JOURNAL OF VIROLOGY, July 1996, p. 4237–4245 Vol. 70, No. 7
0022-538X/96/$04.00+0

Identification and Characterization of a Double-Stranded RNA⁻ Reovirus Temperature-Sensitive Mutant Defective in Minor Core Protein μ2

KEVIN M. COOMBS*

Department of Medical Microbiology and Infectious Diseases, University of Manitoba, Winnipeg, Manitoba, Canada R3E 0W3

Received 27 November 1995/Accepted 28 March 1996

A newly identified temperature-sensitive mutant whose defect was mapped to the reovirus M1 gene (minor core protein μ2) was studied to better understand the functions of this virion protein. Sequence determination of the M1 gene of this mutant (*ts*H11.2) revealed a predicted methionine-to-threonine alteration at amino acid 399 and a change from proline to histidine at amino acid 414. The mutant made normal amounts of single-stranded RNA, both in in vitro transcriptase assays and in infected cells, and normal amounts of progeny viral protein at early times in a restrictive infection. However, *ts*H11.2 produced neither detectable progeny protein nor double-stranded RNA at late times in a restrictive infection. These studies indicate that μ2 plays a role in the conversion of reovirus mRNA to progeny double-stranded RNA.

The mammalian reoviruses have a genome consisting of 10 segments of double-stranded RNA (dsRNA). The RNA is enclosed by nonequivalent amounts of eight different structural proteins (for reviews, see references 59 and 72). The proteins form a double-shelled capsid of approximately 85 nm in diameter (22). Despite the significant amount of detail known about the structure, pathogenesis, and some aspects of replication of this virus, little is known about how mRNA is transcribed from the genomic RNA and about how the mRNA is subsequently used to make progeny dsRNA. After entry into a host cell, the outer capsid of the virus is removed to yield the core, the transcriptionally active inner capsid of the virus. The core is composed of three major proteins λ1, λ2, and σ2, and two minor proteins, λ3 and μ2. The core, which remains intact (9, 81), is a multienzyme complex that contains all necessary components for transcription, methylation, and capping of progeny mRNA (9, 28, 75). The λ2 proteins, which are organized as pentameric spikes at each icosahedral vertex (45, 60), possess guanylyltransferase activity (12, 46). Minor core protein λ3 has been associated with the pH optimum of transcription (20) and has poly(C)-dependent poly(G) polymerase activity (82). Minor core protein μ2 has been associated with the temperature optimum of transcription (90). However, the precise roles of many of these proteins remain unknown.

The segmented nature of the reovirus genome, the known coding capacities of each gene (49, 57), and strain-dependent mobility differences of the genes in polyacrylamide gels allow the generation and identification of intertypic reassortant viruses which have been used to assign particular functions to many of the virus proteins (6, 20, 21, 42, 48, 85). In addition, conditionally lethal *ts* reovirus mutants have been identified (1, 17, 25, 37, 64) and used to understand functions of viral proteins (for examples, see references 15, 17, 18, 27, 38, and 53). Members of the Fields panel of reovirus *ts* mutants have been assigned to 10 reassortant groups which are assumed to represent each of the different gene segments (25, 63). Prototype clones from each group were used to generate reassortants to map the *ts* lesions to their respective genome segments (56, 63, 65).

While analyzing revertants of the assembly-defective mutant *ts*C447, we isolated nine additional spontaneous mutants (15). This report represents the characterization of one of these mutants, clone *ts*11.2, a *ts* mutant that was capable of recombining with all available prototypic reovirus *ts* mutants. Reassortant mapping showed that clone *ts*11.2 contains a *ts* lesion in the M1 gene segment (group H, minor core protein μ2). This mutant was found to produce single-stranded RNA (ssRNA) but not dsRNA and to produce viral proteins only at early times at the nonpermissive temperature. These results indicate that minor core protein μ2 plays an important role in producing dsRNA from viral transcripts and lead to a better understanding of reovirus RNA replication.

MATERIALS AND METHODS

Abbreviations used. The following abbreviations are used in this paper: T1L, reovirus serotype 1 Lang; T3D, reovirus serotype 3 Dearing; *ts*, temperature-sensitive phenotype; ts⁺, wild-type phenotype; EOP, efficiency of plating; MOI, multiplicity of infection; dsRNA, double-stranded RNA; ssRNA, single-stranded RNA; MEM, minimal essential medium; TCA, trichloroacetic acid; and SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Cells and viruses. T1L, T3D, and various prototypic *ts* mutant clones derived from T3D are laboratory stocks which were kindly provided by B. Fields. Clones *ts*11.2 (a *ts* reassortant) and r36.146 (a ts⁺ reassortant with an electropherotype complementary to that of clone *ts*11.2) are clones derived from T3D × tsCr11 and T3D × tsCr36 crosses, respectively (15). Virus clones were plaque purified and grown in mouse L929 cell monolayers in Joklik modified MEM (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, as previously described (15). Large amounts of virus were grown in spinner cultures, extracted with Freon, and purified in cesium chloride gradients as described previously (14).

