

Core Protein $\mu 2$ Is a Second Determinant of Nucleoside Triphosphatase Activities by Reovirus Cores

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NTPase activities in mammalian reovirus cores were examined under various conditions that permitted several new differences to be identified between strains type 1 Lang (T1L) and type 3 Dearing (T3D). One difference concerned the ratio (at pH 8.5) of ATP hydrolysis at 50°C to that at 35°C. A genetic analysis using T1L \times T3D reassortant viruses implicated the L3 and M1 gene segments in this difference, with M1 influencing ATPase activity most strongly at high temperatures. L3 and M1 encode the core proteins $\lambda 1$ and $\mu 2$, respectively. Another difference concerned the absolute levels of GTP hydrolysis by cores at 45°C and pH 6.5. A genetic analysis using T1L \times T3D reassortants implicated the M1 gene as the sole determinant of this difference. The results of these experiments, coupled with previous findings (S. Noble and M. L. Nibert, *J. Virol.* 71:2182–2191, 1997), suggest either that a single type of NTPase in cores is strongly influenced by two different core proteins— $\lambda 1$ and $\mu 2$ —or that cores contain two different types of NTPase influenced by the two proteins. The findings appear relevant for understanding the complex functions of reovirus cores in RNA synthesis and capping.

In the intact virion particle of mammalian reoviruses, the 10 genomic segments of double-stranded RNA (dsRNA) are enclosed by eight viral proteins arranged in two concentric capsids (for a recent review on reoviruses, see reference 27). In vitro proteolysis can be used to cleave distinct subsets of the outer capsid proteins, producing either infectious subvirion particles or cores. The core comprises five proteins— $\lambda 1$, $\lambda 2$, $\lambda 3$, $\mu 2$, and $\sigma 2$ —and contains all the enzymatic functions needed to generate full-length, plus-sense transcripts from each genome segment and to export them from the core (3). The proteins of the core also add a eukaryotic cap 1 structure to the 5' end of each mRNA, designated $^mG(5')ppp(5')G^m pC$. The reovirus core thus represents a simple system for investigating several aspects of transcription and mRNA capping.

The biochemical pathway of transcription and capping by reovirus cores has been elucidated (reviewed in reference 35), and some of the enzymatic activities have been assigned to specific proteins. $\lambda 3$, possibly in concert with protein $\mu 2$ (45), serves as the viral RNA-dependent RNA polymerase (8, 25, 41). $\lambda 2$ provides the guanylyltransferase (5, 10, 21) and possibly also the methyltransferase (19, 34) activities in the capping process. The protein responsible for the first step in capping, the RNA triphosphatase (1, 12), has not yet been identified. Moreover, other enzymatic activities not yet assigned to specific proteins, including an RNA helicase activity, have been postulated to exist in cores and to play important roles in transcription (31, 41).

As part of our efforts to understand reovirus transcription and capping, we recently extended past studies (4, 18) by characterizing an NTPase activity in cores (28). Using cores of two virus strains, type 1 Lang (T1L) and type 3 Dearing (T3D), we investigated the effects of various reaction parameters (pH, temperature, divalent and monovalent cations, etc.) on ATP

hydrolysis. Consequently, we identified several strain-specific differences ATPase activity and exploited two of these to perform genetic analyses with T1L \times T3D reassortant viruses. In each of these analyses, the strain difference in ATP hydrolysis was shown to be genetically determined by the L3 gene segment. L3 encodes protein $\lambda 1$, which is a major structural component of the core shell (44), binds dsRNA (20, 33), and contains sequence motifs (2, 28) conserved in known ATPases (42) including RNA helicases (14). The genetic findings therefore indicate that $\lambda 1$ either mediates or modulates an NTPase activity in cores; however, the precise role of this activity in transcription, capping, or other core functions remains unknown.

In the preceding study, we additionally noted that ATP hydrolysis by T1L and T3D cores exhibited complex behavior in response to pH and temperature, respectively (28). As discussed in that paper, these observations suggested to us that a second viral component, in addition to $\lambda 1$, may contribute to the ATPase activity of reovirus cores. Because of our interest in identifying this second component, we performed the experiments in this study. Following initial experiments to identify proper conditions for comparing the activities of T1L and T3D cores, results of two independent studies with T1L \times T3D reassortant viruses identified the reovirus M1 gene, which encodes core protein $\mu 2$, as a second strong determinant of core NTPase activities. Based on these and the previous findings (28), two explanations seem plausible: either a single type of NTPase in cores is strongly influenced by two different core proteins— $\lambda 1$ and $\mu 2$ —or cores contain two different types of NTPase, which are influenced by the two proteins.

MATERIALS AND METHODS

Cells and viruses. L cells adapted to spinner culture 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 L-glutamine (Irvine Scientific), 100 U of penicillin G (Irvine Scientific) per ml, and 100 μ g of streptomycin (Irvine Scientific) per ml. Reovirus strains T1L and T3D were laboratory stocks. We also used T1L \times T3D reassortant viruses, which have been described previously (reviewed in reference 26). Third-passage L-cell-lysate stocks of twice-plaque-

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purified reovirus clones were used to generate purified virion preparations as previously described (11). Virion concentrations in purified preparations were determined with the equation $1 \text{ OD}_{260} = 2.1 \times 10^{12}$ virions/ml (40), where OD_{260} is optical density at 260 nm.

To produce core particle preparations, purified virions at a concentration of $>2.4 \times 10^{13}$ particles/ml in virion storage buffer (150 mM NaCl, 10 mM MgCl_2 , 10 mM Tris-HCl [pH 7.5]) were digested for 90 min at 37°C with 200 $\mu\text{g}/\text{ml}$ of *N*- α -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 reaction mixtures 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 reaction mixtures (see below). For some experiments (see Fig. 3 to 5), T1L and T3D core preparations were purified on CsCl gradients as described previously (11). Core concentrations in purified preparations were determined with the equation $1 \text{ OD}_{260} = 4.2 \times 10^{12}$ particles/ml (7).

