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Characterization of an ATPase Activity in Reovirus Cores and Its Genetic Association with Core-Shell Protein λ1

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A previously identified nucleoside triphosphatase activity in mammalian reovirus cores was further characterized by comparing two reovirus strains whose cores differ in their efficiencies of ATP hydrolysis. In assays using a panel of reassortant viruses derived from these strains, the difference in ATPase activity at standard conditions was genetically associated with viral genome segment L3, encoding protein λ1, a major constituent of the core shell that possesses sequence motifs characteristic of other ATPases. The ATPase activity of cores was affected by several other reaction components, including temperature, pH, nature and concentration of monovalent and divalent cations, and nature and concentration of anions. A strain difference in the response of core ATPase activity to monovalent acetate salts was also mapped to L3/λ1 by using reassortant viruses. Experiments with different nucleoside triphosphates demonstrated that ATP is the preferred ribonucleotide substrate for cores of both strains. Other experiments suggested that the ATPase is latent in reovirus virions and infectious subviral particles but undergoes activation during production of cores in close association with the protease-mediated degradation of outer-capsid protein μ1 and its cleavage products, suggesting that μ1 may play a role in regulating the ATPase.

The intact virion particles of mammalian reoviruses comprise 10 segments of genomic double-stranded RNA (dsRNA) enclosed in two concentric capsids of eight viral proteins. Virions can be partially disassembled in vitro by removing three of the four outer-capsid proteins to form the transcriptionally active core particle. The core, comprising five proteins, contains all of the enzymatic functions required to transcribe and export nascent reovirus mRNAs, utilizing the 10 genomic dsRNA segments as templates.

In addition to its activities in transcribing the viral mRNAs, the reovirus core contains all of the enzymatic functions needed to add a eukaryotic cap 1 structure to the 5′ end of each mRNA. This cap, which can be designated $m^7G(5')ppp(5')G^mpC$ (13), plays a role both in translation of mRNAs to protein and in assembly of mRNAs into newly forming virions (35). Thus, the reovirus core particle offers an unusual opportunity to study both transcription and capping in a well-defined structural setting.

Current understanding of the first steps in transcription and capping by cores is indicated below (adapted from reference 12). For simplicity, RNA products and substrates are represented as dinucleotides, although it remains unknown when capping occurs on nascent transcripts. Inorganic phosphate (P_i) and pyrophosphate (PP_i) side products are also indicated. To produce the final cap 1 structure on each mRNA, two methyltransferase reactions occur subsequent to the series shown.

$$\text{pppG} + \text{pppC} \xrightarrow{\text{RNA polymerase}} \text{pppGpC} + PP_i \quad (1)$$

$$\text{pppGpC} \xrightarrow{\text{RNA triphosphatase}} \text{ppGpC} + P_i \quad (2)$$

$$\text{pppG} + \text{ppGpC} \xrightarrow{\text{guanylyltransferase}} \text{GpppGpC} + PP_i \quad (3)$$

Most of the enzymatic activities in transcription and capping have been attributed either definitively or tentatively to specific core proteins. Minor core protein λ3, which is present in approximately 12 copies per particle, contains a series of sequence motifs characteristic of RNA polymerases (20, 26) and has been associated with the pH optimum of transcription in genetic studies with reassortant reoviruses (10). In addition, λ3 protein expressed from a recombinant vaccinia virus was shown to possess poly(C)-dependent poly(G)-polymerase activity (37), indicating strongly that this protein is an important component of the reovirus RNA polymerase (reaction 1). It seems, however, that auxiliary proteins, possibly including the minor core protein μ2 (45), may be required as additional subunits of the polymerase to provide specificity and processivity with reovirus-specific RNA templates. Outer-capsid protein λ2, present in 60 copies per core particle, possesses guanylyltransferase activity (reaction 3) (8, 11, 22) and may also act as one or both of the methyltransferases involved in capping (19, 34). Less is known about the initial step in mRNA capping, the RNA triphosphatase activity (reaction 2), and the protein responsible remains unknown.

Proteins λ1 (120 copies per core) and σ2 (120 to 180 copies per core) are the two remaining core proteins which together form the icosahedral lattice of the inner capsid (41, 42). To date, neither of these proteins has been definitively demonstrated to play a role in transcription or capping. Nevertheless, the deduced amino acid sequence of λ1 includes a putative nucleoside triphosphate (NTP)-binding motif, suggesting that it may have a transcription-related enzymatic activity (3), an implication supported somewhat by earlier studies (24, 31). dsRNA-binding activities have also been shown for both λ1 and σ2 (21, 33).

In addition to activities that have been explicitly demonstrated for transcription or capping by reovirus cores, two other activities have been proposed or demonstrated. First, an

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RNA helicase activity in cores was postulated to be the target for inhibition of transcript elongation by ribavirin (32). Second, a nucleoside triphosphatase (NTPase) activity in reovirus cores was firmly demonstrated (4, 18) and was later suggested to represent the capping-associated RNA triphosphatase (2, 13). Notably, however, the NTPase in cores exhibits a preference for ATP, whereas the 5′-terminal nucleotide (on which the RNA triphosphatase acts) in each of the 10 reovirus mRNAs is a guanosine (1), suggesting that the NTPase may represent a separate activity from the RNA triphosphatase.

To learn more about the role of the NTPase in transcription, we undertook studies to further characterize the NTPase activity in reovirus cores with particular regard to the utilization of ATP as a substrate. We investigated the effects of a number of reaction components (temperature, pH, divalent cations, substrate concentration, etc.) on ATP hydrolysis by cores through quantitation of the released inorganic phosphate ion in a colorimetric assay. Using cores from two distinct strains of reovirus, type 1 Lang (T1L) and type 3 Dearing (T3D), we identified several strain-dependent differences in ATPase activity. By exploiting two of these strain differences, we showed through genetic analyses with cores from reassortant viruses that the ATPase phenotype is genetically determined by reovirus genome segment L3, which encodes the core-shell protein λ1. We also confirmed that λ1 possesses sequence motifs conserved in other, known ATPases, including ATP-dependent RNA helicases (16). Lastly, we demonstrated that ATPase activity is substantially greater in cores than in intact virions or infectious subviral particles (ISVPs) and that activation of the ATPase occurs in close parallel with proteolytic degradation of outer-capsid protein μ1 and its cleavage products at the ISVP-to-core particle transition.

MATERIALS AND METHODS

Cells and viruses. Spinner-adapted L cells were grown in Joklik's modified Eagle's minimal essential medium (Irvine Scientific, Santa Ana, Calif.) containing 2% fetal bovine serum (HyClone Laboratories, Logan, Utah), 2% bovine calf serum (HyClone), 2 mM glutamine (Irvine), 100 U of penicillin G (Irvine) per ml, and 100 μg of streptomycin (Irvine) per ml. Reovirus strains T1L and T3D were laboratory stocks obtained from Bernard N. Fields. We also used T1L × T3D reassortant viruses that were described previously (reviewed in reference 29). Third-passage L-cell lysate stocks of twice-plaque-purified reovirus clones were used to generate purified virion preparations as previously described (12). Virion concentrations in purified preparations were determined by assuming that an optical density at 260 nm of 1 equals 2.1×10^{12} virions/ml (36).