Virus titrations and EOP determinations. Serial 10-fold dilutions of viral lysate stocks were prepared in gel-saline, titers were determined in duplicate on L929 cell monolayers in six-well culture dishes, and the monolayers were overlaid with agar-medium 199, incubated, and stained with neutral red at various temperatures between 32 and 40°C as described previously (34). EOP values for each temperature were determined by dividing the apparent titer at the respective temperature by the apparent titer at 32°C (25). EOP values of ≤0.05 of the EOP values for parallel control T3D cultures were considered to indicate the *ts* phenotype, while EOP values of ≥0.1 of the EOP values for the parallel control T3D cultures were considered to indicate the ts⁺ phenotype.

Recombination assays. Assays to determine the reassortant group to which each *ts* mutant belonged were performed as described previously (24). Briefly,

* Phone: (204) 789-3309. Fax: (204) 783-5255. Electronic mail address: coombs@bldghsc.lanl.umanitoba.ca.

subconfluent L cells in 2-dram (ca. 7-ml) glass vials were infected with different combinations of *ts* clones, each at an MOI of 5 PFU per cell. Infected cells were incubated for 33 h at 32°C to generate reassortants. Viral lysates were prepared by two freeze-thaw cycles and further disrupted by sonication, and serial 10-fold dilutions were plated and incubated at 32, 39, and 40°C. Recombination was measured by the following formula (24): $\{[(AB)^{NP} - (A + B)^{NP}]/(AB)^{32}\} \times 100$, where NP corresponds to the nonpermissive temperature, A and B correspond to infections with each individual parent, and AB corresponds to the mixed infection with two parents.

Generation of reassortants for mapping temperature sensitivity. Mixtures for mixed infections and all subsequent plaque purifications and amplifications were incubated at 32°C to ensure there was no selection against either *ts* or ts^+ progeny. A subconfluent L929 monolayer in a 2-dram (ca. 7-ml) vial was infected with clone *ts*11.2 and clone r36.146, each at an MOI of 5 PFU per cell. Infection mixtures were incubated for 33 h at 32°C to generate reassortants. Viral lysates were prepared by freeze-thawing three times, and serial 10-fold dilutions were plated and incubated at 32°C. Individual plaques separated by ≥1 cm were picked, and each plaque was amplified to obtain second-passage stocks. Cytoplasmic extracts were prepared from each second-passage stock, RNA was resolved in polyacrylamide slab gels, and gene segments were identified as described previously (34).

Immunoprecipitations of virus-infected cell extracts. Immunoprecipitations were carried out essentially as described previously (34), but with the following modifications. Subconfluent L cells in six-well culture dishes were infected with either clone *ts*11.2 or T1L at an MOI of 10 PFU per cell. After adsorption for 1 h at 4°C, infection mixtures were overlaid with prewarmed MEM and incubated at various temperatures between 32 and 40°C. To analyze cumulative viral protein production at late times in infection, [^{35}S]methionine-cysteine (ICN Biomedical, Costa Mesa, Calif.) was added, to a final concentration of 25 μCi/ml, at 3 h postattachment for infections at 37 to 40°C or at 5 h postattachment for infections at 32 and 34°C. For each experiment, labeled infection mixtures were harvested after equivalent rounds of replication (32 to 48 h for 39 and 40°C infections, 35 to 56 h for 37 and 38°C infections, and 48 to 72 h for 32 and 34°C infections). Cytoplasmic extracts were prepared, incubated with anti-T3D reovirus-conjugated protein A-Sepharose beads, and processed, and radiolabeled peptides were resolved and measured as described previously (34).

Early viral protein production was analyzed essentially as previously described (17, 95). Briefly, L cells were pretreated for 1 h with 1 μg of actinomycin D per ml and mock infected or infected with T1L or *ts*11.2 at an MOI of 10 PFU per cell in the presence of actinomycin D. After a 1-h adsorption at 4°C, the inoculum was removed and cells were overlaid with prewarmed MEM that contained 1 μg of actinomycin D and 33 μCi of [^{35}S]methionine-cysteine per ml. Cells were incubated at 32 and 39°C, and at various times between 2 and 24 h postinfection, cells were harvested and cytoplasmic extracts were prepared. Aliquots of the cytoplasmic extracts were analyzed for total TCA-precipitable material, examined by SDS-PAGE, and immunoprecipitated and processed as described above.

