and the quantity of protein(s) in a total reaction volume of 60 µL was determined from the standard curve.

For the radiographic RTPase assay reaction conditions were the same as for the colorimetric assay except that the reaction volume was only 20 µL, and 5 µL of [γ-32P]ATP (3000 Ci/mmol) (PerkinElmer Life Sciences) was used instead of 2 mM ATP. After incubating at room temperature for 30 min, the reaction was stopped with 10 mM EDTA. 1 µL of the stopped reaction was spotted onto a polyethyleneimine-cellulose thin-layer chromatography (TLC) plate (EM Science). After drying overnight at 4 °C, the TLC plate was developed in 1.2 M LiCl solvent, and the separated reaction products were visualized by either phosphorimaging (Molecular Dynamics) or exposure to x-ray film (Fuji or Kodak) in the presence of intensified screen.

**RTPase Assay—MAXiScript**—in vitro transcription kit (Ambion) was used to generate γ-labeled 45-nucleotide RNA substrates following the manufacturer’s directions. Each 20-µL reaction mixture contained 30 units of T7 RNA polymerase from the kit, 1× transcription buffer (from the kit), 1 mM ATP, 1 mM CTP, 1 mM UTP, 0.03 mM GTP, 50 µCi of either [γ-32P]GTP (6000 Ci/mmol) or [γ-32P]UTP (3000 Ci/mmol) (Perkin Elmer), 1 µg of PGEM-4Z (Promega) (linearized with Smal and purified from an agarose gel), and 1 unit/µL RNasin (Promega). After a 2-h incubation at 37 °C the reaction was adjusted to 50 µL with H2O. Template DNA was degraded by treatment with 30 min at 37 °C with 2 units of DNase I from the kit. Free nucleotides were removed with a nucleotide removal kit (Qiagen) and a Sephadex G-25 column (Amersham Biosciences). The quality and purity of the purified 45-mer [γ-32P]- or [α-32P]RNA (i.e. appropriate RNA size and essential absence of [γ-32P]- or [α-32P]RNA and small abortive transcripts) were verified on 10% polyacrylamide gels containing 7 % urea (data not shown). Efficient removal of GTP and small abortive transcripts from the [γ-32P]RNA was also verified by TLC, in which [γ-32P]GTP was found to migrate slightly faster than the upper spot of the radiolabeled RNA (data not shown). RNA concentration was estimated by absorbance at 260 nm.

For the RTPase assay reaction conditions were the same as for the radiographic NTPase assay except that specified amounts of [γ-32P]-labeled RNA instead of 2 mM ATP. After incubating for the standard time at room temperature (standard, 1 h), the reaction was stopped with 10 mM EDTA. For Fig. 6, RNAs were resolved on a 10% polyacrylamide gel containing 7 % urea and then visualized by exposure to x-ray film in the presence of an intensifying screen. Otherwise, reaction products were separated by TLC as described for the radiographic RTPase assay, visualized by phosphorimaging. For quantitation of products from TLC, the intensity of the [32P] spot obtained in each µ2- and/or µ3-containing sample was expressed as a fraction of that obtained by treatment of the RNA with calf intestinal phosphatase (data not shown). This upper spot is indeed attributable to RNA, not GTP.

**Microtubule Spin-down Assay**—A spin-down kit (Cytoskeleton, Inc.) was used according to the manufacturer’s suggestions. Briefly, the indicated amounts of purified wt or K415A/K419A µ2 were mixed with microtubules stabilized with 20 µM taxol after polymerizing purified α- and β-tubulin by incubation at 37 °C for 20 min. After 40 min at room temperature, the mixtures were overlaid on a 100-µl sucrose cushion and spun at 100,000 g for 40 min at room temperature in a TL-100 ultracentrifuge with a TLA-100 rotor (Beckman). The supernatant was removed, and the pelleted was resuspended in 50 µL of phosphate-buffered saline (PBS). 10 µL of the resuspended pellet was then separated by SDS-polyacrylamide gel electrophoresis and analyzed by Western blotting with µ2-specific polyclonal antisera (36) and a horse-radish peroxidase-based enhanced chemiluminescence detection method (Pierce).