To produce core particle preparations, purified virions at a concentration $>2.4 \times 10^{13}$ particles/ml in virion buffer (150 mM NaCl, 10 mM $MgCl_2$, 10 mM Tris-Cl [pH 7.5]) were digested for 90 min at 37°C with 200 μg/ml of *N*α-*p*-tosyl-L-lysine chloromethyl ketone (TLCK)-treated bovine α-chymotrypsin (Sigma Chemical Co., St. Louis, Mo.). Digestion was stopped by adding 5 mM phenylmethylsulfonyl fluoride (Sigma) to reactions and cooling to 4°C. Digests containing cores were diluted with virion buffer to a concentration of 1.2×10^{13} particles/ml prior to use in standard reactions (see below). In some cases, T1L and T3D core preparations were purified on CsCl gradients as described previously (12). Core concentrations in purified preparations were determined by assuming that an optical density at 260 nm of 1 equals 4.2×10^{12} particles/ml (9).

NTPase reactions and colorimetric assay for phosphate ion. We performed the NTPase reactions in 1.5-ml microtubes for ease of manipulation and incubation and then transferred aliquots to a 96-well microplate for development and measurement in a phosphate assay (7). Standard NTPase reaction mixtures contained 50 mM Tris-morpholinesulfonic acid (MES) (pH 8.5), 7.5 mM NaCl, 5 mM $MgCl_2$, 6×10^{11} cores per ml, and 1 mM ATP in a total volume of 60 μl. Reaction components were mixed on ice in a cold room, incubated at 35°C for 30 min (standard), and then returned to ice. Termination of each reaction was ensured by the addition of an equal volume of 10% trichloroacetic acid (TCA). To measure the amount of phosphate ion in each sample, 100 μl of the stopped reaction mixture was transferred to a microplate and mixed with an equal volume of colorimetric reagent (3 volumes of 0.8% ammonium molybdate, 1 volume of 6 N sulfuric acid, 1 volume of 10% [wt/vol] ascorbic acid). After all samples in the experiment had been added, the microplate was incubated in a 37°C incubator for 30 min. During development, a reduced phosphomolybdate complex was formed, which was blue in color and quantifiable by A_{655} nm in a microplate reader (Bio-Rad Laboratories, Hercules, Calif.). In each experiment, samples containing ATP but no cores were included to permit correction for background attributable to phosphate released upon nonenzymatic hydrolysis of ATP (usually $<30\ A_{655}$ units per sample). Variations to standard conditions are described in the text.

A_{655} values could be converted to amounts of phosphate ion (HPO_4^{2-}) released per reaction by comparison with a standard curve of K_2HPO_4 (0, 2, 5, 10, 20, 40, and 60 nmol in 100-μl samples assayed as described above) that was included in most experiments. Using these standards, we found the colorimetric assay to distinguish differences in phosphate concentrations of less than 5 μM, or differences of less than 500 pmol of phosphate ion released per reaction (data not shown). The standard curve was found to be highly reproducible from experiment to experiment, yielding a mean value of $0.025 \pm 0.003\ A_{655}$ units per nmol of phosphate ion in a 100-μl sample and implying that little variability was introduced in the detection phase of the assay. As a consequence, raw A_{655} values were generally used for the comparisons between strains.

TLC assay for NTPase products. [α-^{32}P]ATP (400 Ci/mmol) (Amersham Corp., Arlington Heights, Ill.) was added to standard reactions to a concentration of 0.4 μM. Reaction mixtures were incubated at 35°C for 30 min, and then reactions were terminated by addition of an equal volume of 10% TCA. Reaction products (along with known standards) were analyzed by thin-layer chromatography (TLC). One-half microliter of reaction mixture was spotted onto plastic-backed polyethyleneimine-cellulose TLC sheets (EM Separations Technology, Gibbstown, N.J.) and developed by ascending chromatography in solvent (1 M formic acid, 0.5 M LiCl). After drying, reaction products were visualized by using a PhosphorImager (Molecular Dynamics, Inc., Sunnyvale, Calif.).

SDS-PAGE. Discontinuous sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed as previously described (27). Briefly, in preparation for electrophoresis, viral particles in virion storage buffer were mixed 1:1 with 2× sample buffer (250 mM Tris [pH 8.0], 4% 2-mercaptoethanol, 2% SDS, 20% sucrose, 0.02% bromophenol blue) and incubated at 95°C for 1 min. Samples were loaded into wells of 10% (2.6% bis) polyacrylamide gels and subjected to electrophoresis at 12- to 24-mA constant current until the dye front reached the bottom of the gel. Gels were fixed, stained with Coomassie blue, and air dried between cellulose sheets.

Statistics. Statistical analyses were performed by computer with the following programs: Systat 5.2 for the Mann-Whitney *U* test; Excel 4.0 for two-sample *t* test, simple linear regression, and χ^2 analysis; and SPSS 7.0 for multiple regression.

RESULTS

A difference in ATPase activity between reovirus T1L and T3D cores. To analyze the NTPase activity of reovirus cores, we used a sensitive colorimetric assay for phosphate ion (7) to measure the amount of phosphate that is released upon hydrolysis of NTPs. We first studied the NTPase by using ATP as the substrate, given previous findings that ATP is hydrolyzed at a higher rate than other NTPs by type 3 cores (4, 18). ATP hydrolysis was found to depend on both cores and ATP being added to reactions: no phosphate was detected when either cores or ATP was omitted (Fig. 1). When both cores and ATP were added, in contrast, an increasing amount of phosphate was detected over time (Fig. 1).

Cores of reovirus T1L were found to hydrolyze ATP at a higher rate than T3D cores (Fig. 1). The greater rate of hydrolysis by T1L cores was most evident at early time points such that the initial velocity of ATP hydrolysis by T1L cores approximated 2.5 times that by T3D cores in the experiment shown: approximately 0.81 nmol of phosphate ion released per min per 3×10^{10} T1L cores, versus 0.33 nmol phosphate of ion released per min per 3×10^{10} T3D cores. A similar difference in ATPase activity of T1L and T3D cores was confirmed in assays using numerous independent core preparations, suggesting that it was a genetically based difference between these strains that might be exploited to identify which of the reovirus genes determines the level of ATPase activity in cores (see below).

The colorimetric assay for phosphate ion exhibited a cross-reaction with pyrophosphate (data not shown). To estimate the relative contribution of a pyrophosphatase to the activity detected in cores, we used TLC to identify whether ADP or AMP was generated as the product of ATP hydrolysis (8). As previously observed with type 3 cores (4, 18), the product of reac-

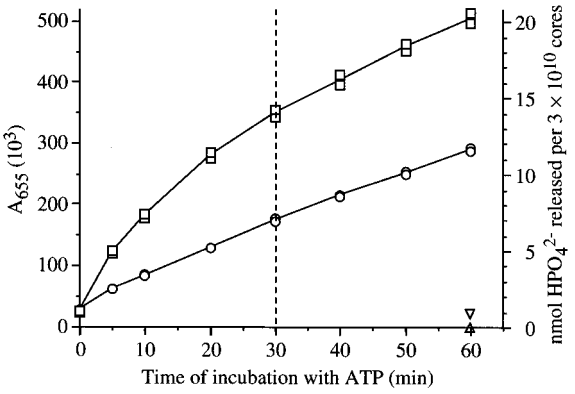

FIG. 1. Time course of ATP hydrolysis by reovirus cores. Standard reaction mixtures (see Materials and Methods) containing reovirus T1L cores plus ATP (□), reovirus T3D cores plus ATP (○), T1L cores but no ATP (▽), or ATP but no cores (△) were incubated at 35°C for different times. After stopping reactions with TCA, we determined the amount of inorganic phosphate ion that had been released by ATP hydrolysis by transferring an aliquot of each reaction mixture to a 96-well plate and performing a colorimetric assay based on the formation of phosphomolybdate complexes (7). The A_{655} for each sample was determined in a microplate reader, as shown on the left axis. A standard curve generated with K_2HPO_4 was used to convert A_{655} values to amounts of HPO_4^{2-} released, as shown on the right axis. Each time point was performed in duplicate, as indicated by the double symbols. Solid lines connect the mean values for each duplicate. The vertical broken line denotes the time point used in the subsequent genetic analysis and typically used in other endpoint analyses.

tions with both T1L and T3D cores was found to be ADP, with only minor production of AMP (Fig. 2). These findings confirm that the product detected in the colorimetric assay was primarily phosphate and that the measured activity was primarily a triphosphatase (NTPase) and not a pyrophosphatase. The strain difference whereby T1L cores hydrolyze ATP more rapidly than T3D cores was evident again in this experiment (Fig. 2).