NTPase reactions and phosphate assay. NTPase reactions were performed as previously described (28). Standard reaction conditions included the use of 50 mM Tris-MES [2-(*N*-4-morpholino)ethanesulfonic acid], 7.5 mM NaCl, 5 mM MgCl_2 , 6×10^{11} cores per ml, and 1 mM ATP or GTP. Temperature and pH were varied for specific experiments as described below. We mixed 50 mM Tris and 50 mM MES solutions to obtain buffers that spanned a wide pH range (pH 5 to 9.5) as measured at room temperature. Reaction mixtures were generated in a cold room and put on ice, incubated at the appropriate temperature for 30 min, returned to ice, and terminated by addition of an equal volume of 10% trichloroacetic acid (TCA).

The amount of released phosphate ion was quantitated as described previously (28). Briefly, 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) was added to an aliquot of the terminated reaction mixture in a microplate and incubated at 37°C for 30 min. Phosphomolybdate complexes formed during development were then detected by A_{655} in a microplate reader (Bio-Rad Laboratories, Hercules, Calif.). Samples containing ATP but no cores were included to permit correction for background attributable to phosphate released upon nonenzymatic hydrolysis of ATP (usually $<0.03 A_{655}$ units per sample).

Samples for each set of variables in an experiment were always generated in duplicate, each of which was then singly analyzed. To reduce the number of nonreliable data values, the values from duplicate samples in an experiment were both discarded if their mean deviation was $>5\%$. The data shown in the figures are means of results from duplicate samples in a single representative experiment.

Statistics. Statistical analyses were performed with the following programs: Systat version 5.2 for the Mann-Whitney U test; Excel 4.0 for χ^2 analysis; and SPSS version 7.0 for multiple regression.

RESULTS

T1L and T3D cores have divergent temperature curves for ATP hydrolysis at pH 8.5. As shown in previous work (28) and again in this study (Fig. 1A), the temperature curves for ATP hydrolysis at pH 8.5 by reovirus T1L and T3D cores differed in several respects. At lower temperatures, the curves were similar in that they included similar relative increases from 25 to 35°C. As a result, the 35°C/25°C activity ratios were nearly identical for the two strains, e.g., 1.50 for T1L and 1.54 for T3D in the experiments shown in Fig. 1A. However, T1L cores showed greater absolute activity from 25 to 35°C than did T3D cores, and it was the difference at 35°C that we previously mapped to the L3 gene using T1L \times T3D reassortants (28). At higher temperatures, the curves of the two strains were more distinct. T1L showed a plateau from 35 to 40°C and then decreased in activity from 40 to 50°C, whereas T3D showed steadily increasing activity from 35 to 50°C. Although the absolute activities at 50°C were similar for T1L and T3D cores, the 50°C/35°C activity ratios were quite different, e.g., 0.71 for T1L and 1.58 for T3D in the experiments shown in Fig. 1A. Thus, the 50°C/35°C activity ratio was a parameter that could differentiate strains and that could be subjected to a genetic analysis to identify viral proteins that influence ATPase activity.

The 50°C/35°C ATPase activity ratios of T1L and T3D cores at pH 8.5 are determined by the L3 and M1 genes. To identify the genetic basis for the distinct 50°C/35°C activity ratios of the two parents, the ATPase activities of cores of 29 T1L \times T3D

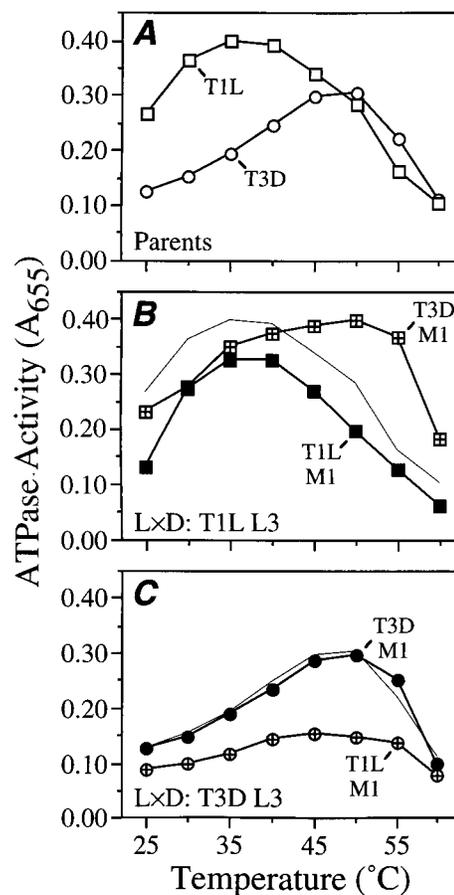


FIG. 1. Temperature curves for ATPase activity at pH 8.5 by T1L, T3D, and reassortant cores that contain the four possible pairings of T1L and T3D (L \times D) L3 and M1 genes ($\lambda 1$ and $\mu 2$ proteins). Cores in chymotrypsin digests were mixed with 1 mM ATP in pH 8.5 reaction buffer and incubated at the appropriate temperature for 30 min. Reactions were stopped by cooling and TCA addition, and the samples were analyzed for NTP hydrolysis by colorimetric assay for free phosphate. (A) T1L and T3D cores; (B) reassortants with T1L L3 and either T1L M1 (EB143) or T3D M1 (EB31); (C) reassortants with T3D L3 and either T1L M1 (H14) or T3D M1 (EB86). In panel B the T1L curve and in panel C the T3D curve are shown by finer lines for comparison with the reassortants.

reassortants were measured at pH 8.5 at both 50 and 35°C. In Table 1, the virus strains are listed in rank order according to these ratios, which span a range including T1L and T3D at values of 0.70 and 1.71, respectively. When these data were subjected to the Mann-Whitney U test, a nonparametric analysis (46), two genes—L3 and M1—were found to deviate from a random distribution within the ranked data set in a significant manner ($P = 0.0001$ and 0.0002 for L3 and M1, respectively) (Table 2). Similar conclusions were reached by multiple regression, a parametric analysis (46). According to the regression model, 98% of the variance in the 50°C/35°C activity ratios was explained by contributions from the 10 gene segments, but only the L3 and M1 genes individually accounted for $>5\%$ of the variance ($r^2 > 0.05$): 57 and 40%, respectively (Table 2). These data indicate that both the L3 gene (which encodes protein $\lambda 1$ and was implicated previously [28]) and the M1 gene (which encodes protein $\mu 2$) influence core ATPase activities.