Analyses of virus-specific RNA synthesis. The abilities of various virus clones to produce both ssRNA and dsRNA in infected cells were determined essentially as described previously (17, 39). Briefly, subconfluent L cells in six-well culture dishes were infected with various clones at an MOI of 10 PFU per cell. After attachment for 1 h at 4°C, infection mixtures were overlaid with prewarmed phosphate-free MEM supplemented with 0.5 μg of actinomycin D per ml and incubated at 39°C. $^{32}P_i$ (New England Nuclear, Mississauga, Ontario, Canada) was added at 30 min postattachment to a final concentration of 12.5 μCi/ml. Labeled infection mixtures were harvested 9 h later (for ssRNA) or 20 h later (for dsRNA). Infected cells were treated with lysis buffer (100 mM NaCl, 50 mM Tris [pH 8.0], 0.5% Nonidet P-40) supplemented with 800 U of RNasin (Boehringer Mannheim, Laval, Québec, Canada) per ml. Nuclei were removed by centrifugation to prepare cytoplasmic extracts, RNA was extracted and deproteinized (70), and ssRNA was separated from dsRNA by two consecutive LiCl precipitations (3). Labeled ssRNAs were precipitated, resuspended in 90% dimethyl sulfoxide–10 mM Tris (pH 7.4)–5 mM EDTA, and hybridized (63% dimethyl sulfoxide, 37°C, 20 h) to a mixture of excess unlabeled T1L and T3D genomic RNAs as described previously (39). Labeled RNA segments were resolved in SDS-PAGE as described below.

In vitro transcriptase activities of cores derived from various virus clones were measured as described previously (90). Briefly, spinner cultures of L cells were infected with appropriate virus stocks, incubated at 33°C for 65 h, harvested, and extracted with Freon, and the virus was purified in CsCl gradients. Purified virus was dialyzed and digested with chymotrypisin, and cores were purified in CsCl gradients. Purified cores were dialyzed, transcriptase assays were performed at various temperatures, and ^{32}P incorporation was measured.

SDS-PAGE. Radiolabeled samples were dissolved in electrophoresis sample buffer (0.24 M Tris [pH 6.8], 1.5% dithiothreitol, 1% SDS). Radiolabeled peptides were heated to 95°C for 3 to 5 minutes and resolved in SDS-PAGE with 5 to 15% polyacrylamide gels (44) (16.0 by 12.0 by 0.1 cm) at 5 mA for 18 h.

TABLE 1. Recombination between selected reovirus *ts* mutants

Mutant	Gene	EOP[a]	% ts^+ recombinants[b] when crossed with:									
			*ts*A201 [M2]	*ts*B352 [L2]	*ts*C447 [S2]	*ts*D357 [L1]	*ts*E320 [S3]	*ts*F556 [(M3)]	*ts*G453 [S4]	*ts*H26/8 [M1]	*ts*I138 [L3]	*ts*J128 [S1]
*ts*A201	M2	0.006	0	1.1	0.5	4.3	1.3	0	1.9	1.4	0	0
*ts*A279		0.01	0	3.0	0.9	5.1	2.4	0	5.9	2.0	0.2	0
*ts*A340		0.001	0	1.5	0.4	2.0	0.9	0	1.2	0.4	0	0
*ts*B271	L2	0.00003	1.8	0	1.3	5.5	12.3	1.6	0	0.7	0.2	0
*ts*B352		0.0002	1.1	0	0.7	1.0	0.1	3.6	2.6	0	1.3	0
*ts*B405		0.000003	3.6	0	3.0	13.6	3.8	3.3	7.6	0	1.4	4.5
*ts*C447	S2	0.00004			0	2.0	1.6	1.0	0.7	0.9	1.1	0
*ts*D357	L1	0.004				0	6.2	4.9	10.5	3.6	2.4	2.7
*ts*E320	S3	0.007					0	0	8.6	1.1	0.4	0
*ts*F556	(M3)	0.01						0	1.6	0.8	0.3	0
*ts*G453	S4	0.00001							0	18.7	0.5	0.2
*ts*H26/8	M1	0.007								0	0.3	0
*ts*I138	L3	0.000002									0	0
*ts*J128	S1	0.1										0
*ts*11.2[c]		0.0002	0.3	7.7	0.6	8.6	12.8	5.9	4.9	10.0	1.5	0
*ts*11.31[c]		0.0006	0.1	2.8	0.3	11.0	5.0	4.7	0	6.5	0	0
*ts*23.10[c]		0.007	3.6	0.6	21.1	25.6	13.3	32.7	4.5	0	6.8	8.6
*ts*23.59[c]		0.00002	0.2	5.9	0	0	0.2	1.1	0.1	3.4	0.2	0.4
*ts*23.66[c]		0.00006	1.4	0.1	1.3	0	2.0	0.5	1.0	1.7	2.0	0.6
*ts*28.22[c,d]		0.0007	1.3	0.9	2.9	2.2	3.0	0.1	0	5.0	0.1	3.6
*ts*31.13[c]		0.0001	2.7	1.3	3.2	12.6	8.4	0.9	0.5	7.3	0.1	0
*ts*31.25[c]		0.0001	11.3	2.3	0	4.4	3.6	1.2	0.4	0	0.2	0
*ts*36.27[c]		0.00007	0	4.2	0.2	0	0	0.6	0	0	0.1	0

[a] Ratio of plaque yield at 39°C to plaque yield at 32°C.
[b] Percent ts^+ reassortants = $\{[(\text{titer } AB^{39}) - (\text{titer } A^{39} + \text{titer } B^{39})]/\text{titer } AB^{32}\} \times 100$. Zero indicates that the calculated value was less than 0.05.
[c] Isolated as described in reference 15.
[d] Mapped to group G in reference 15.

