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The M1 Gene Is Associated with Differences in the Temperature Optimum of the Transcriptase Activity in Reovirus Core Particles†

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The reovirus core is a multienzyme complex that contains five different structural proteins and 10 segments of double-stranded RNA. The core is responsible for transcribing mRNA from the enclosed double-stranded RNA. The reovirus transcriptase has an unusual temperature profile, with optimum transcription occurring at approximately 50°C and little activity occurring below 30 or above 60°C. Purified reovirus serotype 1 Lang (T1L) cores transcribed most efficiently at 48°C. The transcriptase temperature optimum of purified reovirus serotype 3 Dearing (T3D) cores was 52°C. In addition, T1L cores produced more mRNA per particle than did T3D cores at their respective temperature optima. Core particles were purified from T1L × T3D reassortants and were used to map these differences. The M1 gene, which encodes minor core protein μ2, was uniquely associated with the difference in temperature optimum of transcription ($P = 0.0003$). The L1 gene, which encodes minor core protein λ3 (previously implicated as the RNA polymerase), and the M1 gene were associated with the difference in absolute amounts of transcript produced ($P = 0.01$ and $P = 0.0002$, respectively). These data suggest that minor core protein μ2 also plays a role in reovirus transcription.

Syntheses of progeny proteins and nucleic acid are necessary steps in the replication of viruses that precedes assembly and release. Most DNA viruses utilize host cell enzymes to accomplish both functions. RNA viruses require RNA-dependent RNA polymerases to make copies of their genomes. In addition, except for viruses whose genomes can serve directly as message (i.e., members of the family *Picornaviridae*), many RNA viruses also must use RNA-dependent RNA polymerases (transcriptases) to convert their genomic information into mRNA.

The mammalian reoviruses have a genome consisting of 10 segments of double-stranded RNA. The RNA is enclosed by nonequivalent amounts of eight different structural proteins organized as a double capsid (for reviews, see references 24 and 27). The inner capsid (core) is the transcriptionally active form of the virus and is composed of three major proteins (λ1, encoded by the L3 gene; λ2, encoded by the L2 gene; and σ2, encoded by the S2 gene) and two minor proteins (λ3, encoded by the L1 gene; and μ2, encoded by the M1 gene). The segmented nature of the viral genome, knowledge that most segments encode single proteins, and serotype-dependent mobility differences of the genes allow the identification of intertypic reassortant viruses which have been used to assign particular functions to each of the three viral outer capsid proteins (10, 34) and to some of the five core proteins (3, 9, 16, 20).

After entry into a host cell, the outer capsid of the virus is removed to yield the core. This inner capsid has a diameter of about 60 nm. Protein λ2 is organized as pentameric spikes at each icosahedral vertex (17, 25). The spikes extend from the core capsid for an additional 9.5 nm and undergo significant conformational rearrangement during the uncoating process (11). However, the precise locations of λ1, λ3, μ2, and σ2 remain unknown. The core is a multienzyme complex that contains all necessary components for transcription, methylation, and capping of progeny mRNA (5, 12, 29). Little is known about details of core structure and how this particle transcribes mRNA from the enclosed genomic RNA. The λ2 spikes possess guanylyltransferase activity (6, 18). Proteins λ1 and λ2 have been labelled with pyridoxal phosphate in actively transcribing particles (21). Minor core protein λ3 has been associated with the pH optimum of transcription (9). In addition, regions of protein λ3 share sequence homology with some other RNA polymerases (22), and recombinant λ3 has some polymerase activity (33). However, the polymerase activity of the recombinant λ3 lacks specificity and processivity, suggesting that ancillary proteins and/or other components of the core are required for accurate and efficient genomic transcription. To identify these other components, we have examined the abilities of purified core particles to transcribe under a variety of conditions.