**Immunofluorescence Microscopy**—Transfection was performed using 2 µg of plasmid DNA and 6 µL of LipofectAMINE reagent (Invertogen) as previously described (35). At 18 h post-transfection cells were fixed with 100% methanol and stained with either Oregon Green-conjugated µ2-specific polyclonal antibodies, Texas Red-conjugated µNS-specific polyclonal antibodies, or both, as previously described (36). Fluorescence was visualized with a TE-300 inverted light microscope (Nikon), and the collected images were processed and optimized with Photoshop software (Adobe Systems).

**Innectorpantcirus**—Each of the indicated amounts of purified wt or K415A/K419A µ2 was mixed with 300 ng of purified µ3 in binding buffer (20 mM Tris, pH 8.5, 50 mM NaCl, 50 mM KCl, 5 mM MgCl2, 10% glycerol, 0.01% Triton X-100, 2 µM β-mercaptoethanol, 1× protease inhibitor mixture). The mixtures were incubated overnight with rocking at 4 °C. µ2-specific polyclonal antibodies (purified from antisera using a protein A column (36)) were added to a final concentration of 33 µg/mL, and the mixtures were incubated for an additional 2 h at 4 °C. Each mixture was supplemented with 10 µL of protein A Dynabeads (Dynal Biotech) preincubated with binding buffer and then incubated for 30 min at room temperature. Bound-bound antigen-antibody complexes were isolated using the same magnetic bead (Dynal Biotech) and washed twice with binding buffer and twice with washing buffer (binding buffer supplemented with NaCl to 200 mM and Triton X-100 to 0.05%). The washed beads were resuspended in 50 µL of 1× gel sample buffer (125 mM Tris, pH 8.0, 1% SDS, 2% β-mercaptoethanol, 10% sucrose, 0.01% bromphenol blue), boiled, separated by SDS-polyacrylamide gel electrophoresis, and analyzed by immunoblots with either µ2- or µ3-specific polyclonal antisera (36, 40) and an alkaline phosphatase-based color detection method (39).

**RESULTS**

**Purification of mORV µ2 Protein**—A protocol for large scale purification of µ2 protein has not been previously reported, and we, therefore, set out to devise one. Recombinant µ2 was expressed in SF21 cells using a baculovirus vector in which the M1 gene was under control of the polyhedrin promoter. About half of µ2 expressed in this way appeared in the nuclear fraction of lysed cells (data not shown). This was not wholly unexpected since some nuclear localization of recombinant µ2 has been previously noted in mammalian cells by immunofluorescence microscopy (35, 36, 38), although the basis of this localization has remained unclear. The nuleoplasim (postnuclear supernatant) of µ2-expressing SF21 cells was obtained and found to contain larger amounts of µ2 relative to other proteins than did the cytoplasm. The nuleoplasim was, therefore, used for further purification of µ2 on a series of ion-exchange and affinity columns: DEAE, Cibacon Blue, and heparin. SDS-polyacrylamide gel electrophoresis and immunoblotting with µ2-specific polyclonal antisera were used to identify the column fractions containing µ2. Fig. 1A summarizes representative gel results from the series and shows that one major protein band (Mr ~ 80,000) was strongly enriched. This band comigrated with the µ2 protein from mORV cores during electrophoresis (Fig. 1B, left) and was also recognized by the µ2 antisera in immunoblots (Fig. 1B, right). Another band sometimes seen migrating above µ2 (Fig. 1, B, left, and C) was a contaminant found in different amounts in different prepara-
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Fig. 1. Purification of mORV \( \mu_2 \) protein and analysis of its ATPase activity. Denatured proteins were resolved by electrophoresis on SDS-polyacrylamide mini-gels (10% acrylamide), which were then either stained with Coomassie Brilliant Blue R-250 or electroblotted on a colorimetric assay. Samples containing wt or K415A/K419A \( \mu_2 \) were analyzed for their capacity to release Pi (Fig. 1C). We have previously noted a conserved region of the \( \mu_2 \) primary sequence with some similarities to nucleotide binding motifs (28, 42), are also shown. Amino acid position numbers are indicated atop each sequence. Positions of alanine-for-lysine substitutions in \( \mu_2 \) mutant K415A/K419A (K415/9A) are also indicated. B, denatured samples of the purified wt and K415A/K419A \( \mu_2 \) proteins were resolved in adjacent lanes of an SDS-polyacrylamide mini-gel (10% acrylamide), which was then stained with Coomassie Brilliant Blue R-250. Positions of size markers (kDa) (Bio-Rad) are indicated at left. D, all fractions shown in C were analyzed for their capacity to release Pi, from ATP as measured by \( A_{660} \) in a colorimetric assay.