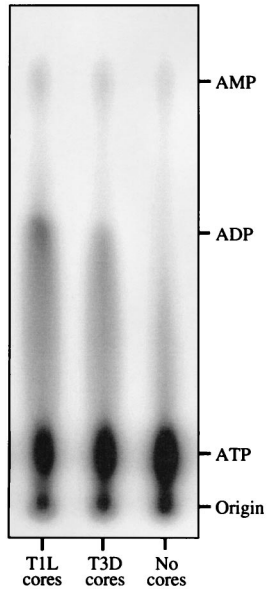

FIG. 2. Nucleotide products of ATP hydrolysis by reovirus cores. Standard reaction mixtures containing either T1L cores, T3D cores, or no cores were supplemented with [α-^{32}P]ATP and incubated at 35°C for 30 min. After the reactions were stopped with TCA, reaction products were resolved by TLC and detected on a PhosphorImager. The positions of nonradiolabeled ATP, ADP, and AMP markers are indicated and were determined by UV absorption.

Genetic mapping of ATPase activity by using reassortant viruses. Reassortant viruses derived from wild-type T1L and T3D parents were characterized previously (reviewed in reference 29) and used for genetic analyses of other phenotypes that differ between these strains. We chose 24 of these reassortants, each with a unique mixture of T1L and T3D genes, for determining the genetic basis of the different rates of ATP hydrolysis by T1L and T3D cores. After purification from infected cells, reassortant and parental virions were subjected to chymotrypsin treatment to generate cores, as confirmed by SDS-PAGE. Equivalent numbers of cores (3×10^{10}, according to A_{260} [36]) were then tested for ATPase activity by incubation for 30 min at standard reaction conditions. In this manner, reassortant cores were found to exhibit characteristic ATPase activities that spanned a range of values encompassing those of the T1L and T3D parents (Table 1).

To ascertain whether one or more specific genes might play a role in determining the different ATPase activities of the 24 reassortant and two parent viruses, we subjected the data in Table 1 to a number of statistical tests (Table 2). Because of uncertainties about the normality and variance of the activity data with respect to parental origin of each gene segment, the nonparametric Mann-Whitney U test (46) is an appropriate univariate method for these data and was applied to each gene segment in the 26 viruses ranked by their mean activity values. According to this test, the parental origins of two gene segments, L3 and S1, correlated with ATPase activity to significant levels ($P < 0.05$): L3 to a very high level of significance ($P = 2 \times 10^{-5}$) and S1 to a moderate level of significance ($P = 0.008$). Parametric methods, assuming normal distributions and equal variances among samples, were also used. The two-sample t test was performed as a univariate method for each gene segment with respect to the mean activity values and again showed that the parental origins of gene segments L3 and S1 correlated with ATPase activity to significant levels ($P < 0.05$): L3 to an extremely high level of significance ($P = 8 \times 10^{-12}$) and S1 to a mild level of significance ($P = 0.02$). Simple linear regression was performed as another univariate method and showed that the parental origins of four gene segments, L3, S1, L2, and S3, can individually account for ≥5% of the variance in ATPase activities among the 26 viruses ($r^2 \geq 0.05$): 86, 22, 9, and 5% for L3, S1, L2, and S3, respectively. When multiple regression was performed, however, a simpler model was identified by which 94% of the variance in ATPase activities can be explained by contributions from the 10 gene segments, and only 1 gene segment accounts for >5% of this variance ($r^2 > 0.05$): L3 for 86%.

When the 26 viruses were listed in rank order according to ATPase activity as in Table 1, a segregation of viruses and activities into two distinct groups was evident, permitting another method of analysis that is more typical of the older reovirus literature. In this case, all viruses with the L3 gene derived from T1L had an ATPase activity of ≥286 A_{655} units, whereas all viruses with the L3 gene derived from T3D had an ATPase activity of ≤231 A_{655} units. Using this empirical cutoff to distinguish between viruses with high (T1L-like) and low (T3D-like) activity, each of the nine gene segments other than L3 exhibited at least eight exceptions to segregation with the different levels of ATPase activity (Table 1). With the data divided in this fashion, χ^2 analysis for each gene segment demonstrated that parental origin of the L3 gene alone correlated with ATPase activities among the 26 viruses to a very high level of significance ($P = 3 \times 10^{-6}$).

From these tests, we conclude that the L3 genome segment is the primary genetic determinant of the differences in ATPase activity observed for T1L, T3D, and reassortant cores.

TABLE 1. Genetic analysis of absolute ATPase activity by T1L, T3D, and reassortant cores at standard conditions (study 1)

Virus strain	Parental origin of gene segment (core protein)[a]										ATP hydrolysis (A_{655} [10^3])[c]:	
	L1 (λ3)	L2 (λ2)	**L3[b] (λ1)**	M1 (μ2)	M2	M3	S1	S2 (σ2)	S3	S4	Mean	SD[d]
EB120	D	D	**D**	L	L	D	D	D	L	L	101	10
EB136	D	D	**D**	L	D	L	D	D	D	D	111	6
EB62	D	D	**D**	D	D	D	D	L	D	L	116	7
EB39	L	D	**D**	L	D	D	D	D	D	D	138	18
EB137	D	D	**D**	D	L	L	D	L	L	L	141	3
H15	L	D	**D**	L	D	D	D	D	D	L	147	7
T3D	D	D	**D**	D	D	D	D	D	D	D	152	12
E3	D	D	**D**	D	L	D	D	D	D	D	167	10
EB129	D	D	**D**	D	D	L	D	L	L	D	172	3
EB86	L	D	**D**	D	D	L	D	D	D	L	173	7
EB147	D	D	**D**	D	D	L	L	D	L	L	200	3
H14	L	L	**D**	L	L	L	L	D	D	L	201	5
EB145	D	D	**D**	D	D	L	L	D	D	D	231	4
EB143	D	L	**L**	L	L	L	D	L	L	L	286	8
EB87	L	D	**L**	L	D	L	L	D	L	L	328	6
G2	L	D	**L**	L	L	L	D	L	L	L	340	5
T1L	L	L	**L**	L	L	L	L	L	L	L	362	7
EB144	L	L	**L**	L	D	D	L	L	D	L	364	4
KC13	D	D	**L**	D	D	D	D	L	L	D	370	28
EB146	L	L	**L**	D	L	L	L	L	L	D	383	7
H9	D	D	**L**	D	L	L	D	D	D	D	387	10
EB15	D	D	**L**	L	L	D	L	D	L	D	389	17
H41	D	D	**L**	L	L	D	L	L	D	L	397	31
EB31	L	L	**L**	D	L	L	L	D	D	L	410	2
KC34	L	D	**L**	D	D	D	L	D	D	L	416	25
EB123	D	D	**L**	D	D	D	D	D	L	D	512	5

[a] Parental origins of gene segments and proteins in each reassortant strain were recently confirmed by SDS-PAGE (28). D, gene segment derived from T3D; L, gene segment derived from T1L.
[b] Segregation of the L3 gene segment (λ1 core protein) with high and low ATPase activity by cores is highlighted with boldface and with a space separating the T3D and T1L L3-containing strains.
[c] ATP hydrolysis was determined as A_{655} by colorimetric assay for released phosphate ion as described in the text.
[d] SD, standard deviation for mean ATPase activity from four determinations for each virus strain.