The in vitro transcriptase reaction conditions that allow reovirus to generate acid-insoluble nucleic acid have been described elsewhere (9, 32). Stocks of reovirus serotype 1 Lang (T1L), reovirus serotype 3 Dearing (T3D), and T1L × T3D reassortant viruses (originally isolated as described elsewhere [4, 7, 10]) were grown in mouse L929 cell monolayers in Joklik modified minimal essential medium (GIBCO, Grand Island, N.Y.) supplemented to contain 2.5% fetal calf serum (Intergen, Purchase, N.Y.), 2.5% VSP neonate bovine serum (Biocell, Carson, Calif.), 2 mM glutamine, 100 U of penicillin per ml, 100 μg of streptomycin sulfate per ml, and 1 μg of amphotericin B per ml, essentially as previously described (8). The gene patterns of all reassortants used were verified by electropherotyping as described elsewhere (15, 28). Reovirus core particles were purified as described elsewhere (7). Briefly, virus was grown in suspension cultures at 34°C for 65 h, harvested by freon extraction, and banded in cesium chloride gradients. Gradient-purified virus was dialyzed and digested with chymotrypsin, and core particles were purified in cesium chloride gradients. The core bands were harvested and dialyzed against

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† This study is dedicated to the memory of Bernard N. Fields.

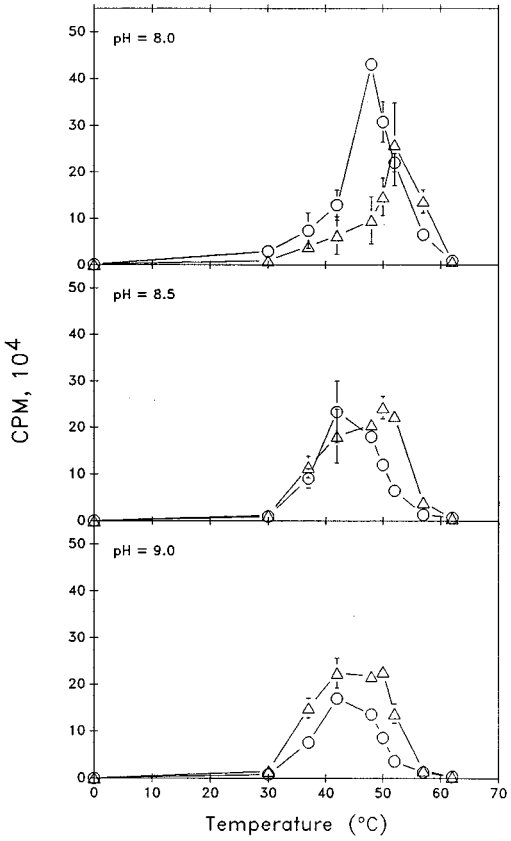

FIG. 1. Transcriptase activities of purified reovirus cores at different temperatures and pH values. Aliquots of 3.5×10^{11} total core particles in 50-μl volumes were incubated with 0.1 μCi of [α-^{32}P]UTP per μl at the indicated temperatures and pH values for 1 h and precipitated with 5% TCA, and precipitates were collected on filters and counted. Results are the averages of duplicate experiments. ○, T1L; △, T3D. Error bars indicate variability between experiments.

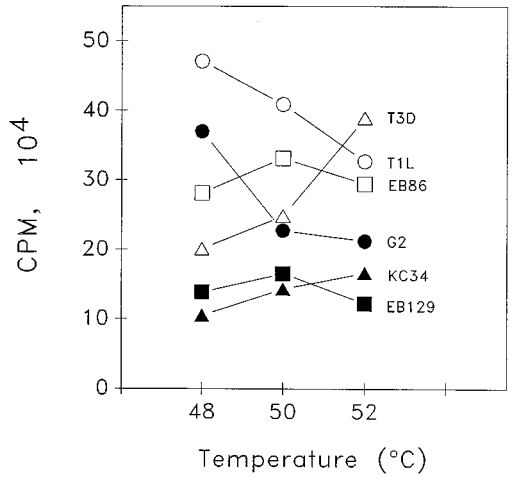

FIG. 2. Transcriptase activities of T1L, T3D, and selected T1L × T3D reassortant cores at 48, 50, and 52°C (pH 8.0). Purified cores from the indicated clones were assayed for transcriptase activities at the indicated temperatures as described in the legend to Fig. 1. Results are the averages of three experiments (except for results for T1L and T3D, which are from five experiments).