 revelations that fraction numbers 11–13 in this experiment had a level of ATPase activity substantially above the background level of other fractions (Fig. 1D). These same fractions were shown to contain the highest amounts of \( \mu_2 \) protein by gel analysis (Fig. 1C). Moreover, the level of ATPase activity of each fraction was proportional to the amount of \( \mu_2 \) the fraction contained (fraction 12 > 13), strongly suggesting that \( \mu_2 \) is required for the activity. Other colorimetric assays with the peak fractions provided evidence that \( \mu_2 \) is able to hydrolyze GTP, CTP, and UTP as less preferred substrates (data not shown), suggesting that \( \mu_2 \) is a nonspecific NTPase.

Mutant \( \mu_2 \) Protein (K415A/K419A) with Alanine Substitutions in a Putative Nucleotide Binding Motif—After the preceding results we remained concerned that the NTPase activity might be attributable to a contaminant and not \( \mu_2 \) in the purified preparation. To prove that NTP hydrolysis was mediated by \( \mu_2 \) we sought to create a \( \mu_2 \) mutant that lacked this activity. We have previously noted a conserved region of the \( \mu_2 \) primary sequence with some similarities to nucleotide binding A and B motifs (24) (Fig. 2A). Comparison with recently reported sequences for the homologous VP5 proteins of Aquareovirus (41) showed that these motifs are conserved despite an overall \( \mu_2 \)-VP5 homology of only 24% (42) (Fig. 2A). Moreover, the putative A motif of Ortho- and Aquareovirus is very similar to that of Aquareovirus VP5, a known NTPase and RTPase (28, 42). Nsp2, a known NTPase and RTPase (28, 42) (Fig. 2B). Comparison with recently reported sequences for the homologous VP5 proteins of Aquareovirus (41) showed these motifs are conserved despite an overall \( \mu_2 \)-VP5 homology of only 24% (42) (Fig. 2A). Moreover, the putative A motif of Ortho- and Aquareovirus is very similar to that of Aquareovirus VP5, a known NTPase and RTPase (28, 42). Nsp2, a known NTPase and RTPase (28, 42) (Fig. 2B). Comparison with recently reported sequences for the homologous VP5 proteins of Aquareovirus (41) showed these motifs are conserved despite an overall \( \mu_2 \)-VP5 homology of only 24% (42) (Fig. 2A). Moreover, the putative A motif of Ortho- and Aquareovirus is very similar to that of Aquareovirus VP5, a known NTPase and RTPase (28, 42). Nsp2, a known NTPase and RTPase (28, 42) (Fig. 2B). Comparison with recently reported sequences for the homologous VP5 proteins of Aquareovirus (41) showed these motifs are conserved despite an overall \( \mu_2 \)-VP5 homology of only 24% (42) (Fig. 2A). Moreover, the putative A motif of Ortho- and Aquareovirus is very similar to that of Aquareovirus VP5, a known NTPase and RTPase (28, 42). Nsp2, a known NTPase and RTPase (28, 42) (Fig. 2B). Comparison with recently reported sequences for the homologous VP5 proteins of Aquareovirus (41) showed these motifs are conserved despite an overall \( \mu_2 \)-VP5 homology of only 24% (42) (Fig. 2A). Moreover, the putative A motif of Ortho- and Aquareovirus is very similar to that of Aquareovirus VP5, a known NTPase and RTPase (28, 42).

\( \mu_2 \) (K415A/K419A):
tions at positions 415 and 419 in the putative A motif (Fig. 2A) in an effort to eliminate the apparent NTPase activity of \( \mu_2 \).