Radiolabeled RNA samples were heated to 65°C for 5 min and resolved in SDS–10% PAGE (16.0 by 16.0 by 0.15 cm) slab gels at 18 mA per slab for 45 h. Gels were fixed, and protein gels were impregnated with Enlightning (DuPont, Mississauga, Ontario, Canada), and dried. RNA gels were autoradiographed, and protein gels were fluorographed (X-AR X-ray film; Kodak, Rochester, N.Y.) (33). Multiple film exposures of the gels were made and scanned with an LKB Ultroscan XL laser densitometer. Band intensities were scaled to each film exposure to determine the range of linear film response for each sample and the amount of material present in each band.

Sequencing the M1 gene. Genomic dsRNA was extracted from gradient-purified T1L and clone *ts*11.2 virions with phenol-chloroform (70). Oligonucleotide primers corresponding to the 5′ forward-sense end of the gene, the 5′ reverse-sense end, and at approximately 400-base intervals in both directions (about a 200-base overlap) were designed on the basis of the known T1L and T3D M1 sequences (86, 92) and synthesized with a Beckman Oligo1000 apparatus according to the manufacterer's instructions (Beckman Instruments, Fullerton, Calif.). Additional oligonucleotides for sequencing were designed as needed. cDNA copies of the M1 genes of each virus were constructed by using the 5′ oligonucleotide primers and reverse transcriptase (Boehringer Mannheim). The cDNAs were amplified by PCR (69) and resolved in 0.9% agarose gels (70), and the bands corresponding to the 2.3-kb gene were excised and eluted with Prep-a-gene according to the manufacterer's instructions (Bio-Rad, Richmond, Calif.). Sequences of the eluted cDNAs were determined in both directions by dideoxynucleotide cycle sequencing (16, 55, 71) and analyzed with DNASTAR (DNASTAR, Inc., Madison, Wis.).

RESULTS

Recombination between prototypic reovirus *ts* mutants and a panel of newly isolated *ts* mutants. We recently isolated nine additional *ts* mutants while screening revertants of *ts*C447 (15). To determine which gene segment(s) in these new mutants contained lesions, each was crossed with each of the prototypic reovirus *ts* mutants. The percentages of ts^+ reassortants generated from each of these recombination crosses and from crosses between each of the prototypic clones are shown in Table 1. As has been previously shown (8, 25, 63), most of the prototypic clones were capable of recombining efficiently with each other. The only exceptions were *ts*H26/8, which behaved like a group B (L2 gene) mutant, and *ts*F556, *ts*I138, and *ts*J128, which showed little recombination with other clones. Many of the newly isolated mutants could be easily placed into one of the preexisting reassortant groups by this test. For example, the inability of clone *ts*23.10 to recombine significantly with either *ts*B352 or *ts*H26/8 and the concurrent ability to recombine with other *ts* mutants indicated that clone *ts*23.10 had a *ts* lesion in the L2 gene. Similarly, the inability of clone *ts*28.22 to recombine with *ts*G453 indicated that clone *ts*28.22 had a *ts* lesion in the S4 gene, where it had previously been mapped (15). Some mutants appeared to have a *ts* defect in more than a single gene. Clone *ts*23.66 appeared to be a double mutant, with lesions in both the L1 and the L2 genes. Clones *ts*23.59 and *ts*36.27 appeared to be incapable of recombining with prototypic mutants in at least five different groups, suggesting that these clones had numerous defective genes. Clone *ts*11.2 was capable of recombining with all prototypic mutants. This implies that this mutant may represent a new reassortment group. Low recombination values obtained from crosses between clone *ts*31.13 and either *ts*G453 or *ts*I138 suggest that *ts*31.13 has lesions in both the L3 and S4 genes. Alternatively, these low, but positive, values might represent recombination, suggesting that this mutant also represents a new reassortment group. Clones *ts*11.2 and *ts*31.13 were able to recombine with each other (data not shown), indicating they belong to different reassortment groups.