A histogram plot of the 50°C/35°C activity ratios gave further insight into the genetic basis of ATPase activity (Fig. 2). The histogram shows that the activity ratios clustered into three

TABLE 1. Genetic analysis of ATPase activity ratios (50°C/35°C) in T1L, T3D, and reassortant cores at pH 8.5

| Virus strain | Parental origin of gene segment (core protein) ^a : | | | | | | | | | | Mean 50°C/35°C ratio ± SD of ATP hydrolysis (n) ^c |
|--------------|---|---------|----------------------|----------------------|----|----|----|---------|----|----|--|
| | L1 (λ3) | L2 (λ2) | L3 (λ1) ^b | M1 (μ2) ^b | M2 | M3 | S1 | S1 (ε2) | S3 | S4 | |
| EB144 | L | L | L | L | D | D | L | L | D | L | 0.58 ± 0.15 (6) |
| H60 | D | D | L | L | D | D | D | D | D | L | 0.59 ± 0.03 (4) |
| EB15 | D | D | L | L | L | D | L | D | L | D | 0.61 ± 0.03 (4) |
| H41 | D | D | L | L | L | D | L | L | D | L | 0.62 ± 0.01 (2) |
| KC2 | D | D | L | L | D | D | L | D | D | D | 0.63 ± 0.05 (4) |
| EB87 | L | D | L | L | D | L | L | D | L | L | 0.64 ± 0.13 (4) |
| G2 | L | D | L | L | L | L | D | L | L | L | 0.67 ± 0.10 (4) |
| T1L | L | L | L | L | L | L | L | L | L | L | 0.70 ± 0.02 (4) |
| EB143 | D | L | L | L | L | L | D | L | L | L | 0.72 ± 0.10 (4) |
| EB39 | L | D | D | L | D | D | D | D | D | D | 0.97 ± 0.10 (6) |
| H15 | L | D | D | L | D | D | D | D | D | L | 0.98 ± 0.09 (4) |
| EB123 | D | D | L | D | D | D | D | D | L | D | 1.04 ± 0.02 (4) |
| KC13 | D | D | L | D | D | D | D | L | L | D | 1.04 ± 0.08 (6) |
| KC55 | L | D | L | D | D | D | L | D | L | D | 1.07 ± 0.12 (4) |
| KC12 | D | D | L | D | D | L | L | D | D | D | 1.09 ± 0.11 (4) |
| EB138 | D | L | L | D | D | L | D | D | L | L | 1.10 ± 0.14 (4) |
| EB146 | L | L | L | D | L | L | L | L | L | D | 1.12 ± 0.04 (4) |
| KC34 | L | D | L | D | D | D | L | D | D | D | 1.12 ± 0.02 (4) |
| EB136 | D | D | D | L | D | L | D | D | D | D | 1.14 ± 0.16 (6) |
| EB31 | L | L | L | D | L | L | L | D | D | L | 1.16 ± 0.06 (4) |
| H9 | D | D | L | D | L | L | D | D | D | D | 1.17 ± 0.04 (4) |
| H14 | L | L | D | L | L | L | L | D | D | L | 1.21 ± 0.07 (4) |
| EB120 | D | D | D | L | L | D | D | D | L | L | 1.35 ± 0.05 (4) |
| EB86 | L | D | D | D | D | L | D | D | D | L | 1.60 ± 0.06 (4) |
| EB62 | D | D | D | D | D | D | D | L | D | L | 1.62 ± 0.27 (6) |
| EB129 | D | D | D | D | D | L | D | L | L | D | 1.63 ± 0.12 (4) |
| EB137 | D | D | D | D | L | L | D | L | L | L | 1.65 ± 0.05 (2) |
| EB145 | D | D | D | D | D | L | L | D | D | D | 1.67 ± 0.06 (4) |
| EB147 | D | D | D | D | D | L | L | D | L | L | 1.67 ± 0.11 (4) |
| E3 | D | D | D | D | L | D | D | D | D | D | 1.68 ± 0.07 (4) |
| T3D | D | D | D | D | D | D | D | D | D | D | 1.71 ± 0.13 (4) |

^a Parental origins of gene segments and/or proteins in each reassortant strain were recently confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (26). D, derived from reovirus strain T3D; L, derived from reovirus strain T1L.

^b Data indicate segregation of the L3 and M1 gene segments (λ1 and μ2 core proteins) with high and low 50°C/35°C activity ratios for ATP hydrolysis at pH 8.5.