The \( \mu_2 \) mutant K415A/K419A was expressed using a baculovirus vector and purified through the same procedures as wt \( \mu_2 \). Gels showed no mobility difference between the purified K415A/K419A and wt proteins (Fig. 2B; data not shown). Over the series of columns in the purification, the mutant behaved almost identically to wt protein, except that K415A/K419A \( \mu_2 \) was eluted at slightly lower salt concentration from the Cibacron Blue column (data not shown). The similar purification patterns suggest the mutant is folded and structurally similar to wt protein (see Fig. 3 and “Microtubule Binding and \( \mu_2 \)NS Association by wt and K415A/K419A \( \mu_2 \)” below for more evidence). The colorimetric assay revealed that the ATPase activity of purified wt \( \mu_2 \) increased in concert with protein concentration, whereas the activity of K415A/K419A \( \mu_2 \) remained at background level across the range of concentrations (Fig. 2C).

These results indicate that \( \mu_2 \) is itself an ATPase and suggest that its putative nucleotide binding A motif is involved in this activity. Based on the comparison with Alphavirus Nsp2 (Fig. 2A), we consider it likely that the conserved Lys-419 residue in mORV \( \mu_2 \), whereas the activity of K415A/K419A \( \mu_2 \) remained at background level across the range of concentrations (Fig. 2C).

Microtubule Binding and \( \mu_2 \)NS Association by wt and K415A/K419A \( \mu_2 \)—The preceding results with the K415A/K419A mutant would be most convincing if the effect were limited to NTP hydrolysis, whereas other activities of \( \mu_2 \) are spared. Such findings would indicate that the K415A/K419A mutations do not globally disrupt the folding and structure of \( \mu_2 \). The fact that K415A/K419A \( \mu_2 \) could be purified in a similar manner to wt \( \mu_2 \) provided some evidence in this regard, but we sought further evidence. Recent work from our laboratory has shown the capacity of \( \mu_2 \) to associate with both microtubules and mORV \( \mu_2 \)NS protein in infected and transfected cells (35, 36).

We, therefore, tested the mutant for these activities. In transfected CV-1 cells, K415A/K419A \( \mu_2 \) was coexpressed with microtubules similarly to wt \( \mu_2 \) (Fig. 3A). A substantial portion of both \( \mu_2 \) proteins localized to the nucleus (Fig. 3A), as previously noted for wt \( \mu_2 \) (35, 36). The exact role if any of this nuclear localization remains unknown. To complement the in vitro results demonstrating association between \( \mu_2 \) and microtubules, we performed an in vitro spin-down assay with a mixture containing taxol-stabilized microtubules (44, 45) and increasing concentrations of either wt or K415A/K419A \( \mu_2 \). After proteins in the pellets were separated on SDS-polyacrylamide gels, immunoblots revealed that the K415A/K419A mutant was spun down with microtubules as well as wt \( \mu_2 \), whereas neither was spun down in the absence of microtubules (Fig. 3B). These results provide strong evidence that \( \mu_2 \) binds directly to microtubules and also show that the K415A/K419A mutant has a microtubule binding capacity similar to that of wt protein. When transiently expressed in CV-1 cells, the \( \mu_2 \)NS protein, a major component of viral factories in mORV-infected cells (36), formed globular inclusions within the cytoplasm (Fig. 3C, bottom), as previously reported (36). When wt \( \mu_2 \) was coexpressed with \( \mu_2 \)NS, on the other hand, \( \mu_2 \)NS localized to microtubules along with \( \mu_2 \) (Fig. 3C, upper left), as also previously reported (36). When K415A/K419A \( \mu_2 \) was coexpressed with \( \mu_2 \)NS, \( \mu_2 \)NS again localized to microtubules along with \( \mu_2 \) (Fig. 3C, upper right). In sum, these results demonstrate that K415A/K419A \( \mu_2 \) behaves like wt \( \mu_2 \) with regard to microtubule binding both in vivo and in vitro, nuclear localization, and interaction with \( \mu_2 \)NS in vivo, indicating that the two forms of \( \mu_2 \) are functionally similar in many regards. We conclude that the K415A/

K419A mutations have little or no effect on the overall conformation of \( \mu_2 \) but specifically abrogate its ATPase activity.