Clone *ts*11.2 is *ts* at temperatures of 39 and higher. We and others have demonstrated that some reovirus mutants are only weakly *ts* at 39°C (25, 34) and that the choice of restrictive temperature can influence both the magnitude of apparent temperature sensitivity and resultant interpretations in *ts* mutant studies (34). Therefore, the expression of temperature

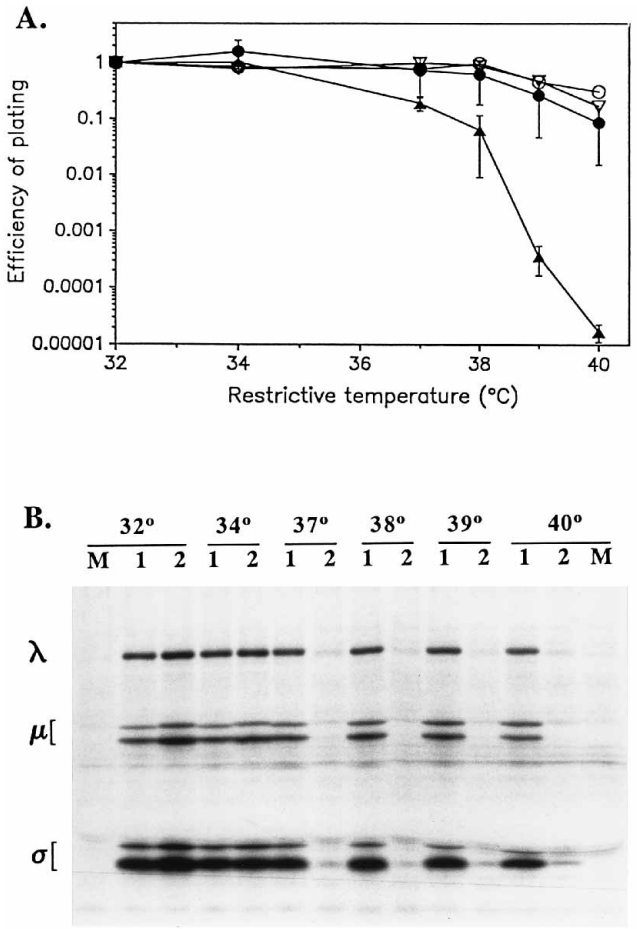

FIG. 1. Expression of temperature sensitivity by clone *ts*11.2 at different temperatures. (A) EOP values of wild-type reovirus serotypes T1L (○) and T3D (●), of ts^+ reassortant r36.146 (▽), and of *ts* reassortant *ts*11.2 (▲). EOP values for each restrictive temperature were determined by dividing the apparent titer at each temperature by the apparent titer at 32°C. Values represent averages from three or more experiments. Error bars, representing standard deviations from multiple experiments, are shown only for T3D and clone *ts*11.2 for clarity. (B) Immunoprecipitation fluorograms of [^{35}S]methionine-cysteine-labeled cell extracts mock infected (M) or infected with either T1L (lanes 1) or clone *ts*11.2 (lanes 2) and incubated at the indicated temperatures. Extracts were precipitated with Sepharose-conjugated protein A to which had been attached anti-T3D whole virus polyvalent antiserum. Labeled peptides were resolved by SDS-PAGE with 5 to 15% polyacrylamide gels (16.0 by 12.0 by 0.1 cm) at 5 mA for 18 h, and the gels were fixed, fluorographed, and exposed to X-ray film. Lanes were scanned with a densitometer to determine the amount of viral protein production at each temperature.

sensitivity of clone *ts*11.2 by EOP and progeny protein production over a range of temperatures from 34 to 40°C was determined. There was little difference between the EOP values of *ts*11.2 and various ts^+ clones at temperatures of 37°C and below (Fig. 1A). At 38°C the mutant had an EOP value almost 10-fold lower than that of the ts^+ clones. At higher temperatures, the EOP values of clone *ts*11.2 were progressively reduced, by between 1 and 2 orders of magnitude for each 1°C increase in temperature up to 40°C. The EOP value of the mutant at 39°C was about 1,000-fold lower than the EOP values of the ts^+ clones. Furthermore, electron microscopic analyses of cells infected with clone *ts*11.2 at the restrictive temperature of 39°C and examined at 24 h postinfection or later did not reveal any viral inclusions (data not shown). The *ts* mutant produced about the same amount of radiolabeled