^c ATP hydrolysis was determined as A_{655} by colorimetric assay for released phosphate ion as described in the text. *n*, number of determinations for each virus strain.

discrete groups of low, intermediate, and high values. Comparing the genotypes of different viruses to their positions in the histogram revealed that all viruses in the low-activity-ratio cluster (activity at 50°C < activity at 35°C) contained both L3 and M1 from T1L, all viruses in the high-activity-ratio cluster

(activity at 50°C > activity at 35°C) contained both L3 and M1 from T3D, and all viruses in the intermediate-activity-ratio cluster (activity at 50°C ≈ activity at 35°C) contained the two different heterologous pairings of L3 and M1. Since L3 was previously identified as the primary determinant of the differ-

TABLE 2. Statistical values for influence of gene segments on NTPase phenotype

| Data set from Table | Test ^a | <i>P</i> or <i>r</i> ² value for gene segment (core protein) ^b : | | | Value for all 10 genes ^c |
|---|------------------------------|--|---------|---------|-------------------------------------|
| | | L1 (λ3) | L3 (λ1) | M1 (μ2) | |
| 1 | U (<i>P</i>) | | 0.0001 | 0.0002 | 0.98 |
| | MR (<i>r</i> ²) | | 0.57 | 0.40 | |
| 1 (T1L-L3-containing reassortants only) | U (<i>P</i>) | | NA | 0.0003 | 0.99 |
| | χ ² (<i>P</i>) | | NA | 0.0002 | |
| | MR (<i>r</i> ²) | | NA | 0.96 | |
| 1 (T3D-L3-containing reassortants only) | U (<i>P</i>) | 0.02 | NA | 0.003 | 0.98 |
| | χ ² (<i>P</i>) | | NA | 0.003 | |
| | MR (<i>r</i> ²) | | NA | 0.88 | |
| 3 | U (<i>P</i>) | | | 0.0002 | 0.78 |
| | MR (<i>r</i> ²) | | | 0.67 | |

^a The following statistical tests were used: Mann-Whitney U test (U), multiple regression (MR), and χ² test by 2 × 2 contingency analysis.

^b For the seven reovirus gene segments not listed in this table, *P* values of >0.05 or *r*² values of <0.05 were obtained in every case. Blank positions for the L1 and L3 genes indicate that *P* values of >0.05 or *r*² values of <0.05 were obtained in those cases. For data from Table 1 for T1L-L3-containing reassortants only and for T3D-L3-containing reassortants only, statistical values are not applicable (NA) to L3 because the origin of L3 was fixed to be T1L for the T1L-L3-containing reassortants and T3D for the T3D-L3-containing reassortants.

^c An *r*² value for all 10 genes is applicable to multiple regression only.

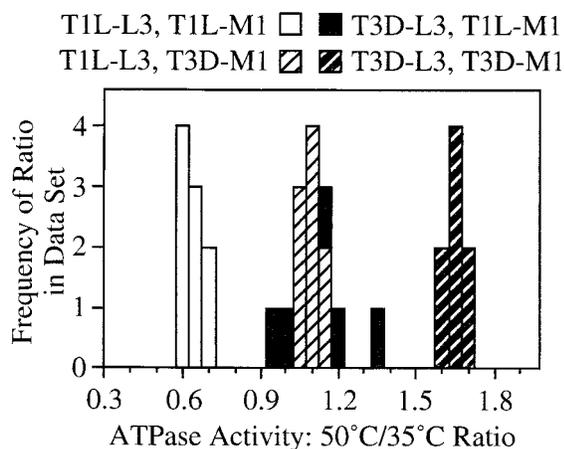


FIG. 2. Histogram of 50°C/35°C ATPase activity ratios for T1L, T3D, and reassortant cores. Activity ratios from Table 1 were used to generate a histogram in which the frequencies of viruses with activity ratios in consecutive 0.05-unit intervals are graphed. Contributions to the histogram by viruses with different pairings of the T1L and T3D L3 and M1 genes ($\lambda 1$ and $\mu 2$ proteins) are indicated by different styles of shading as labeled above the histogram.

ence in levels of hydrolysis of ATP by T1L and T3D cores at 35°C, with no evident contribution by M1 (28), we reanalyzed the new data for 50°C/35°C activity ratios by first dividing the viruses into two groups according to the origin of L3 and then subjecting these groups to separate statistical analyses.

For viruses containing the T1L L3 gene, the Mann-Whitney U test identified the M1 gene as the sole significant determinant ($P = 0.0003$) of the variation in 50°C/35°C activity ratios (Table 2). Similarly, multiple regression showed that 99% of the variance in 50°C/35°C activity ratios was explained by contributions from the 10 gene segments in these viruses, but only the M1 gene accounted for >5% of the variance, specifically, 96% (Table 2). As can be seen in the histogram (Fig. 2), viruses containing the T1L L3 gene clustered into two discrete groups whose 50°C/35°C activity ratios were determined by the M1 gene. Viruses containing T1L L3 and T1L M1 clustered tightly around a ratio of 0.64 (± 0.05), whereas viruses containing T1L L3 and T3D M1 clustered tightly around a ratio of 1.10 (± 0.04). There were no exceptions to this segregation with M1, and no other gene showed a similar distribution. In addition, the T1L-L3-containing reassortants chosen for these analyses permitted all genes but M1 to be eliminated from segregation with the 50°C/35°C activity ratio at least four times each, twice in the setting of each M1 allele (Table 1). χ^2 analysis (46) of these data for segregation of the T1L-L3-containing reassortants into groups of low and intermediate ATPase activity ratios again implicated M1 to a high degree of significance ($P = 0.0002$) (Table 2).

For viruses containing the T3D L3 gene, similar results were obtained; however, the smaller available number of these reassortants, particularly ones containing the T1L M1 gene (five only [26]) limited some of the statistical analyses. For viruses containing the T3D L3 gene, the Mann-Whitney U test identified M1 as the main significant determinant of the variation in 50°C/35°C activity ratios ($P = 0.003$) but also showed a contribution by the L1 gene ($P = 0.02$) (Table 2). Multiple regression showed that 98% of the variance in 50°C/35°C activity ratios for these viruses was explained by contributions from the 10 gene segments, but only the M1 gene accounted for >5% of the variance, specifically, 88% (Table 2). Again as can be seen in the histogram (Fig. 2), viruses containing the T3D L3 gene

clustered into two discrete groups whose 50°C/35°C activity ratios were determined by the M1 gene. Viruses with T3D L3 and T1L M1 clustered tightly together with a ratio of 1.13 (± 0.14), whereas viruses with T3D L3 and T3D M1 clustered around a ratio of 1.65 (± 0.03). There were no exceptions to this segregation with M1 and no other gene showed a similar distribution. It should be noted, however, that because the available panel of T3D-L3-containing reassortants was limited, the L1 gene could be eliminated from segregation with the 50°C/35°C activity ratio only once in the setting of the T3D M1 allele, which may explain its statistical association in the Mann-Whitney U test. In addition, the L2 gene could not be eliminated from segregation even once in the setting of the T3D M1 allele, because no reassortants containing the T1L L2, T3D L3, and T3D M1 genes are available, probably due to nonrandom segregation during reassortment (26). Despite these limitations, χ^2 analysis of these data for segregation of the T3D-L3-containing reassortants into groups of intermediate- and high-activity ratios again implicated M1 to a high degree of significance ($P = 0.003$) (Table 2).