Specificity of wt \( \mu_2 \) for Release of \( \gamma \)-Phosphate from NTPs—

The \( \mu_2 \)- and \( \lambda_1 \)-influenced NTPase activities in mORV cores are specific for triphosphorylated nucleotides and release only the \( \gamma \)-phosphate (21-24, 30). We, therefore, tested for such specificity with the purified \( \mu_2 \) proteins. [\( \alpha\)-\( ^{32} \)P]ATP was used as substrate in a radiographic assay in which the reaction products were analyzed by TLC and fluorography. In this assay

\[ \text{Fig. 3. Associations of wt and K415A/K419A } \mu_2 \text{ with microtubules and } \mu_2 \text{NS. Proteins in CV-1 cells were visualized by immunofluorescence microscopy. Cell nuclei were stained with 4,6-diamidino-2-

phenylindole (blue). Scale bars, 10 } \mu \text{m. A, the wt or K415A/K419A (K415/9A) } \mu_2 \text{ protein was expressed in cells transfected with pCI-neo plasmids containing the respective M1 genes. At 18 h post-transfection, cells were fixed, and } \mu_2 \text{ was immunostained with Oregon Green-conjugated } \mu_2 \text{-specific polyclonal antibodies (green). B, increasing amounts of purified wt } \mu_2 \text{ (top) or K415A/K419A } \mu_2 \text{ (bottom) (0, } 0.84, 1.8, \text{ or } 4.2 \text{ pmol each in lanes 1–4) were mixed with microtubules (MT) stabilized by taxol after tubulin polymerization in vitro and sedimented through a sucrose cushion. Control samples containing no microtubules (lane 5, } 4.2 \text{ pmol of } \mu_2 \text{) were also analyzed. Proteins in the sedimented pellets were resolved in adjacent lanes of an SDS-polyacrylamide mini-gel (10% acrylamide), which was then subjected to immunoblot analysis with } \mu_2 \text{-specific polyclonal antiserum. C, the wt } \mu_2 \text{, K415A/K419A } \mu_2 \text{, and/or wt } \mu_2 \text{NS proteins were expressed in cells transfected with pCI-neo plasmids containing the respective M1 or M3 genes. } \mu_2 \text{NS was separately coexpressed with each } \mu_2 \text{ protein (top) and was also expressed in the absence of } \mu_2 \text{ (bottom). At 18 h post-transfection, cells were fixed, and } \mu_2 \text{ and } \mu_2 \text{NS were respectively immunostained with Oregon Green-conjugated } \mu_2 \text{-specific polyclonal antibodies (green) and Texas Red-conjugated } \mu_2 \text{NS-specific polyclonal antibodies (red).} \]
wt \( \mu_2 \) generated ADP as the only radiolabeled product (Fig. 4A, lane 2), indicating that \( \mu_2 \) specifically attacks the \( \beta\gamma \) phosphodiester linkage of ATP, generating ADP (labeled) and P\(_i\) (not labeled) as products. As expected from previous results, K415A/K419A \( \mu_2 \) showed no activity in this assay (Fig. 4A, lane 3). The addition of EDTA to the reaction containing \( \mu_2 \) completely abolished the ATPase activity (Fig. 4A, lane 4), indicating that the activity is dependent on divalent cations, as are many other NTPases (25–29) including those in mORV cores (23) (see below for additional evidence). Specific cleavage of the \( \beta\gamma \) phosphodiester linkage was further indicated by experiments in which the colorimetric assay was used to examine the capacity of \( \mu_2 \) to hydrolyze GTP, GDP, or GMP and which showed that only GTP could serve for P\(_i\) release (Fig. 4B).

**Others Characteristics of the NTPase Activities of wt \( \mu_2 \)**—NTPase activities are commonly sensitive to the type and concentration of divalent cations in the reaction mixture (25–29). We, therefore, tested the ATPase activity of \( \mu_2 \) in the presence of increasing concentrations of different cations. The results demonstrated that mM concentrations of a divalent cation are necessary for the activity and that Ca\(^{2+}\), Mg\(^{2+}\), and Mn\(^{2+}\) can each serve similarly well in this capacity (Fig. 4C). Synergistic effects of Mg\(^{2+}\) and Mn\(^{2+}\) (26) were not observed (data not shown). In other experiments we found that the pH optimum for ATP hydrolysis by \( \mu_2 \) was 6.5–7.0 (Fig. 4D). At higher temperatures (45 or 55°C) little activity was seen, and the activity at 25°C was higher than that at 35°C (Fig. 4D). Experiments to estimate the \( V_{\text{max}} \) apparent \( K_m \), and \( K_{\text{cat}} \) values of \( \mu_2 \) in hydrolysis of each NTP were performed with increasing concentrations of ATP, GTP, CTP, or UTP (Fig. 4E). The results confirmed ATP as the preferred substrate for hydrolysis and suggested this preference is based in modest increases in both substrate binding affinity and catalytic efficiency relative to GTP or CTP (Table II). UTP was a relatively poorer substrate for hydrolysis (Table II). These estimated values for the kinetic properties of \( \mu_2 \) in NTP hydrolysis are similar to published findings for several other viral NTPases (see “Discussion”).