TABLE 2. Electropherotypes and EOPs of *ts*11.2 × r36.146 reassortants

Clone	Parental source for the following gene segment[a]:										EOP[b]
	L1	L2	L3	M1	M2	M3	S1	S2	S3	S4	
*ts*11.2	3	1	1	1	3	3	1	1	1	1	
r36.146	1	3	3	3	1	1	3	3	3	3	
*ts*11.2	○	○	○	○	○	○	○	○	○	○	0.00021 ± 0.000031
r36.146	●	●	●	●	●	●	●	●	●	●	0.77 ± 0.29
11/36.04	○	●	○	○	○	●	○	○	○	●	0.0000056 ± 0.00000074
11/36.75	○	●	○	○	○	○	○	○	○	○	0.000011 ± 0.0000012
11/36.41	○	●	○	○	●	○	○	●	●	●	0.000017 ± 0.0000014
11/36.65	●	○	○	○	●	●	●	●	○	●	0.000019 ± 0.0000055
11/36.17	●	●	○	○	●	○	●	○	○	●	0.000020 ± 0.0000024
11/36.42	●	●	●	○	●	●	●	●	○	●	0.000024 ± 0.0000010
11/36.07	○	○	○	○	●	●	○	○	●	○	0.000026 ± 0.000018
11/36.47	●	●	○	○	●	●	●	●	○	●	0.000034 ± 0.0000026
11/36.32	●	●	○	○	○	○	○	○	○	○	0.000044 ± 0.000028
11/36.53	○	●	○	○	○	○	○	○	○	●	0.000061 ± 0.000041
11/36.44	○	●	○	○	○	○	○	○	●	○	0.000071 ± 0.000079
11/36.26	○	○	○	○	●	○	○	●	●	●	0.00010 ± 0.0000072
11/36.62	●	○	○	○	●	○	●	●	○	○	0.00013 ± 0.00012
11/36.64	●	●	●	○	●	○	○	●	○	○	0.00024 ± 0.000086
11/36.01	●	○	○	○	○	○	○	○	●	●	0.00041 ± 0.00029
11/36.66	○	○	○	○	●	○	○	○	○	○	0.00076 ± 0.00047
11/36.46	○	○	○	○	○	○	○	○	○	●	0.0024 ± 0.0017
11/36.61	●	●	○	●	●	●	●	●	○	○	0.065 ± 0.020
11/36.40	●	●	○	●	○	●	○	○	●	○	0.171 ± 0.038
11/36.03	●	○	○	●	○	○	○	○	○	○	0.247 ± 0.040
11/36.73	○	○	○	●	○	○	○	○	○	○	0.270 ± 0.0026
11/36.02	●	●	●	●	●	●	○	●	●	○	0.275 ± 0.0026
11/36.15	○	○	●	●	●	●	○	○	○	○	0.370 ± 0.16
11/36.34	○	●	●	●	●	●	○	○	○	○	0.654 ± 0.37
11/36.68	●	○	○	●	●	●	○	●	●	○	0.691 ± 0.34
11/36.16	●	●	●	●	●	●	●	○	●	●	0.722 ± 0.40
11/36.19	●	○	●	●	●	●	○	●	●	○	0.798 ± 0.069
11/36.09	○	○	○	●	●	●	○	●	●	○	0.897 ± 0.27
11/36.28	○	○	●	●	●	●	○	●	●	○	1.100 ± 0.50
Total no. of exceptions	13	17	8	0	13	7	15	13	10	21	

[a] Parental origin of gene. ○, *ts*11.2; ●, r36.146.
[b] Yield at 39°C/yield at 32°C. Values represent the averages obtained from two or more experiments ± standard deviations.

protein as T1L at 32 and 34°C (Fig. 1B). However, the mutant produced progressively less protein than did T1L at increasing temperatures. At 37°C the mutant produced about 10% of the amount of radiolabeled protein produced by T1L, and at temperatures of ≥38°C the mutant produced about 2 to 3 orders of magnitude less protein than did T1L.

The *ts* lesion in *ts*11.2 resides in the M1 gene. Clone *ts*11.2 was crossed with clone r36.146. A total of 75 clones from the mixed infection were selected, amplified, and electropherotyped. Of these, 30 had reassortant electropherotypes (reassortment efficiency = 40%; 29 unique electropherotypes). EOP analyses of the resultant unique reassortants indicated that they could be divided into two groups (Table 2). Every reassortant with a *ts*-like EOP value (more than 100-fold lower than the EOP values of T1L, T3D, or clone r36.146) contained the M1 gene derived from the mutant. Every reassortant with a ts^+-like EOP value (within an order of magnitude of the EOP values of T1L and T3D) contained the M1 gene derived from clone r36.146. One of these latter clones (clone 11/36.73) was an M1 monoreassortant. Finally, each of the other nine genes was randomly associated with respect to temperature sensitivity. These observations clearly indicate that clone *ts*11.2 contains a *ts* lesion in the M1 gene, which encodes minor core protein μ2. These data would place this mutant in recombination group H. This mutant will subsequently be referred to as *ts*H11.2.

The mutant *ts*H11.2 M1 gene contains two alterations. The sequence of the mutant M1 gene was determined and compared with the T1L M1 sequence. The mutant M1 gene contained two changes in the μ2 open reading frame, a U-to-C change at nucleotide position 1209 and a C-to-A substitution at nucleotide position 1254 (Fig. 2A). Each of these alterations lead to predicted amino acid changes; a methionine-to-threonine substitution at amino acid 399 and a change from proline to histidine at amino acid 414, respectively (Fig. 2B).