In summary, statistical analyses identified the L3 and M1 genes as the primary genetic determinants of the different 50°C/35°C ratios for ATPase activity at pH 8.5 by T1L and T3D cores. Since L3 determines the different levels of ATP hydrolysis by these cores at 35°C (28), we conclude that the M1 gene, which encodes the $\mu 2$ protein, determines the distinct behaviors of T1L and T3D cores at temperatures near 50°C.

Reassortants with heterologously paired L3 and M1 genes have chimeric temperature curves for ATP hydrolysis at pH 8.5. The similar ATPase activities at 35 and 50°C for reassortants with heterologously paired L3 and M1 genes (Fig. 2) suggested that these reassortants might have temperature curves distinct from either parent. To test this possibility, we generated temperature curves with reassortants containing each of the four pairings of the T1L and T3D L3 and M1 genes. The results were striking. Reassortant EB143, which has both L3 and M1 from T1L, displayed a curve very similar to that for T1L (Fig. 1B), whereas reassortant EB86, which has both L3 and M1 from T3D, displayed a curve almost identical to that for T3D (Fig. 1C). In contrast, reassortants EB31 (Fig. 1B) and EB146 (data not shown), which have the T1L L3 and T3D M1 genes, showed curves that were distinct from either parent but contained a mixture of elements from each, including high activities at both 35 and 50°C. Reassortants H14 (Fig. 1C) and EB120 (data not shown), which have the T3D L3 and T1L M1 genes, also showed curves that were distinct from those for either parent but contained a mixture of elements from each, including low activities at both 35 and 50°C.

These temperature curves with reassortant cores confirm that both the L3 and M1 genes influence the capacity of reovirus T1L and T3D cores to hydrolyze ATP. Moreover, they suggest that the effects of these two genes on ATP hydrolysis are independent of each other. One peak of ATPase activity occurs near 35°C and differs between T1L cores (high activity) and T3D cores (low activity) in a manner determined by the L3 gene ($\lambda 1$ protein). Another peak of ATPase activity occurs near 50°C and differs between T1L cores (low activity) and T3D cores (high activity) in a manner determined by the M1 gene ($\mu 2$ protein). While it is possible that a single type of ATPase in cores has such a complex response to temperature, perhaps because it is strongly influenced by two different proteins, another explanation is that $\lambda 1$ and $\mu 2$ either mediate or modulate two different types of ATPase in cores, which differ in their temperature optima and whose combined activities give rise to the temperature curves that we observed (see Discussion).

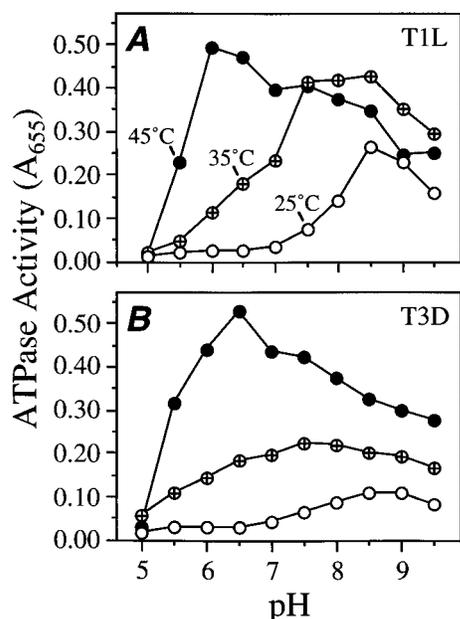


FIG. 3. pH curves for ATPase activity at different temperatures by T1L and T3D cores. T1L (A) or T3D (B) cores were mixed with 1 mM ATP in reaction buffer adjusted to different pH values and incubated at 25°C (○), 35°C (◐), or 45°C (●) for 30 min. Reactions were stopped by cooling and TCA addition, and the samples were analyzed for NTP hydrolysis by colorimetric assay for free phosphate.

ATPase activities of T1L and T3D cores exhibit complex behavior with respect to pH at different temperatures. In the previous study (28), we observed that the pH curve of ATPase activity at 35°C by T1L cores also appeared biphasic, suggesting that the ATPase activity of cores may exhibit complex behavior with respect to pH as well as temperature. In an effort to relate the effects of both pH and temperature on core ATPase activity, we generated pH curves for T1L and T3D cores at 25, 35, and 45°C. For both strains, the pH optima of ATPase activity underwent large shifts toward lower pH values as temperature was increased (Fig. 3). For T1L cores, for example, the pH optimum was near 8.5 at 25°C, between 7.5 and 8.5 at 35°C, and between 6.0 and 6.5 at 45°C (Fig. 3A). Similar values were observed for T3D cores (Fig. 3B). In addition, the pH curves for T1L and T3D cores at 45°C appeared biphasic. Thin-layer chromatography was used to demonstrate that cores of both strains cleaved [α - 32 P]ATP to yield almost exclusively [α - 32 P]ADP at pH 6.5 and 45°C (data not shown), confirming that the core enzyme being studied at these conditions was indeed a triphosphatase, as was previously shown for the activity at pH 8.5 and 35°C (28).