\( \lambda_3 \) Interaction with wt and K415A/K419A \( \mu_2 \)—The two stoichiometrically minor core proteins, \( \mu_2 \) and \( \lambda_3 \), have been hypothesized to interact within cores (46–48). Direct evidence has remained lacking, but evidence from electron cryomicroscopy has suggested that the two proteins are at least juxtaposed in the core interior (5). In addition, the genetic evidence that \( \mu_2 \) can influence core transcriptase behaviors has sug-

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**Table II**

| Exp. no. | Substrate ± pmol of added \( \lambda_3 \) | \( V_{\text{max}} \) | \( K_m \) | \( K_{\text{cat}} \) |
|----------|---------------------------------|-----------------|--------|--------|
|          | nMol or fmol min\(^{-1}\)      | nMol or \( \mu \) | min\(^{-1}\) |
| 1        | ATP                             | 2.4             | 2.5    | 830    |
| 2        | ATP                             | 1.7             | 2.9    | 590    |
| 3        | GTP                             | 1.4             | 3.7    | 480    |
| 4        | GTP                             | 1.3             | 3.8    | 440    |
| 5        | UTP                             | 0.29            | 9.1    | 73     |
| 6        | ATP + 0.87                      | 0.93            | 2.5    | 320    |
| 7        | ATP + 4.4                       | 1.0             | 2.6    | 350    |
| 8        | RNA                             | 1.3             | 2.6    | 450    |

\(^{a}\) Estimated from hyperbolic fits to the Michaelis-Menten equation by nonlinear regression with Prism 4.0 (GraphPad Software). \( V_{\text{max}} \) values are expressed in nMol min\(^{-1}\) unless otherwise indicated. \( K_m \) values are expressed in mM unless otherwise indicated. Differing \( V_{\text{max}} \) values for ATP hydrolysis between experiments reflect variation in the levels of activity observed with different purified preparations of wild-type \( \mu_2 \) and after different times of protein storage at 4°C. \( K_m \) values for ATP hydrolysis were much less variable (2.6 ± 0.2 nM) in these experiments. \(^{b}\) Turnover number calculated from \( V_{\text{max}} \) and amount of wild-type \( \mu_2 \) in each reaction.
K419A mutations do not affect this interaction, providing further evidence that the interaction is not driven by the nucleotide interactions but is specific to the protein-protein interaction. 

B - ATPase Activity of wt μ2

Increased concentrations of bovine serum albumin had no effect on the ATPase activity of wt μ2 (Fig. 5C), providing evidence that the stimulatory effect was specific to λ3. An experiment to estimate the effects of λ3 on the kinetic properties of μ2 in ATP hydrolysis (data not shown) suggested that increasing amounts of λ3 progressively increased the $K_m$ of the reaction but had little effect on its apparent $K_m$ (Table II). This last finding suggests that λ3 acted to increase the catalytic efficiency of μ2 and not its substrate binding affinity.