L3 encodes the λ1 protein, which is one of the major structural components of the core shell (42), plays a speculative role in transcription by cores (24, 31), and contains sequence motifs characteristic of an NTP-binding protein near its N terminus (3). Although some of the statistical tests suggest a role for the S1 gene segment as well, we consider it unlikely that S1 is truly a determinant of the ATPase activity of cores since the S1 gene products σ1 and σ1s are not found in cores (see below for additional data and discussion).

Kinetic basis for the difference in ATPase activity between T1L and T3D cores. In an effort to explain the difference in activity between T1L and T3D cores, we studied the kinetic

TABLE 2. Statistical values for influence of gene segments on ATPase activity

Test[a]	P or r^2 value for each gene segment (core protein)[b]										Value for all 10 genes[c]
	L1 (λ3)	L2 (λ2)	L3 (λ1)	M1 (μ2)	M2	M3	S1	S2 (σ2)	S3	S4	
Study 1											
U (P)			2×10^{-5}				0.008				
t (P)			8×10^{-12}				0.02				
SR (r^2)		0.09	0.86				0.22		0.05		
MR (r^2)			0.86								0.94
χ^2 (P)			3×10^{-6}								
Study 2											
U (P)		0.03	8×10^{-4}								
t (P)			7×10^{-9}								
SR (r^2)	0.05	0.19	0.91	0.09	0.19		0.18		0.21		
MR (r^2)			0.90								0.99
χ^2 (P)			5×10^{-4}								

[a] The following statistical tests were used: Mann-Whitney U test (nonparametric), two-sample t test (parametric), simple linear regression (SR) (parametric), multiple regression (MR) (parametric), and χ^2 2 by 2 contingency analysis (nonparametric). Study 1, genetic mapping study presented in Table 1 (absolute ATPase activity at standard conditions). Study 2, genetic mapping study presented in Table 3 (ratio of ATPase activity in the presence and absence of 150 mM KOAc).
[b] For each gene segment, blank spaces denote P values of >0.05 or r^2 values of <0.05.
[c] An r^2 value for all 10 genes is applicable to multiple regression only.

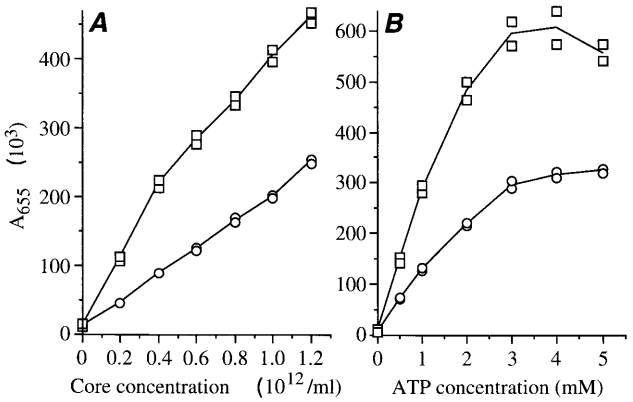

FIG. 3. ATP hydrolysis by cores as a function of core or ATP concentration. T1L cores (□) or T3D cores (○) were incubated at 35°C for 30 min in reaction mixtures containing standard components except for different core concentrations (A) or ATP concentrations (B). After reactions were stopped with TCA, the amount of released phosphate ion was measured as A_{655} by colorimetric assay. Each set of conditions was performed in duplicate, as indicated by the double symbols. Solid lines connect the mean values for each duplicate.

behavior of the ATPase. ATP hydrolysis by cores of each strain increased in an approximately linear fashion with increasing core (i.e., enzyme) concentration (Fig. 3A). T1L cores, however, showed a more rapid increase in activity as a function of core concentration than did T3D cores. ATP hydrolysis by cores of each strain also increased in an approximately linear fashion with increasing, low concentrations of ATP (i.e., substrate), with T1L cores again showing a more rapid increase in activity than T3D cores (Fig. 3B). In addition, T1L and T3D cores approached distinct maxima of ATPase activity as ATP concentration neared 3 mM in the presence of 5 mM $MgCl_2$ (Fig. 3B). A similar dependence of activity on ATP concentration, with a similar difference in activity maxima between strains, was observed when the concentration of $MgCl_2$ was increased to 15 mM, indicating that the activity maxima of the two strains were not a consequence of limiting Mg^{2+} ion (see below). These findings (different activity maxima reached at similar ATP concentrations) suggest that T1L and T3D cores exhibit similar affinities for ATP and that their different activities result instead from different turnover numbers for ATP hydrolysis. To confirm this tentative conclusion, we attempted to perform more formal kinetic analyses to determine K_m and V_{max} values for T1L and T3D ATPases; however, initial studies indicated that ATPase activities for both T1L and T3D cores did not obey Michaelis-Menten kinetics, making the K_m and V_{max} values of questionable validity and utility (see Discussion).

Specificity for nucleotide substrates. To study the substrate specificity of the core NTPase, we performed experiments using different nucleotide substrates (Fig. 4). ADP and AMP were hydrolyzed to a negligible extent by both T1L and T3D cores, confirming that the triphosphate substrate is required (also see Fig. 2). AMP-PNP, an ATP analog with a nonhydrolyzable β-γ phosphoanhydride bond, was hydrolyzed negligibly as well, indicating that the β-γ bond, and not the α-β bond, is the usual site of ATP hydrolysis by cores in these assays. Different NTPs were hydrolyzed at different efficiencies by both T1L and T3D cores, in the order ATP > GTP > CTP > UTP. Notably, GTP, which one might expect to be the preferred NTP substrate for the capping RNA triphosphatase since all reovirus mRNAs have an encoded guanosine at their 5′ ends (see Discussion), was hydrolyzed at only 40% of the efficiency of ATP by T3D cores and even less efficiently, 12% of the efficiency of ATP, by T1L cores. dNTPs were also hydrolyzed by cores of these strains, in order of efficiency dATP > dGTP > dCTP ≈ dTTP. In fact, dATP, dGTP, and dCTP were cleaved somewhat more efficiently than their ribonucleotide counterparts, confirming previous results (4, 18). The explanation for this finding remains unclear although similar results have been obtained with other ATPases (39). T3D cores were consistently more efficient than T1L cores at hydrolyzing other NTP and dNTP substrates relative to ATP or dATP.