core buffer (1 M NaCl, 100 mM $MgCl_2$, 25 mM *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] [HEPES]; pH 8.0). The concentration of purified cores was measured (with the relationship 1 optical density unit at 260 nm = 4.2×10^{12} particles per milliliter), glycerol was added to a final concentration of 25%, and core aliquots were frozen at −80°C. This treatment had no detectable effect on core structure or function compared with nontreated, nonfrozen cores. Core purity was monitored by characteristic protein profiles in sodium dodecyl sulfate-polyacrylamide gels and by electron microscopy. Aliquots of cores were thawed and diluted into core buffer, diethylpyrocarbonate-treated distilled H_2O, and transcription reaction buffer such that the final reaction components were 2 mM each ATP, CTP, GTP, and UTP; 3.3 mM phosphoenolpyruvate; 100 ng of pyruvate kinase, 0.8 U of RNAsin (Boehringer Mannheim, Laval, Québec, Canada), and 0.1 μCi of [α-^{32}P]UTP (New England Nuclear, Mississauga, Ontario, Canada) per μl; 9.5 mM $MgCl_2$; and 100 mM HEPES [pH 8.0]. To reduce pipeting errors, a single reaction mixture was prepared for each type of core tested and divided into aliquots, and each aliquot was incubated at various temperatures for appropriate periods of time. Transcriptase reactions were terminated by placing the reaction tubes on ice and adding 8 volumes of ice-cold 5% trichloroacetic acid (TCA) to precipitate macromolecules. Precipitates were collected onto filters and washed with 5% TCA and ethanol, and radioactivity was measured in a Beckman model 5000LS scintillation counter.

Previous investigators have shown that when purified virus is digested with chymotrypsin, the digestion is stopped by the addition of phenylmethylsulfonyl fluoride, and the mixture of cores, digested outer capsid proteins, and phenylmethylsulfonyl fluoride-stopped chymotrypsin is used, reovirus transcribes most efficiently between pH values of 8.0 and 9.0 (9, 32). In addition, Drayna and Fields showed that there were subtle strain-specific differences in the pH optimum within this range (9). Thus, our initial reactions with purified cores were conducted at multiple temperatures and at several pH values between 8.0 and 9.0. Purified T1L and T3D cores had little detectable transcriptase activity at temperatures of ≤30°C, and both strains showed transcriptase activity at higher temperatures, with the peak of activity at about 45 to 50°C at all tested pH values, in agreement with previous reports (9, 32).

However, at pH 8.0, purified T1L cores transcribed most efficiently at 48°C and purified T3D cores transcribed most efficiently at 52°C (Fig. 1). At higher pH values, the activity peaks of cores derived from both strains were lower and broader, and strain-specific differences were less obvious (Fig. 1). These results differ slightly from those in previous reports, possibly because we used purified cores, smaller changes in temperature near the peaks, or both. Neither type of core produced significant amounts of product at temperatures of ≥62°C at any tested pH. Additional studies indicated that at core concentrations of $>7.5 \times 10^9$/μl, cores produced less TCA-precipitable material per particle than at lower concentrations (data not shown). Therefore, in subsequent experiments, core concentrations were kept at about 7×10^9/μl, and transcriptase reactions were performed at pH 8.0.