RTPase Activity of wt μ2 - The capacity of wt μ2 to release the γ-phosphate from an NTP led us to hypothesize that μ2 may also release the 5′-γ-phosphate from an RNA molecule. mORV cores mediate such an RTPase activity, which yields a diphosphorylated RNA 5′ end (11) for linkage to GMP by the λ2-associated guanylyltransferase (4, 11, 14, 15, 17, 18), as part of the mRNA capping process. The RTPase activity has been previously attributed to the λ1 shell protein (12). We nevertheless considered it possible that this activity may instead be represented by the RTPase activity of μ2, and we, therefore, tested purified μ2 for its capacity to release the γ-phosphate from RNA. An in vitro transcription reaction driven by T7 RNA polymerase was performed in the presence of $[\gamma-32P]GTP$ to generate RNA transcripts 45 nucleotides in length in which only the 5′-terminal γ-phosphate was radiolabeled. Alternatively, the reaction was performed in the presence of $[\alpha-32P]GTP$ to generate 45-mer transcripts with the radiolabel in internal positions. The quality of these substrate RNAs and their essential lack of contamination with $[\gamma-32P]$- or $[\alpha-32P]$-labeled RNA were confirmed by electrophoresis in denaturing polyacrylamide gels (data not shown). After incubation in the presence of purified wt μ2 followed by denaturing gel analysis, loss of radiolabel from the 45-mer RNA was observed with the $[\gamma-32P]GTP$-labeled substrate but not the $[\alpha-32P]GTP$-labeled substrate (Fig. 6). These results indicate that μ2 indeed displays RTPase activity and not a nonspecific nuclease activity that would have degraded both substrates.

The RTPase activity of μ2 was further analyzed by TLC. This assay revealed that wt μ2 in the presence of increasing concentrations of $[\gamma-32P]GTP$-labeled substrate RNA released increasing amounts of $[\alpha-32P]P$-labeled P (Fig. 7A, lanes 3–7). The addition of EDTA abolished the γ-phosphate release from RNA by wt μ2 (Fig. 7A, lane 8), indicating that the RTPase activity is dependent on divalent cations, as is the NTPase activity. K415A/K419A μ2 showed no activity in the RTPase assay (Fig. 7A, lanes 8–11).
RNA was visualized by phosphorimaging after separation of reaction products by TLC. The radioactivity from each TLC lane was determined by phosphorimaging and expressed as counts per minute (cpm) per unit of substrate RNA. A control sample containing 0.4 pmol of substrate RNA and 32P-labeled 45-mer poly(A) was included to demonstrate complete release of the 32P-labeled γ-phosphate.

**Discussion**

Results in this report demonstrate that mORV μ2 protein in the presence of divalent cations can catalyze removal of the γ-phosphate from either an NTP or the 5′ end of an RNA. When a purified protein is shown to exhibit a new enzymatic activity, it is always possible that the activity is attributable to a contaminant in the preparation and not the protein of interest. To rule out that possibility we introduced alanine substitutions in μ2 that we expected to reduce its NTPase and/or RTPase activities because the substitutions were located in a putative nucleotide binding region of μ2. These substitutions did not substantially affect the purification profile of the protein but did abolish its capacity for γ-phosphate removal from either type of substrate. Thus, both activities are attributable to the wt μ2 protein. Moreover, both activities involve the same putative nucleotide binding region of μ2 in which the alanine substitutions were located. An asparagine substitution for Lys-219 in Semliki Forest virus (SFV) 3C protease has been shown to abrogate both its NTPase and its RTPase activities (28). Similarly, alanine substitutions that inactivate the baculovirus and vaccinia virus RTPases also abrogate their NTPase activities (25, 26, 29).

Further evidence that the NTPase and RTPase activities are present in the μ2 protein unless it is always possible that the activity is attributable to a contaminant in the preparation and not the protein of interest.
attributable to μ2 is that λ3 mildly stimulated both activities of wt but not mutant, μ2. A viral component such as λ3 would be unlikely to have a stimulatory effect on the activities of a cellular contaminant, but since λ3 interacts with μ2 (Fig. 5), its stimulatory effect on the NTPase and RTPase activities of μ2 is not surprising. Because (i) λ3 alone did not show these activities, (ii) no increase in activities was seen when λ3 was incubated with mutant μ2, and (iii) λ3 interacts with both wt and mutant μ2, the increase in activities when λ3 was incubated with wt μ2 cannot be attributed to an effect of μ2 binding on latent activities of λ3. The new evidence for μ2-λ3 interaction in vitro also supports the hypothesis that λ3 and μ2 interact within mORV cores, as previously suggested by electron cryomicroscopy and genetic results (5, 31). Such an interaction would further manifest the close juxtaposition of different transcription and capping enzymes through protein-protein contacts within viral particles (4, 5) (see Table I), as has been recently shown for analogous cellular enzymes (for review, see Ref. 50 and 51).