***ts*H11.2 produces ssRNA, but not dsRNA, at the restrictive temperature.** The capacities of various virus clones to produce progeny ssRNA were measured by two different methods. Gradient-purified core particles derived from T1L, T3D, and various *ts* mutants were used in in vitro transcriptase assays. All clones produced comparable, and increasing, levels of product as the reaction temperature was raised from 30 to 50°C (Fig.

A

```
          1190                                                                                                      1273
T1L       AGG CAU ACA AUC GAU GUC AUG CCU GAU AUA UAU GAC UUC GUU AAA CCC AUU GGC GCU GUG CUG CCU AAG GGA UCA UUU AAA UCA
tsH11.2   --- --- --- --- --- --- -C- --- --- --- --- --- --- --- --- --- --- --- --- --- --- -A- --- --- --- --- --- ---
```

B

```
          393                     399                                                           414                     420
T1L       Arg His Thr Ile Asp Val Met Pro Asp Ile Tyr Asp Phe Val Lys Pro Ile Gly Ala Val Leu Pro Lys Gly Ser Phe Lys Ser
tsH11.2                           Thr                                                           His
```

FIG. 2. Comparison of the nucleotide and predicted amino acid sequences of the T1L and *ts*H11.2 M1 genes. For clarity, only those regions of the sequences that contain differences are shown. (A) Coding-strand nucleotide sequences of T1L and *ts*H11.2. Nucleotide positions are numbered above the sequences, and residues in *ts*H11.2 identical to those in T1L are indicated by dashes. (B) Deduced amino acid sequences of the T1L and *ts*H11.2 μ2 proteins corresponding to the region of nucleotide sequence shown in panel A. Amino acid positions are numbered above the sequence, and only altered amino acids are shown for *ts*H11.2.

3). *ts*G453 produced less TCA-precipitable material per core particle than did the other clones, as previously demonstrated (17). *ts*H11.2 produced nearly the same amount of material as did T3D at all tested temperatures.

To determine if various virus-specific ssRNAs were produced in cells incubated at the restrictive temperature, ssRNA was purified from ^{32}P-labeled 39°C infection mixtures and hybridized to a mixture of excess unlabeled T1L and T3D genomic RNAs. The two prototype wild-type clones T1L and T3D, the *ts* clone *ts*H11.2, and the ts^+ clone r36.146 all were capable of producing virus-specific ssRNA (Fig. 4A). T1L, T3D, clone r36.146, and the ts^+ M1 monoreassortant 11/36.73 also produced dsRNA (Fig. 4B). However, *ts*H11.2 and the two *ts* reassortants 11/36.41 and 11/36.42 (which collectively rule out all non-M1 genes) produced undetectable amounts of dsRNA. Collectively, these results indicate that the M1 *ts* defect in *ts*H11.2 allows the generation of ssRNA but not dsRNA at the nonpermissive temperature.

***ts*H11.2 produces normal amounts of viral protein at early times, but little detectable protein at late times, in restrictive infections.** Reovirus proteins are translated from plus-sense transcripts that are produced both early and late in infection (83; for a review, see reference 76), and the production of late transcripts requires prior protein production (43, 77, 84). Thus, to determine whether the significant decrease in cumulative viral protein production seen at late times in infection (Fig. 1B) resulted from a decrease in early and/or late translation, viral protein production was examined at various times during infection. The kinetics of virus protein production appeared to be similar for T1L and *ts*H11.2 at 32°C. Virus proteins were detected by 4 to 6 h postinfection, and the amount of protein increased with increased incubation times (Fig. 5). The kinetics of virus protein production also were similar for T1L and *ts*H11.2 at early times at 39°C. Virus proteins were detected by 2 to 4 h postinfection. The two clones produced nearly equivalent amounts of protein until 8 h postinfection. However, in contrast to T1L infections, in which the total amount of viral protein continued to increase with time, there appeared to be little additional increase in the amount of protein produced by *ts*H11.2 after 8 to 12 h. In addition, the mutant showed altered μ1/μ1c ratios at this time. These data indicate that in the restrictive mutant infections, proteins were produced only from early transcripts.