The different transcriptase temperature optima of cores derived from the two different virus strains (Fig. 1) suggested that reassortants could be used to determine which gene(s) was responsible for the phenotypic differences. Some reassortants (i.e., G2) behaved like T1L; cores derived from such clones possessed more transcriptase activity at 48 than they did at 50°C and more activity at 50 than at 52°C (Fig. 2). Other reassortants (i.e., KC34) behaved like T3D; they had more activity at 52 than at 50°C and more activity at 50 than at 48°C. A few reassortants (i.e., EB86 and EB129) behaved like neither parent; they had virtually the same activity at 48 as at

TABLE 1. Genotypes of T1L × T3D intertypic reassortants tested for transcriptase temperature optimum

Clone	Parental source for the following gene segments[a]:										Ratio[b]	Rank[c]
	L1	L2	L3	M1	M2	M3	S1	S2	S3	S4		
G2	1	3	1	1	1	1	3	1	1	1	1.77 ± 1.15	19
H17	3	3	3	1	3	3	1	3	3	1	1.63 ± 0.66	18
EB120	3	3	3	1	1	3	3	3	1	1	1.57 ± 0.47	17
T1L	1	1	1	1	1	1	1	1	1	1	1.54 ± 0.35	16
EB39	1	3	3	1	3	3	3	3	3	3	1.46 ± 0.60	15
EB136	3	3	3	1	3	1	3	3	3	3	1.33 ± 0.30	14
H14	1	1	3	1	1	1	1	3	3	1	1.29 ± 0.18	13
EB47	1	3	1	1	1	1	1	1	1	1	1.26 ± 0.55	12
KC10	1	1	1	1	1	1	1	3	1	1	1.24 ± 0.35	11
EB129	3	3	3	3	3	1	3	1	1	3	1.21 ± 0.26	10
EB97	3	3	1	3	3	3	3	3	3	1	1.02 ± 0.17	9
EB86	1	3	3	3	3	1	3	3	3	1	0.99 ± 0.05	8
EB62	3	3	3	3	3	3	3	1	3	1	0.90 ± 0.09	7
EB146	1	1	1	3	1	1	1	1	1	3	0.68 ± 0.12	6
KC309[d]	3	1	3	3	3	1	3	3	3	1	0.65 ± 0.32	5
G16	1	1	1	3	1	1	1	3	1	1	0.63 ± 0.15	4
KC34	1	3	1	3	3	3	1	3	3	3	0.61 ± 0.22	3
EB96	1	3	1	3	1	1	1	1	3	1	0.57 ± 0.15	2
T3D	3	3	3	3	3	3	3	3	3	3	0.54 ± 0.22	1

[a] Numbers indicate parental sources of gene. 1, T1L; 3, T3D.
[b] Ratios were derived by dividing activity at 48°C by activity at 52°C (averages ± standard deviations for three or more experiments).
[c] Ranking used for statistical analyses (Table 2).
[d] Clone was derived from a T3D × H14 cross (7).

TABLE 2. Nonparametric and parametric analyses of the 10 reovirus gene segments involved in transcriptase temperature optimum

Gene	Mean transcriptase optimum[a]		
	Univariate[b]		Multivariate (r^2)[c]
	Wilcoxon rank sum (P value)	Linear regression (r^2)	
L1	0.97	0.0001	
L2	0.69	0.03	
L3	0.6	0.01	
M1	0.0003	0.74	0.74
M2	0.44	0.03	
M3	0.1	0.0002	
S1	0.71	0.01	0.06
S2	0.9	0.004	
S3	0.23	0.09	
S4	0.36	0.05	
All 10 genes			0.90

[a] Determined from gene rankings of averaged clones shown in Table 1.
[b] Univariate tests of each individual gene were the nonparametric Wilcoxon rank sum analysis and the parametric linear regression analysis.
[c] Multivariate test used was the all-subset multiple regression analysis. Numbers shown are the r^2 values of genes adjusted for all other genes in the selected model. A blank position means r^2 of <0.04, not statistically significant, and not included in the model.