In sum, these data indicate that core ATPase activity exhibits complex behavior with respect to pH, with two peaks of activity discernible: one at higher pH values (near pH 8.5 for both strains) and one at lower pH values (near pH 6.5 for both strains). It also appears that the lower-pH activity is very low at 25°C but increases more rapidly and to higher values than the higher-pH activity as the temperature is raised from 35 to 45°C. Thus, the component of ATPase activity with a higher pH optimum contributes the most to ATP hydrolysis at lower temperatures whereas the component with a lower pH optimum contributes the most to ATP hydrolysis at higher temperatures (Fig. 3).

A genetic analysis in the previous study (28) involved an absolute difference in levels of hydrolysis of ATP by T1L and

T3D cores at a lower temperature (35°C) and higher pH (pH 8.5). Its results implicated the L3 gene in determining this difference and thus suggested that ATPase activity is strongly influenced by the λ 1 protein under those conditions. In contrast, the above-described findings suggest that ATPase activity under other conditions—higher temperature and possibly lower pH—is strongly influenced by the μ 2 protein. As an additional test of these conclusions about the influence of μ 2, we wished to perform a genetic analysis specifically under conditions of lower pH. Comparing the T1L and T3D pH curves in Fig. 3, however, it was evident that these two strains exhibited only minor differences (less than twofold) in their absolute ATPase activities at lower pH values and none that were large enough to justify a genetic study. The similar ATPase activities of T1L and T3D cores at pH 6.5, near the peak of lower-pH activity, are emphasized by their temperature curves for ATP hydrolysis at that pH (Fig. 4A). Given these findings, we turned to other nucleoside triphosphates (NTPs) to identify a larger difference between T1L and T3D cores under conditions of lower pH that might be useful for genetics.

T1L and T3D cores exhibit differences in GTPase activity at lower pH and higher temperature values. Since GTP is hydrolyzed next most efficiently to ATP by both T1L and T3D cores at pH 8.5 (28), we performed additional studies using GTP as the substrate at pH 6.5. In an experiment identical to the one for ATP, whose results are shown in Fig. 4A, a sizeable difference was noted for T1L and T3D cores in GTP hydrolysis over a range of temperatures at pH 6.5 (Fig. 4B). The largest difference was seen at 45°C, where GTP hydrolysis by T3D cores was more than twice that by T1L cores. To characterize the difference in levels of GTP hydrolysis by T1L and T3D cores more fully, we also generated pH curves at the temperatures 25, 35, and 45°C (Fig. 5). As in analogous experiments with ATP (Fig. 3), we observed a large shift in the pH optima of GTP hydrolysis with increasing temperatures. For T3D cores,

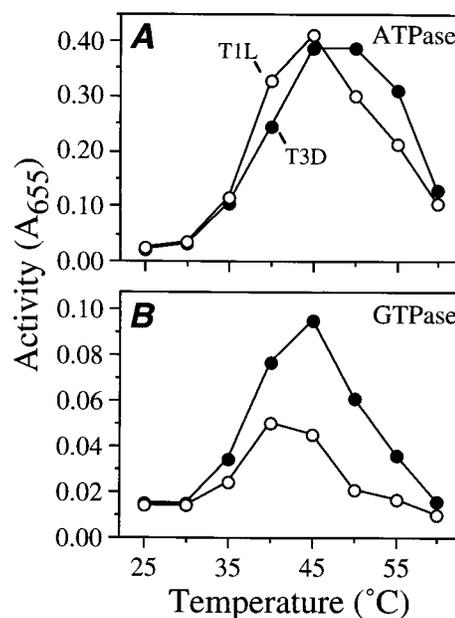


FIG. 4. Temperature curves for ATPase and GTPase activity at pH 6.5 by T1L and T3D cores. T1L (○) or T3D (●) cores were mixed with 1 mM ATP (A) or GTP (B) in pH 6.5 reaction buffer and incubated at the appropriate temperature for 30 min. Reactions were stopped by cooling and TCA addition, and the samples were analyzed for NTP hydrolysis by colorimetric assay for free phosphate.

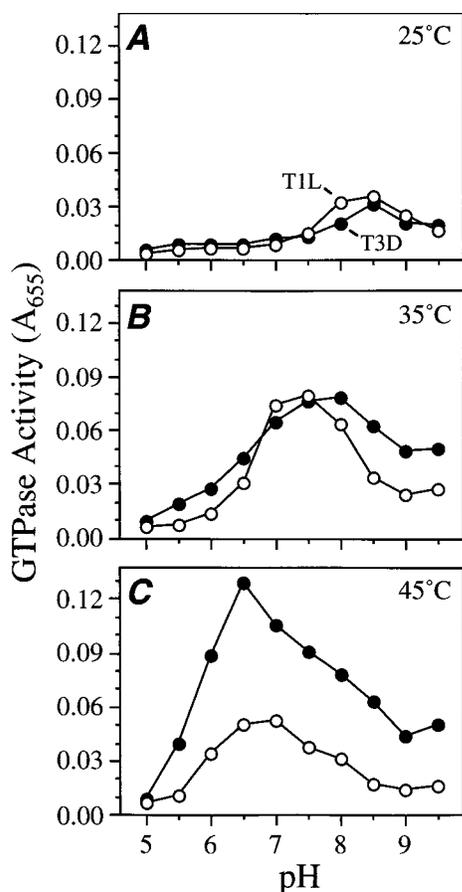


FIG. 5. pH curves for GTPase activity at different temperatures by T1L and T3D cores. T1L (○) or T3D (●) cores were mixed with 1 mM GTP in reaction buffer adjusted to different pH values and incubated at 25, 35, or 45°C for 30 min. Reactions were stopped by cooling and adding TCA, and the samples were analyzed for NTP hydrolysis by colorimetric assay for free phosphate.

for example, the pH optimum for GTPase activity was near pH 8.5 at 25°C, pH 7.5 at 35°C, and pH 6.5 at 45°C (Fig. 5). Similar values were observed for T1L cores. Importantly for our goal of extending the genetic studies, the GTPase activities of T1L and T3D cores were found to be distinct at 45°C, particularly at the lower pH values (Fig. 5C). The largest difference was noted at pH 6.5, with the activity of T3D being more than twice that of T1L, sufficiently large to justify a genetic study.