In an effort to explain why T1L and T3D cores cleave ATP with greater efficiency than other ribonucleotides, we analyzed the hydrolysis of GTP or CTP by cores over a range of substrate concentrations. Results were similar to those described for ATP above, namely, that hydrolysis of GTP or CTP by cores of either strain increased in an approximately linear fashion with increasing low concentrations of NTP but reached distinct maxima of activity as the concentration neared 4 mM for GTP or 3 mM for CTP (data not shown). Given difficulties in making valid determinations for K_m and V_{max} with each substrate, however, we cannot draw firm conclusions regarding the kinetic basis of these strain differences.

Effects of temperature and pH on the ATPase. Previous studies examined the effects of a variety of other reaction components on the ATPase activity of reovirus type 3 cores (4, 18). We performed related experiments in an effort to compare T1L and T3D cores and to clarify the nature of their differences in ATPase activity.

Temperatures at 5°C intervals between 25 and 60°C were tested for their effects on ATP hydrolysis by T1L and T3D cores. The profiles of the two reoviruses were found to be very different (Fig. 5A). One difference was that T3D cores exhibited a substantially lower ATPase activity than T1L cores at 25 to 35°C, and it is this difference that was examined in the preceding genetic analysis (Tables 1 and 2). In addition, T1L and T3D cores showed distinct temperature optima, 35 to 40°C for T1L versus 50°C for T3D, and the temperature curve for T3D, but not T1L, cores had a distinctly biphasic appearance, with a phase transition near 35°C. The biochemical bases for

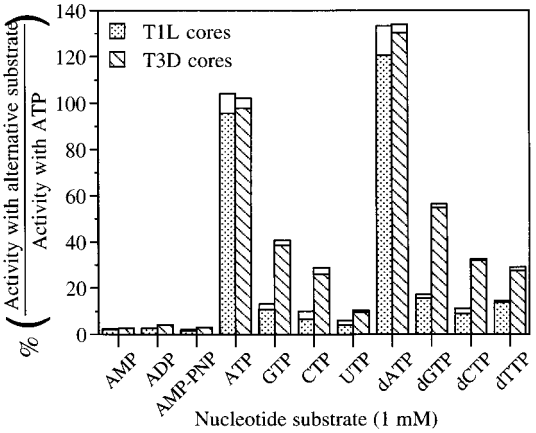

FIG. 4. Utilization of alternative nucleotide substrates by the core NTPase. Otherwise standard reaction mixtures containing the substrate indicated at 1 mM and either T1L or T3D cores were incubated at 35°C for 30 min. After reactions were stopped with TCA, the amount of phosphate ion released from each substrate was measured as A_{655} by colorimetric assay. For each strain, activity is presented as a percentage of that observed in the presence of ATP. Each set of conditions was performed in duplicate, as indicated by the cap on each bar. AMP-PNP is an ATP analog that contains a nonhydrolyzable β-γ phosphoanhydride bond.

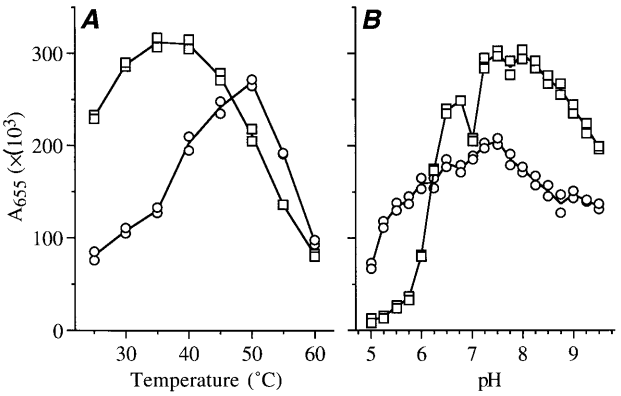

FIG. 5. ATP hydrolysis by cores as a function of temperature or pH. Otherwise standard reaction mixtures containing either T1L cores (□) or T3D cores (○) were incubated for 30 min at pH 8.5 and different temperatures (A) or 35°C and different pH values achieved with 50 mM Tris-MES buffers (B). After reactions were stopped with TCA, the amount of released phosphate ion was measured as A_{655} by colorimetric assay. Each set of conditions was performed in duplicate, as indicated by the double symbols. Solid lines connect the mean values for each duplicate. The same tick labels for A_{655} apply to both panels.

the different temperature optima of the T1L and T3D ATPases and the biphasic nature of the T3D temperature curve remain unknown (see Discussion).

To examine the effects of pH, the ATPase activities of T1L and T3D cores were tested at intervals of 0.25 units between pH 5 and 9.5. Cores of the two reoviruses were similar in having activity over a broad pH range, with a global optimum near pH 7.5 (Fig. 5B). Differences were noted, however, in the specific characters of their profiles. One difference was that T1L cores showed higher activity than T3D cores at pH values of >7, and it is this difference that was examined in the preceding genetic analysis (Tables 1 and 2). At pH values of <6.25, in contrast, T3D cores showed higher activity than T1L cores. In addition, the pH curve for T1L, but not T3D, cores showed evidence for two peaks of activity, near pH 6.5 to 6.75 and pH 7.5 to 8.5, respectively. The biochemical bases for the higher activity of T3D cores at low pH and the complex nature of the T1L pH curve remain unknown (see Discussion).

Effects of salts on the ATPase. Divalent cations were confirmed to be required for ATPase activity by both T1L and T3D cores. In the absence of added divalent cation, ATPase activity by both T1L and T3D cores was minor (Fig. 6A) and was further abrogated by the addition of 1 mM EDTA (data not shown). In the presence of 1 mM ATP, ATP hydrolysis by cores of both strains approached their respective maxima as the $MgCl_2$ concentration neared 3 mM (Fig. 6A). Both T1L and T3D cores exhibited their highest ATPase activities when Mg^{2+} was used as the divalent cation, as opposed to Ca^{2+}, Mn^{2+}, Zn^{2+}, or Cu^{2+} (Fig. 6B). Substantial levels of activity were nonetheless also obtained in the presence of either Ca^{2+} or Mn^{2+}, particularly with T3D cores. For either T1L or T3D cores, the profile of ATP hydrolysis was nearly identical with $CaCl_2$ or $MnCl_2$ as with $MgCl_2$ in that maximal activity was reached as the divalent cation concentration neared 3 mM in the presence of 1 mM ATP (data not shown). With 5 mM ATP as the substrate, the profiles of ATPase activity were changed for both T1L and T3D cores in that low activities were observed with $MgCl_2$ concentrations below 3 mM and maximal activities were obtained only when $MgCl_2$ concentrations exceeded 5 mM (data not shown), suggesting that the formation of complexes between ATP and divalent cations is an important determinant of activity levels.

A slightly higher level of ATPase activity was obtained with cores of each strain when 5 mM magnesium acetate was substituted for $MgCl_2$ (data not shown), suggesting that the Cl− anion may act as a mild inhibitor. Stronger evidence for inhibition by Cl− was obtained when the ATPase activities of T1L and T3D cores were determined by using 100 mM Tris buffers that had been adjusted to pH 8.5 with either hydrochloric or acetic acid (data not shown). Inhibition of ATPase activity specifically attributable to Cl− was also demonstrated by inhibition seen when equivalent concentrations of KCl or potassium acetate (KOAc) were added to T1L or T3D cores (Fig. 7A). Similar conclusions were reached from experiments using Na+ or Li+ salts of these anions (data not shown). The ATPase activities of T1L and T3D cores were inhibited to nearly identical extents by Cl−, compared to OAc−, anion (Fig. 7B). These findings indicate that the inhibition of ATPase activity by 10 to 100 mM KCl or NaCl observed for type 3 cores by Borsa et al. (4) was likely attributable to the Cl− anion and not to the respective monovalent cations.