52°C, with a minor peak of activity at 50°C. The different reassortant behaviors prevented all clones from easily being placed into only two distinct groups (because some of the reassortants had intermediate behaviors). Therefore, the relative transcriptase activities at various temperatures were determined in order to facilitate reassortant mapping analyses. This relative activity could be represented as a ratio of the transcriptase activity at 48°C to the transcriptase activity of the same clone at 52°C. The average ratio values obtained for each reassortant clone from three or more experiments generated a continuum from approximately 1.8 to approximately 0.5 (Table 1). Many of the reassortants near the top of the table (i.e., G2, H17, and EB120) behaved like T1L; the 48/52°C ratio from every experiment was >1.0. (The relatively large errors associated with G2 and H17 resulted from single experiments in which the activity ratios were significantly higher.) Many reassortants near the bottom of the table (i.e., EB62, G16, and KC34) behaved like T3D; the 48/52°C ratio was always less than 1.0. A few reassortants (i.e., EB47, EB97, and KC309) gave variable results, with 48/52°C ratios of less than 1.0 in some experiments and greater than 1.0 in other experiments. However, the average ratio obtained for these reassortants placed them within the continuum. The reassortants were ranked according to the average activity ratio values obtained. The relative contribution of each of the genes was determined, with SAS (1), by both nonparametric and parametric statistical methods to ensure that the conclusions derived were independent of the method used. A nonparametric Wilcoxon rank sum analysis (a distribution-free univariate method which can be used when the test population does not follow normal distribution [14]) of the ranked reassortants shown in Table 1 indicated that the M1 gene was strongly associated ($P = 0.0003$) and that no other genes were associated with the difference in temperature optimum (Table 2). A linear regression analysis (which assumes a normal distribution) of each of the 10 genes indicated that the M1 gene accounted for 74% of the variance ($r^2 = 0.74$), the S3 gene accounted for 9% of the variance ($r^2 = 0.09$), and each of the other genes accounted for 5% or less of variance. To examine the possible role of multiple genes, all subset multiple regression analysis (which provides a multivariate tool to examine independent sets of genes) showed that 90% of the variance ($r^2 = 0.9$) could be attributed to the 10 gene segments and that 74% of the total variance ($r^2 = 0.74$) could be attributed solely to the viral M1 gene. The viral S1 gene added a contribution of 6% to the explained variance ($r^2 = 0.06$), whereas each of the other genes added less than 4% to the explained variance. When the entire data set of individual experimental results was examined by each of the above tests, the significance of the M1 gene was increased and no other viral genes were found to be associated (data not shown).

The cumulative transcriptase activity of T1L cores was consistently higher than the cumulative activity of T3D cores near their respective optima (Fig. 1 and 2). To identify the viral gene(s) responsible for this difference, we examined the cumulative activity of various reassortants in this temperature range. Preliminary analyses that involved summing and ranking the activities of each clone in Table 1 at each tested temperature failed to implicate any single gene as a determinant of transcriptase efficiency but did suggest that multiple genes are involved (data not shown). Therefore, additional reassortant cores were prepared. In addition, to determine the cumulative transcriptase activity of each clone at multiple temperatures between 42 and 57°C, reactions were carried out in a programmable thermal controller (MJ Research model PTC-100) that had been programmed to increase temperature from 42 to 57°C over a 2-h incubation (0.1°C increase every 48 s). T3D cores generated about 60% of the amount of product that T1L cores generated (Table 3). Reassortants generated various amounts of product, from about twice the amount produced by T1L (clones H15 and H41) to about 25% of the amount produced by T1L (KC309). A Wilcoxon rank sum analysis of the relative contributions of each of the genes of all ranked reas-

TABLE 3. Genotypes of T1L × T3D intertypic reassortants used to measure transcriptase efficiency