GTPase activities of T1L and T3D cores at pH 6.5 and 45°C are determined by the M1 gene. To identify the genetic basis for the different levels of GTP hydrolysis by T1L and T3D cores at pH 6.5 and 45°C, we measured the GTPase activities of cores of 19 T1L \times T3D reassortants. In Table 3, the virus strains are listed in rank order according to these activities, which are seen to span a range of A_{655} values that includes those for T1L and T3D, i.e., 0.061 and 0.138, respectively. When these data were subjected to the Mann-Whitney U test, only the M1 gene was found to deviate from a random distribution within the ranked data set in a significant manner ($P = 0.0002$) (Table 3). A similar conclusion was reached by using multiple regression, according to which 78% of the variance in GTPase activities was explained by contributions from the 10 gene segments, but only M1 accounted for >5% of the variance, specifically, 67% (Table 3). In addition, 10 of 10 viruses with a T1L M1 gene exhibited GTPase activities below 0.1 A_{655}

units whereas 10 of 11 viruses with a T3D M1 gene exhibited GTPase activities above 0.1 A_{655} units. This pattern suggests segregation of the GTPase phenotype with the M1 gene, with only one virus, EB62, representing an exception. In sum, these data and statistical analyses implicate the M1 gene ($\mu 2$ protein) as the primary genetic determinant of the difference in levels of hydrolysis of GTP by T1L and T3D cores at pH 6.5 and 45°C.

DISCUSSION

This paper demonstrates that the L3 and M1 genes—which encode core proteins $\lambda 1$ and $\mu 2$, respectively—are the primary determinants of specific differences in the NTPase activities of reovirus T1L and T3D cores. The findings for L3 are consistent with those from a previous study (28) implicating the $\lambda 1$ protein as either a mediator or a modulator of core NTPase activity. The findings for M1, on the other hand, are new and implicate the $\mu 2$ protein in a similar fashion. Since $\lambda 1$ was discussed in the previous paper, $\mu 2$ will be the focus here. Sequences for the T1L and T3D M1 genes were previously reported (43, 47) and indicate that the $\mu 2$ proteins of these strains differ at only 10 amino acid positions scattered through most of the protein sequence. Assuming that the clones of T1L and T3D used in this study have the same $\mu 2$ sequences as those previously reported, 1 or more of the 10 amino acid differences between the T1L and T3D $\mu 2$ proteins must determine the relevant differences in core NTPase activities.

Does M1 ($\mu 2$) influence core NTPase activities by affecting core stability? One potential explanation for their different patterns of ATPase activity at temperatures above 35°C might be that T1L cores become subject to heat-induced inactivation at lower temperatures (near 45°C) than T3D cores (near 55°C) (Fig. 1). In that case, the contribution of M1 to determining strain differences at higher temperatures might be that $\mu 2$ influences core stability, or resistance to heat-induced inactivation, at those temperatures. To test that possibility, we preheated T1L and T3D cores to 50°C for 30 min and then measured their ATPase activities at 35 and 50°C. The activity of each was found to be nearly identical to that determined before preheating (data not shown). These findings indicate that cores of neither strain become subject to an irreversible, heat-induced inactivation at 50°C and therefore that the M1 contribution to their different ATPase activities at higher temperatures does not reflect an effect of $\mu 2$ on core stability. This experiment also ruled out the possibility that T3D cores exhibit low ATPase activity at lower temperatures because they are uniquely subject to an inhibitory factor that can be irreversibly inactivated by heating to 50°C (17). Thus, it appears that $\mu 2$ influences the NTPase activities of T1L and T3D cores by some more direct effect on the NTPase enzyme(s).

Is $\mu 2$ an NTPase? One simple interpretation of the genetic data for M1 is that the $\mu 2$ protein is an NTPase. In an effort to demonstrate this activity, we recently assayed $\mu 2$ that had been expressed in insect cells from a recombinant baculovirus, bound to an immunoaffinity column, and eluted by low pH (a gift from D. Noah and B. Sherry, North Carolina State University School of Veterinary Medicine); however, no ATPase activity by this partially purified, recombinant $\mu 2$ protein was seen under the conditions tested (data not shown). Although these findings suggest that $\mu 2$ is not an NTPase, other possibilities are that the protein was inactivated during purification, that the recombinant protein failed to adopt a functional conformation, or that necessary RNA and/or protein cofactors were not added. We also failed to demonstrate NTP binding to $\mu 2$ by radiolabeling of core proteins with oxidized [32 P]ATP in

TABLE 3. Genetic analysis of GTPase activities by T1L, T3D, and reassortant cores at 45°C and pH 6.5