Our estimated values for the kinetic properties of μ2 in NTP hydrolysis (Table II) are similar to published ones for several other divergent cation-dependent viral NTPases. For example, the apparent $K_m$ for ATP hydrolysis by NTPase/RNA helicase NPH-II from vaccinia virus has been reported as 1.2 mM (52), and the $K_{cat}$ for ATP hydrolysis by NTPase/RTPase D1 from vaccinia virus has been reported as 606 min$^{-1}$ (27). In addition, we found apparent $K_m$ and $K_{cat}$ values for the triphosphatase activities of μ2 to be much lower for RNA than for NTP substrates (Table II), and this trend has also been seen with other viral proteins. For example, the apparent $K_m$ for the RTPase activity of vaccinia virus D1 has been reported as 1.0 μM compared with 0.8 μM for ATP hydrolysis (27), and the $K_{cat}$ for the RTPase activity of Semliki Forest virus Nsp2 has been reported as 5.5 min$^{-1}$, compared with 230 min$^{-1}$ for GTP hydrolysis (28).

The literature indicates that it is common for RTPases to exhibit NTPase activities (25–29). The reciprocal statement is harder to support, however, because NTPases have been less routinely tested for RTPase activity. We nonetheless expect that many NTPases could act as RTPases in vitro without the latter being a normal aspect of their functions in cells. Thus, our demonstration that μ2 exhibits RTPase activity in vitro does not necessarily mean that it acts as an RTPase in cores. The same logic applies to the in vitro RTPase activity reported for the mORV λ1 protein (12).

The findings in this report corroborate previous genetic evidence for a role of μ2 in mORV core NTPase activities (24). Future investigations can now be focused on the specific role(s) of the triphosphatase activities of μ2 in core functions. For example, it will be important to dissect whether μ2 functions as an NTPase, an RTPase, or both within cores. The λ1 shell protein has been proposed to be the capping RTPase in cores based on its reported in vitro RTPase activity (12). However, given our new evidence for in vitro RTPase activity of μ2, there appears to be little reason from enzymatic or genetic data to conclude that λ1 is more likely than μ2 to represent the capping RTPase. The apparent $K_m$ values for RNA substrate reported for λ1 and μ2 are similarly low (0.26 and 0.32 μM, respectively), although the reported $K_{cat}$ of the λ1-RNA reaction (3.1 min$^{-1}$) is 10-fold higher than that of the μ2-RNA reaction (0.3 min$^{-1}$) (Ref. 12; this study, Table II). An in vitro system for coupled transcription and capping reconstituted from wt or mutant mORV proteins would be useful for dissecting the relative roles of λ1 and μ2 but has not been reported to date. We are currently testing core-like particles assembled in insect cells from baculovirus-expressed mORV proteins (39)$^8$ for this purpose. New information about the precise structural positions and orientations of proteins within the core may also be informative since whether the catalytic regions of λ1 or μ2 have access to the triphosphorylated 5’ end of nascent mRNA within the crowded core interior may be a key determinant of whether either protein truly acts as an RTPase during viral mRNA synthesis (4, 9, 10).4

What other types of functions might μ2 or λ1 mediate that could be associated with NTP hydrolysis? Many enzymes are known to couple NTP hydrolysis to protein conformational changes; RNA and DNA helicases, myosins, kinesins, and G proteins are well known examples. In many cases the conformational changes allow movement of the NTPase along a nucleic acid or protein polymer track (53, 54). The λ3 crystal structure suggests some such possible roles for μ2 or λ1 in melting, translocating, and/or reannealing the genomic minus-strand RNA as it is passed around the outside of the λ3 polymerase, whereas the genomic minus-strand RNA is passed through the central cavity (and catalytic site) of λ3 during transcript elongation (9). Alternatively, an NTP-dependent activity by either μ2 or λ1 could be required during a limited part of each transcription cycle, such as for template melting or translocation before the transcript has reached a certain length or for reinitiation after termination. To understand the functions of the mORV core as a molecular machine for mRNA synthesis, the roles of μ2 and λ1 must be characterized in more detail. The capacity of μ2 to bind to microtubules (this study, Fig. 3E; also see Refs. 35 and 36) dictates that we must also be alert to possible NTP-dependent functions of μ2 during mORV genome replication and assembly in infected cells.

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Nucleoside and RNA Triphosphatase Activities of Orthoreovirus Transcriptase Cofactor μ2
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