Yet another strain difference in ATPase activity of T1L and T3D cores was found when KOAc was added to the reaction mixtures. At 150 mM KOAc, the ATPase activity of T1L cores was slightly inhibited whereas that of T3D cores was stimulated in comparison to the respective activities in the absence of KOAc. We exploited this new strain difference to perform a second genetic analysis, in an attempt to confirm the preceding finding that L3 is a critical determinant of a difference in ATPase activity between cores of these strains. For this study we used a panel of 14 reassortant viruses (Table 3), including several that were not included in the first mapping (Table 1). After chymotrypsin treatment to generate cores, the ATPase activity of each reassortant and parent strain was determined in the presence and absence of 150 mM KOAc. When the 16 viruses were listed in rank order according to the ratio of their ATPase activities with and without 150 mM KOAc (Table 3), it was evident that the strain difference segregated with parental derivation of the L3 gene such that all viruses with L3

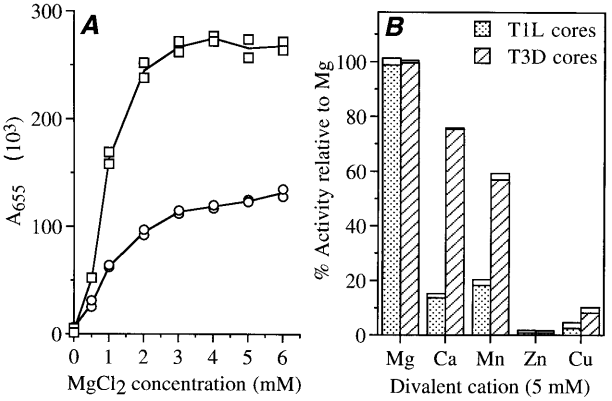

FIG. 6. Effects of divalent cations on core ATPase activity. (A) T1L cores (□) or T3D cores (○) were incubated at 35°C for 30 min in reaction mixtures containing standard components except for varying concentrations of $MgCl_2$. After reactions were stopped with TCA, the amount of released phosphate ion was measured as A_{655} by colorimetric assay. Each set of conditions was performed in duplicate, as indicated by the double symbols. Solid lines connect the mean values for each duplicate. (B) T1L or T3D cores were incubated at 35°C for 30 min in otherwise standard reaction mixtures containing the indicated divalent cation as a chloride salt at 5 mM. After reactions were stopped with TCA, the amount of phosphate ion released from each substrate was measured as A_{655} by colorimetric assay. For each strain, ATPase activity is presented as a percentage of that observed in the presence of 5 mM $MgCl_2$. Each set of conditions was performed in duplicate, as indicated by the cap on each bar.

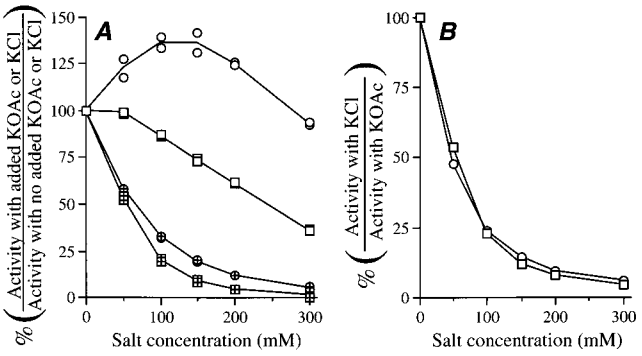

FIG. 7. Effects of acetate and chloride anions on core ATPase activity. T1L cores or T3D cores were incubated at 35°C for 30 min in reaction mixtures containing standard components plus various concentrations of KOAc or KCl. After reactions were stopped with TCA, the amount of released phosphate ion was measured as A_{655} by colorimetric assay. Each set of conditions was performed in duplicate, as indicated by the double symbols. Solid lines connect the mean values for each duplicate. (A) Percent ATPase activity in the presence of different concentrations of KCl or KOAc compared to no added salt. Values are shown for T1L cores plus either KOAc (□) or KCl (⊞) and T3D cores plus either KOAc (○) or KCl (⊕). (B) For either T1L cores (□) or T3D cores (○), ATPase activity in the presence of various concentrations of KCl is shown as a percentage of the activity observed with an equivalent concentration of KOAc.

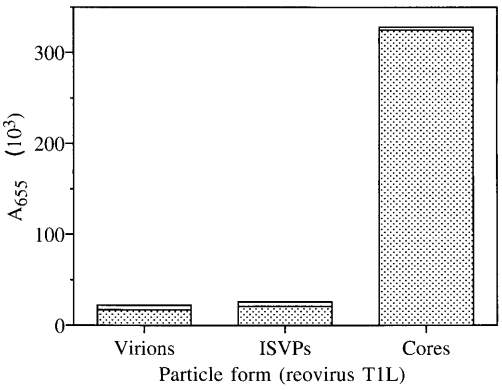

FIG. 8. ATPase activity by different reovirus particle forms. Particles of reovirus T1L were incubated at 35°C for 30 min in standard reaction mixtures. Particles took the form of purified virions, chymotrypsin digests in which virions had been converted to ISVPs, or chymotrypsin digests in which virions had been converted to cores. In each case, particles were added to reactions to the standard final concentration of 6×10^{11} particles per ml. After reactions were stopped with TCA, the amount of phosphate ion released from ATP was measured as A_{655} by colorimetric assay. Each particle form was tested in duplicate, as indicated by the cap on each bar.

derived from T1L exhibited an activity ratio of ≤0.89, whereas all viruses with L3 derived from T3D exhibited an activity ratio of ≥1.24. To formalize the analysis, the same statistical tests as used in the first genetic study were applied to these data and confirmed that the L3 gene (λ1 protein) was the sole highly significant determinant of this strain difference (Table 2).

ATPase activity by different reovirus particle forms. In previous studies with type 3 reoviruses (4, 18), NTPase activity was found to be substantially less in intact virions than in cores. In addition, although explicit evidence was not presented, NTPase activity appeared to be low in ISVPs as well (5, 6). The ISVP is more clearly defined as a distinct particle form now (reviewed in reference 28) than when previous studies of the NTPase were performed. Thus, to clarify and extend the earlier work, we measured ATPase activities for the different particle forms of reovirus T1L and T3D.