| Virus strain | Parental origin of gene segment (core protein) ^a : | | | | | | | | | | Mean $A_{655} \pm$ SD for GTP hydrolysis (n) ^c |
|--------------|---|-------------------|-------------------|----------------------------|----|----|----|------------------|----|----|---|
| | L1 (λ 3) | L2 (λ 2) | L3 (λ 1) | M1 (μ 2) ^b | M2 | M3 | S1 | S2 (σ 2) | S3 | S4 | |
| H14 | L | L | D | L | L | L | L | D | D | L | 0.023 \pm 0.005 (4) |
| H15 | L | D | D | L | D | D | D | D | D | L | 0.043 \pm 0.004 (4) |
| EB136 | D | D | D | L | D | L | D | D | D | D | 0.047 \pm 0.003 (4) |
| H5 | D | D | L | L | L | D | L | D | L | D | 0.055 \pm 0.002 (4) |
| KC15 | L | L | L | L | L | D | L | D | D | L | 0.058 \pm 0.002 (4) |
| T1L | L | L | L | L | L | L | L | L | L | L | 0.061 \pm 0.003 (4) |
| H27 | L | D | L | L | D | L | L | L | L | L | 0.062 \pm 0.008 (4) |
| EB62 | D | D | D | D | D | D | D | L | D | L | 0.078 \pm 0.002 (4) |
| F18 | L | D | L | L | D | L | L | L | D | D | 0.089 \pm 0.013 (4) |
| EB120 | D | D | D | L | L | D | D | D | L | L | 0.092 \pm 0.005 (4) |
| EB39 | L | D | D | L | D | D | D | D | D | D | 0.095 \pm 0.004 (4) |
| EB129 | D | D | D | D | D | L | D | L | L | D | 0.104 \pm 0.002 (4) |
| KC55 | L | D | L | D | D | D | L | D | L | D | 0.109 \pm 0.005 (4) |
| EB86 | L | D | D | D | D | L | D | D | D | L | 0.125 \pm 0.009 (4) |
| KC12 | D | D | L | D | D | L | L | D | D | D | 0.126 \pm 0.010 (4) |
| EB147 | D | D | D | D | D | L | L | D | L | L | 0.129 \pm 0.003 (4) |
| EB146 | L | L | L | D | L | L | L | D | L | D | 0.134 \pm 0.001 (2) |
| T3D | D | D | D | D | D | D | D | D | D | D | 0.138 \pm 0.010 (4) |
| EB145 | D | D | D | D | D | L | L | D | D | D | 0.138 \pm 0.005 (4) |
| EB138 | D | L | L | D | D | L | D | D | L | L | 0.147 \pm 0.041 (4) |
| EB123 | D | D | L | D | D | D | D | D | L | D | 0.167 \pm 0.011 (4) |

^a Parental origins of gene segments and/or proteins in each reassortant strain were recently confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (26). D, derived from reovirus strain T3D; L, derived from reovirus strain T1L.

^b Data indicate segregation of the M1 gene segment (μ 2 core protein) with the absolute level of GTP hydrolysis at 45°C and pH 6.5.

^c GTP hydrolysis was determined as A_{655} by colorimetric assay for released phosphate ion as described in the text. n , number of determinations for each virus strain.

cross-linking experiments (data not shown) (32). Thus, with no NTPase or NTP-binding activity yet directly demonstrated by μ 2, the hypothesis that it directly mediates NTP hydrolysis remains speculative. Nevertheless, in support of this hypothesis, we have noted that sequences in μ 2 between amino acids 410 and 420 (GAVLPKGSFKS) and 445 and 449 (DEVG) bear limited resemblance to the A and B motifs, respectively, of known ATPases (42) and have learned that other sequence similarities between μ 2 and ATP-dependent RNA helicases were also recently noted (36).

Is there more than one type of NTPase in reovirus cores?

The complex patterns with which core NTPase activity responds to temperature and pH and the genetic evidence that this activity is strongly influenced by two different genes—L3 and M1—suggest a hypothesis that cores contain two different types of NTPase which have different temperature and pH optima and are mediated or modulated by the λ 1 and μ 2 proteins. There are in fact numerous conceivable roles for more than one type of NTPase in cores. The possibility that a core NTPase is the RNA triphosphatase that mediates the first step in mRNA capping (1, 12, 35) was discussed for λ 1 in the previous paper (28) and can be restated for μ 2 here. The previous paper also raised the idea that a core NTPase might represent an NTP-dependent RNA helicase (15, 30), the most evident role for which would be to unwind the two strands of each genomic dsRNA segment so that an unpaired region of minus strand is continually presented to the viral RNA polymerase as the template for transcription. There are other possibilities for core NTPases to act at specific steps in the transcription cycle, such as promoter clearance (see reference 13 regarding a similar role for protein complex TFIIH in eukaryotic transcription) or the process by which the genomic template is repositioned for transcript initiation in each new round of mRNA synthesis. An NTPase might also be involved in RNA packaging during viral assembly (29) and might remain active in cores in a vestigial fashion. Given the number of

conceivable roles for NTPases in cores, a hypothesis that cores contain two types of NTPase seems reasonable. By being localized with RNA polymerase protein λ 3 to internal structures in reovirus particles, as was recently shown (9), regions of μ 2 and λ 1 are apparently well-positioned to effect most of these functions relating to RNA metabolism. Moreover, findings for μ 2 in this study appear likely to relate to its recently proposed effects on synthesis of reovirus RNA (37), including both transcription (45) and minus-strand synthesis (6).

Is there a relationship between the roles of M1 (μ 2) in NTPase phenotypes and in reovirus growth and injury to cells? Numerous genetic studies have demonstrated a role for M1 in determining the extent of reovirus growth in and/or injury to cells in culture or infected animals (16, 22–24, 37–39). The molecular basis of these effects remains undefined, although a recent study showed that reovirus-induced myocarditis, as determined by the M1 gene, correlates with the levels of synthesis of reovirus RNA in cardiac myocytes (37). It is difficult to relate the current observations regarding core NTPase activity to more complicated phenotypes in cells and animals, since the conditions in this study were chosen to permit identification of the genetic determinants of NTPase activity and not to reflect physiological conditions. Nonetheless, the evidence that M1 influences core NTPase activities and the many conceivable roles for NTPases in reovirus RNA synthesis are consistent with the hypothesis that M1 affects reovirus growth and/or injury to cells through its role(s) in RNA metabolism (6, 22, 37, 45).

Do the ATPase and GTPase activities represent different enzymes? It is possible that ATP and GTP are hydrolyzed by different enzymes in reovirus cores. Evidence for that hypothesis was previously presented (18), but evidence to the contrary has been presented by other investigators (4). Although our data do not directly address this issue, the hydrolysis of the four common NTPs across wide pH and temperature ranges (Fig. 3 and 5 and data not shown) suggests that each type of

NTPase in reovirus cores is nonspecific and simply hydrolyzes ATP more efficiently than the others.

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