Clone	Parental source for the following gene segments[a]:										Incorporation[b]	Rank[c]
	L1	L2	L3	M1	M2	M3	S1	S2	S3	S4		
H15	1	3	3	1	3	3	3	3	3	1	204.3 ± 20.5	40
H41	3	3	1	1	1	3	1	3	3	1	175.3 ± 37.9	39
EB144	1	1	1	1	3	3	1	1	3	1	155.7 ± 6.7	38
KC10	1	1	1	1	1	1	1	3	1	1	152.3 ± 12.3	37
KC26	3	3	1	1	1	3	3	1	3	3	151.6 ± 20.0	36
EB39	1	3	3	1	3	3	3	3	3	3	138.7 ± 4.4	35
EB85	1	1	1	1	1	3	1	3	1	1	137.3 ± 5.9	34
EB96	1	3	1	3	1	1	1	1	3	1	131.3 ± 46.9	33
H14	1	1	3	1	1	1	1	3	3	1	126.0 ± 35.3	32
KC34	1	3	1	3	3	3	1	3	3	3	110.5 ± 43.4	31
KC19	1	1	1	1	3	1	3	1	3	1	107.1 ± 42.9	30
EB93	1	1	1	1	1	1	1	1	3	1	103.0 ± 8.4	29
T1L	1	1	1	1	1	1	1	1	1	1	100.0	28
G2	1	3	1	1	1	1	3	1	1	1	96.1 ± 7.7	27
EB120	3	3	3	1	1	3	3	3	1	1	94.7 ± 9.3	26
EB47	1	3	1	1	1	1	1	1	1	1	93.9 ± 28.4	25
E3	3	3	3	3	1	3	3	3	3	3	89.8 ± 21.5	24
EB129	3	3	3	3	3	1	3	1	1	3	89.6 ± 10.9	23
KC15	1	1	1	1	1	3	1	3	3	1	88.9 ± 3.4	22
EB73	3	3	3	3	3	1	3	3	1	1	86.7 ± 23.1	21
KC79	3	1	1	3	1	1	1	3	3	1	82.8 ± 19.6	20
H17	3	3	1	1	3	3	1	3	3	1	80.9 ± 6.5	19
KC298	1	1	1	3	1	1	1	3	3	3	78.6 ± 33.6	18
KC257	1	1	1	3	1	1	1	1	1	3	75.1 ± 24.4	17
EB138	3	1	1	3	3	1	3	3	1	1	74.7 ± 10.5	16
KC59	1	1	1	3	3	3	1	3	3	3	72.2 ± 18.2	15
EB86	1	3	3	3	3	1	3	3	3	1	70.9 ± 12.9	14
EB143	3	1	1	1	1	1	3	1	1	1	69.7 ± 32.5	13
EB136	3	3	3	1	3	1	3	3	3	3	66.4 ± 31.3	12
KC252	3	1	3	3	3	1	1	1	3	3	64.9 ± 19.5	11
T3D	3	3	3	3	3	3	3	3	3	3	62.8 ± 11.3	10
KC3	1	3	1	3	1	3	1	1	1	1	62.0 ± 2.6	9
KC9	3	3	1	3	3	3	1	3	3	3	58.6 ± 1.1	8
EB62	3	3	3	3	3	3	3	1	3	1	58.1 ± 5.6	7
G16	1	1	1	3	1	1	1	3	1	1	57.8 ± 9.7	6
EB146	1	1	1	3	1	1	1	1	1	3	56.4 ± 6.6	5
KC36	3	1	1	1	1	1	1	3	1	3	53.8 ± 0.4	4
EB97	3	3	1	3	3	3	3	3	3	1	45.4 ± 4.9	3
H30	3	3	1	3	3	3	3	3	1	3	45.2 ± 4.9	2
KC309	3	1	3	3	3	1	3	3	3	1	23.3 ± 16.7	1

[a] 1, T1L; 3, T3D.
[b] Incorporation was determined by incubating each clone (42 to 57°C [0.1°C increase every 48 s] over a 2-h period) in a programmable thermal controller as detailed in the text. Data are averages expressed as percentages of T1L activity ± standard deviations for three experiments.
[c] Ranking used for statistical analyses (Table 4).