Comparable to findings in previous studies, we found that purified virions of both T1L (Fig. 8) and T3D (data not shown) display only a low level of ATPase activity. To control more precisely for particle number between samples, we subjected the purified virions to in vitro digestion with chymotrypsin to generate either ISVPs or cores as products and confirmed their identities by SDS-PAGE (data not shown). Upon testing the subvirion particles for ATPase activity, we found that ISVPs of both T1L (Fig. 8) and T3D (data not shown) display a low level of ATPase activity similar to that of virions, which is substantially less than that displayed by cores (T1L, Fig. 8; T3D, data

TABLE 3. Genetic analysis of ATPase activity in the presence and absence of 150 mM KOAc by T1L, T3D, and reassortant cores (study 2)

Virus strain	Parental origin of gene segment (core protein)[a]										ATP hydrolysis (activity with KOAc/activity with no KOAc)[c]
	L1 (λ3)	L2 (λ2)	**L3[b] (λ1)**	M1 (μ2)	M2	M3	S1	S2 (σ2)	S3	S4	
EB146	L	L	**L**	D	L	L	L	L	L	D	0.83
KC15	L	L	**L**	L	L	D	L	D	D	L	0.84
EB123	D	D	**L**	D	D	D	D	D	L	D	0.85
T1L	L	L	**L**	L	L	L	L	L	L	L	0.87
KC12	D	D	**L**	D	D	L	L	D	D	D	0.88
EB138	D	L	**L**	D	D	L	D	D	L	L	0.88
H5	D	D	**L**	L	L	D	L	D	L	D	0.89
F18	L	D	**L**	L	D	L	L	L	D	D	0.89
EB136	D	D	**D**	L	D	L	D	D	D	D	1.24
EB39	L	D	**D**	L	D	D	D	D	D	D	1.29
H14	L	L	**D**	L	L	L	L	D	D	L	1.37
EB86	L	D	**D**	D	D	L	D	D	D	L	1.47
T3D	D	D	**D**	D	D	D	D	D	D	D	1.49
EB129	D	D	**D**	D	D	L	D	L	L	D	1.55
EB62	D	D	**D**	D	D	D	D	L	D	L	1.56
EB145	D	D	**D**	D	D	L	L	D	D	D	1.65

[a] For explanation of genotype designation, see Table 1, footnote a.
[b] Segregation of the L3 gene segment (λ1 core protein) with distinctive ratios of core ATPase activity in the presence and absence of 150 mM KOAc is highlighted with boldface and with a space separating the T1L and T3D L3-containing strains.
[c] ATP hydrolysis was determined as A_{655} by colorimetric assay for released phosphate ion as described in the text. Values are presented as ratio of ATP hydrolysis in presence of 150 mM KOAc divided by ATP hydrolysis in absence of KOAc and represent the means of two determinations.

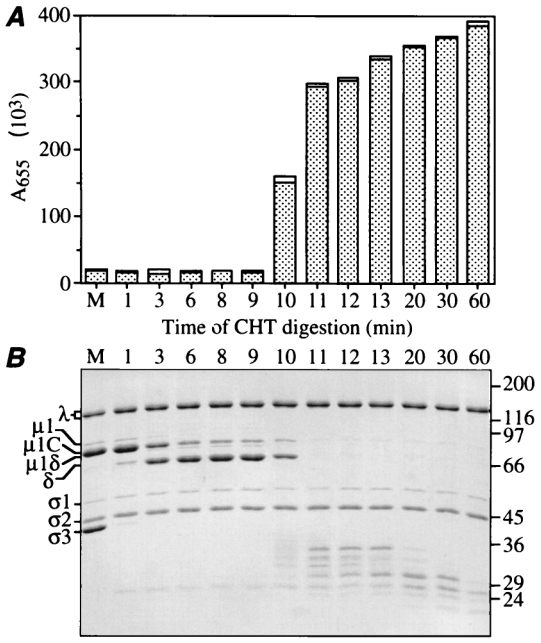

FIG. 9. Activation of ATPase activity and degradation of outer-capsid protein μ1 in a time course of chymotrypsin (CHT) treatment generating cores as products. A sample containing an initial mixture of virion buffer, T1L virions (3×10^{13} per ml), and chymotrypsin (200 μg per ml) was incubated at 37°C, and aliquots were removed at different times to tubes containing phenylmethylsulfonyl fluoride to quench the chymotrypsin digestion. Each aliquot was further split to permit determination of ATPase activity under standard conditions as A_{655} by colorimetric assay for released phosphate ion (A) and determination of viral protein content by SDS-PAGE (B). A mock-digested sample (M, no chymotrypsin added) was also included and analyzed in the same fashions. Positions of viral proteins (left) and molecular weight markers (right; molecular weights indicated in thousands) are labeled in panel B.

not shown). When we repeated these experiments with gradient-purified preparations of T1L ISVPs, T1L cores, or T3D cores, ATPase activities essentially identical to those for the analogous nonpurified particles were demonstrated (data not shown). Gradient-purified T3D ISVPs were not tested because of their poor stability in high-concentration stocks (data not shown).

The preceding findings suggest that an ATPase in reovirus particles undergoes activation from a latent state at the ISVP-to-core transition during generation of subvirion particles in vitro. To confirm this interpretation, we performed a time course of digestion with chymotrypsin in which T1L virions were converted first to ISVPs and then to cores. Proteolytic events across the time course were monitored by subjecting aliquots of samples to SDS-PAGE (Fig. 9B). Another aliquot from each time point was used to measure ATPase activity (Fig. 9A). ATPase activation occurred in close temporal association with the degradation of outer-capsid protein μ1 and its cleavage products μ1C, μ1δ, and δ (27) that accompanies the ISVP-to-core transition. Key events were noted between 9 and 11 min after addition of chymotrypsin to virions, when rapid proteolysis of μ1-derived fragments (μ1C, μ1δ, and δ) yielded degradation products (M_rs of 20,000 to 40,000) accompanied by a concomitantly rapid increase in ATPase activity. In addition, a continued increase in ATPase activity with times of digestion between 11 and 60 min was accompanied by continued cleavage of the μ1-derived degradation products into even smaller fragments. These findings may indicate a role for μ1 and its cleavage products in negatively regulating the ATPase. Essentially identical results were obtained in a similar experiment with chymotrypsin treatment of T3D virions (data not shown).

DISCUSSION

Genetics of ATPase activity in T1L and T3D cores. We subjected the core particles of two reovirus strains, T1L and T3D, to detailed comparisons of their ATPase activities and identified several marked differences. Preliminary work with five other serotype 1 and six other serotype 3 strains showed that the cores of each possess ATPase activity with primarily T1L-like characteristics (data not shown), indicating that the T1L and T3D ATPases exhibit strain- and not serotype-specific differences. A two- to threefold difference in levels of ATP hydrolysis by T1L and T3D cores under our defined standard conditions was exploited to perform a genetic analysis using T1L × T3D reassortant viruses.

Two separate reassortant analyses (Tables 1 and 3) indicated that differences in ATPase activity between T1L and T3D cores are determined by genome segment L3, which encodes the major core-shell protein λ1. In each study, segregation of the L3 segment with ATPase phenotype was confirmed by multiple statistical analyses, which combine to support this conclusion. Although at least two statistical tests identified moderately significant associations between ATPase phenotype and the S1 segment in study 1 and the L2 segment in study 2 (Table 2), we consider it unlikely that these segments actually contribute to defining that activity since each segment appears to contribute in only one of the genetic studies. In addition, neither of the S1 gene products, outer-capsid protein σ1 and nonstructural protein σ1s, is known to reside in core particles. Instead, we suggest that the apparent contribution of S1 and L2 to ATPase phenotypes results from a restricted panel of reassortant genotypes that was available for analysis in these studies due to nonrandom segregation of the S1, L2, and L3 segments during generation of T1L × T3D reassortant viruses (29). The fact that an S1 or L2 contribution was not seen with multiple regression analysis, which can account for such confounding influences, supports our conclusion to discount the influence of S1 and L2 on ATPase activity.