TABLE 4. Nonparametric and parametric analyses of the 10 reovirus gene segments involved in transcriptase efficiency

Gene	Mean transcriptase optimum[a]		
	Univariate[b]		Multivariate (r^2)[b]
	Wilcoxon rank sum (P value)	Linear regression (r^2)	
L1	0.01	0.13	0.06
L2	0.72	0.01	
L3	0.77	0.0005	
M1	0.0002	0.33	0.33
M2	0.44	0.01	
M3	0.15	0.03	
S1	0.12	0.06	
S2	0.52	0.001	
S3	0.44	0.05	0.05
S4	0.34	0.03	
Total for 10 genes			0.51

[a] Determined from gene rankings of averaged clones shown in Table 3.
[b] Univariate and multivariate tests used as described in footnotes *b* and *c* of Table 2.

sortants shown in Table 3 indicated that the L1 gene was significantly associated ($P = 0.01$), that the M1 gene was strongly associated ($P = 0.0002$), and that no other genes were associated with the difference in transcriptase efficiency (Table 4). Linear regression analysis of each of the 10 genes indicated that the M1 gene accounted for 33% of the variance ($r^2 = 0.33$), the L1 gene accounted for 13% of the variance ($r^2 = 0.13$), the S1 gene accounted for 6% of the variance ($r^2 = 0.06$), and each of the other genes accounted for 5% or less of variance. All subset multiple regression analysis of the average transcriptase efficiencies showed that 51% of the variance ($r^2 = 0.51$) could be attributed to the 10 gene segments and that 33% of the total variance ($r^2 = 0.33$) could be attributed solely to the viral M1 gene. The viral L1 gene added a contribution of 6% to the explained variance, and the S3 gene added an additional contribution of 5% to the variance, whereas each of the other genes added less than 4% to the explained variance. When the entire data set of individual experimental results was examined by each of the above tests, the significance of both the L1 and M1 genes was increased and no other viral genes were found to be associated (data not shown).

These results indicate that, in addition to the known role of λ3 as the polymerase (9, 22, 33), the viral M1 gene also is involved in transcription. The M1 gene is 2,304 bp long (35, 36) and encodes minor reovirus core protein μ2. Depending on where translation of this gene's mRNA initiates, μ2 is predicted to be either 687 or 736 amino acids in length (26, 35, 36). There is about 98% amino acid identity between the T1L and T3D μ2 proteins (36). However, the protein shares no similarity with other proteins in GenBank (35). Little is known about the function(s) of this minor protein. Reassortant mapping experiments suggest that μ2 plays a role in determining the level of virus growth in cardiac cells (20) and in endothelial cells (19). It is also involved in myocarditis (31) and in organ-specific virulence in SCID mice (13). Such observations led to the speculation that μ2 may play a role in RNA metabolism (19, 20, 31). The protein is present within the reovirus particle at a rate of approximately 12 copies per particle, as is the other minor core protein λ3 (27). Because of the small copy number, which corresponds with the number of vertices on an icosahedral structure such as the core, it seems likely that both minor proteins may reside at or near the core vertices. Such a location would place these proteins near the λ2 spikes, which are known to possess guanylyltransferase activity (6, 18). The λ2 spikes possess channels (11), and electron microscopy of transcribing particles has suggested that nascent mRNA may be extruded through the spikes (2). Recent detailed reassortant analyses of reovirus strains capable of inducing acute myocarditis (30), as well as of reassortants capable of inducing organ-specific virulence in SCID mice (13), suggest that proteins λ2, λ3, and μ2 may interact. The concept that these three proteins may form a structural and/or functional unit within the core particle also is supported by recent cryoelectron microscopy and image reconstruction of T1L top-component particles (23) and by the observation that both λ3 and μ2 are associated with strain-dependent differences in transcription efficiency (Table 4).

Collectively, these observations suggest that proteins λ2, λ3, and μ2 may represent the structural and functional enzymatic complex that directs the transcription and capping of mRNA from the double-stranded RNA genome. More detailed structural and functional analyses may determine the precise locations of these minor core proteins and elucidate the roles played by these proteins in converting the genomic information of reovirus into mRNA.

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