An obvious extrapolation from the genetic analysis is that the λ1 protein itself is an ATPase, but this hypothesis must be formally tested in assays using λ1 expressed in isolation from other core proteins. No enzymatic activity has yet been assigned to λ1, although it was shown to contain N-terminal sequences (residues 8 to 14) similar to an NTP-binding motif (3) and was suggested to bind pyridoxal phosphate in association with that compound's inhibition of reovirus transcription (24) and capping (25). In addition, λ1 has been demonstrated to undergo a change in conformation upon the onset of transcription by cores (31), to bind dsRNA (21, 33), to interact with σ2, the other major core protein, in forming the core shell (41, 42), and to bind independently to core proteins λ2 and λ3 (37). The last two proteins have been shown to play important roles in reovirus transcription (λ3, RNA polymerase subunit) and capping (λ2, guanylyltransferase), and their interaction with λ1, coupled with the putative ATPase activity of λ1 defined in this study, suggests that λ1 too may play an important role in reovirus RNA synthesis.

Possible role(s) of the ATPase in reovirus transcription and capping. Soon after the NTPase activity in reovirus cores was first described (4, 18), it was suggested and has since become widely accepted that this activity represents the RNA triphosphatase, the enzyme responsible for mediating the first step in the mRNA capping reaction (2, 13, 22, 25). In fact, this con-

clusion is speculative, and we suggest that it may be unlikely for two reasons. First, since each of the reovirus mRNAs possess a 5′-terminal guanosine (1), it seems unusual that the RNA triphosphatase would exhibit a preference for ATP, and not GTP, as its NTP substrate. This preference for ATP was demonstrated for the NTPase in type 3 cores previously (4, 18) and was shown again in this study, even more profoundly with T1L cores. Second, another previous study demonstrated that the reovirus RNA triphosphatase activity is active in virions as well as in cores, in that the abortive transcripts produced by both types of particle are fully hydrolyzed to the 5′-diphosphorylated form (ppGpC, ppGpCpU, etc.) (44). This fact contrasts with the current observation that the ATPase is only minimally active in virions.

So, if it does not represent the capping RNA triphosphatase, then what role(s) might the NTPase play in the reovirus life cycle? Joklik and colleagues have speculated that cores might contain an RNA helicase required to open the genomic dsRNA templates for transcription by the viral polymerase (32, 37). Known RNA helicases are ATP-dependent enzymes that hydrolyze ATP as a source of energy for RNA unwinding. They often contain a series of conserved sequence motifs (15, 16, 17, 30), including the ATPase A motif (A/G)xxxxGK(S/T), which is involved in ATP binding (38, 40); the ATPase B motif, which is also involved in ATP binding (38, 40) and commonly contains the sequences DEAD or DExH in RNA and DNA helicases; and the sequence (Q/H)RxGRxxR, which is involved in RNA binding (30) or unwinding (17). We observe that the T3D λ1 contains sequences similar to these three motifs: PRKT KGKS at amino acids 5 to 12, DEAD at amino acids 100 to 103, and NRVGRFDR at amino acids 430 to 436. Thus, it seems possible that the ATPase activity tentatively assigned to λ1 may in fact represent its role as an RNA helicase that is required for one or more steps in reovirus RNA synthesis. Additional experiments are clearly needed to address this possibility. Since a sequence is not yet available for the T1L λ1 protein, it is not possible to compare the T1L and T3D sequences in an effort to explain the differences in ATPase activity observed with cores of these strains.

Regulation of the ATPase in reovirus particles. Observations concerning the ATPase activities of different reovirus particle forms suggest that there exists some mechanism for regulating the ATPase within the different particles. One possibility is that the decreased ATPase activities of virions and ISVPs result from an exclusion of the NTP substrates from catalytic sites within these particles, but this seems unlikely since NTPs are small and since virions are already known to utilize NTPs to produce abortive oligonucleotide transcripts (44). A more likely explanation would seem to be that the catalytic activity of the ATPase is inhibited by protein-protein or protein-RNA interactions within virions and ISVPs, which are absent or altered in cores. Since activation of the ATPase occurs in close association with degradation of μ1 and its cleavage products in time course experiments, μ1 is clearly one candidate for inhibiting the ATPase. A direct interaction between proteins λ1 and μ1 was previously suggested by genetic analysis of an extragenic suppressor of a temperature-sensitive mutation in μ1 (23).

Transcription by reovirus cores involves excessive initiation to produce large amounts of abortive transcripts (43). With cores, these abortive transcripts are generated in great molar excess to the full-length mRNAs. With virions, only the abortive transcripts are generated (44), suggesting that the RNA polymerase in virions is able to form productive initiation complexes but not to engage in elongation. Thus, the activation of transcription that occurs between virions and cores actually involves an activation of transcript elongation, which parallels an activation of the ATPase. In light of our suggestion that the ATPase may reflect an RNA helicase in reovirus cores, it is interesting that the eukaryotic protein TFIIH has been demonstrated to have ATPase and ATP-dependent DNA helicase activities and has been proposed to function at the promoter clearance step of transcription, permitting elongation to proceed (14). A similar function may be involved in reovirus transcription and may be a source of regulation.

Effects of reaction components on the ATPase. The ATPase activities of T1L and T3D cores are influenced by many components of the reaction mixtures in addition to cores and substrate, including temperature, pH, divalent cations, and anions. In some cases, the biological relevance of these effects remains unclear; however, we anticipate that characterizations of these effects will prove important as we continue to study the role(s) of NTPase activity in reovirus transcription and capping and the molecular basis of the differences in activity noted between T1L and T3D cores. Particularly for pH and temperature, the complex dependence of ATPase activity on these parameters (reflected in the biphasic nature of the curves) raises the possibilities that there may in fact be either (i) one activity modulated by two proteins (one of which is λ1) or (ii) two or more distinct ATPases or NTPases within cores. Preliminary evidence supports the existence of a second NTPase in reovirus cores, and further studies are in progress to confirm this hypothesis. The possible existence of a second activity may explain the difficulty that we encountered in determining valid K_m and V_{max} values.

Figure 7B shows that Cl− anions are more inhibitory to the core ATPase activity than OAc− anions; moreover, the similarity of the curves for T1L and T3D cores suggests that there is no difference in the anion effect between these two strains. It is also evident that 150 mM KOAc stimulates the ATPase activity of T3D cores but inhibits that of T1L cores (Fig. 7A). This strain difference, which forms the basis for the second genetic study (Table 3), suggests that either (i) K+ inhibits and OAc− stimulates or (ii) K+ stimulates and OAc− inhibits ATPase activity and that the relative levels of these antagonistic effects differ for the two strains. Although we have not formally shown that OAc− anions inhibit ATPase activity, the available evidence (that one small anion, Cl−, is inhibitory and that there is no strain difference when Cl− and OAc− effects on ATPase are compared) would appear to support the second conclusion, namely, that K+ cations stimulate and OAc− ions inhibit ATPase activity. If K+ cations do indeed stimulate ATPase activity, then it is interesting to note that the peak of this stimulation for T3D cores occurs near 150 mM, consistent with the intracellular K+ ion concentration.

Colorimetric assay for NTPase activity. A final word can be said about the colorimetric assay for phosphate ion (7) used to measure NTPase activity in this study. We adapted this assay, a modification of one commonly used in clinical chemistry laboratories, to a microplate format and found it to be sensitive, reliable, and extremely convenient to use. It compares favorably to related assays using α-^{32}P- or γ-^{32}P-labeled NTPs and clearly surpasses those assays in safety by not involving a radiolabeled compound. We can recommend this assay for use in studies of other NTPases.

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