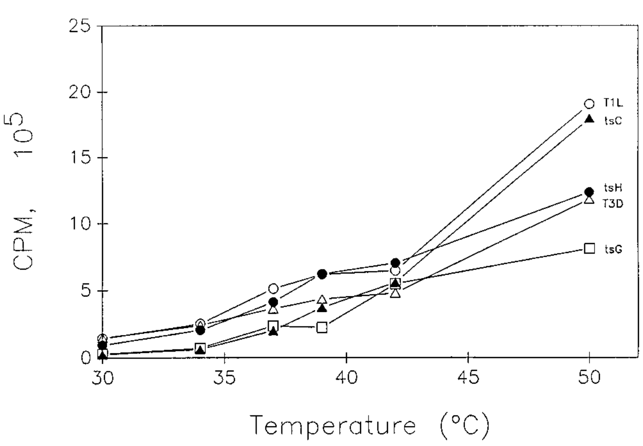

FIG. 3. Transcriptase activities of T1L (○), T3D (△), and the *ts* mutants *ts*C447 (σ2 core protein) (▲), *ts*G453 (σ3 outer capsid protein) (□), and *ts*H11.2 (●). Aliquots of 3.5×10^{11} total core particles in 50-μl volumes were incubated at the indicated temperatures for 1 h and precipitated with TCA, precipitates were collected on filters, and radioactivity was counted. Results are averages from triplicate experiments.

DISCUSSION

Minor reovirus core protein μ2 is involved in producing dsRNA. A *ts* reovirus mutant that appeared to be capable of producing normal amounts of virus-specific ssRNA at various temperatures (Fig. 3 and 4) and normal amounts of viral proteins at early times in a restrictive infection (Fig. 5) was identified. However, this mutant produced little detectable dsRNA or protein at later times at the nonpermissive temperature (Fig. 1B, 4, and 5). This suggests that *ts*H11.2 represents a new $dsRNA^-$ mutant which is not able to use the ssRNA produced as a template for second-strand synthesis. The mechanisms by which the enclosed reovirus genomic dsRNA is transcribed to produce mRNA and by which the mRNA is used to generate progeny dsRNA are among the most poorly understood processes in viral replication. The use of conditionally lethal *ts* mutants has allowed the elucidation of protein-folding pathways (51), viral pathogenesis (74, 79), genomic integration (74, 87) and replication (11, 50, 58), and virus morphogenesis and assembly (5, 13, 23, 67). Thus, the availability and study of a *ts* mutant defective in a poorly understood reovirus process might prove useful for elucidating that process. Reassortant mapping experiments (Table 2) clearly indicated that the *ts* inability of *ts*H11.2 to utilize the ssRNA resided in the viral M1 gene.

The reovirus M1 gene is 2,304 bp long (86, 92) and encodes

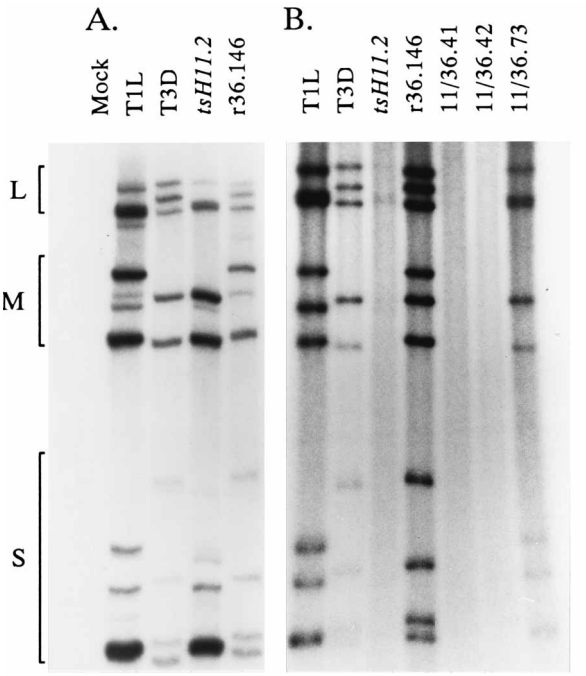

FIG. 4. SDS–10% PAGE analysis of ^{32}P-labeled viral RNA. Cells were infected with the indicated clones, labeled with $^{32}P_i$, and incubated at 39°C, as detailed in Materials and Methods. (A) At 9 h postinfection, ssRNA was purified and hybridized to a mixture of excess unlabeled T1L and T3D genomic RNAs as detailed in Materials and Methods. Mock, labeled T1L-infected cells processed for ssRNA but not hybridized. (B) At 20 h postinfection, dsRNA was purified. Gels were run at 18 mA for 45 h, fixed, dried, and autoradiographed.

minor reovirus core protein μ2. Depending upon where translation of this gene's mRNA initiates, μ2 is predicted to be either 687 or 736 amino acids in length (68, 86, 92). Recent evidence suggests that the initial in-frame AUG at nucleotide position 14 is used (94), which would result in a protein 736 amino acids in length. The use of this AUG, rather than the next in-frame AUG at nucleotide position 161, would generate a 5′ nontranslated region consistent in length with the 5′ nontranslated regions of each of the other reovirus genes whose sequences have been determined (reference 19 and references therein). The *ts*H11.2 μ2 protein contains two predicted amino acid changes. Secondary-structure predictions, based on the Garnier-Robson algorithms (29), suggest that the first amino acid alteration, a relatively conservative change from methionine to threonine at position 399, induces minor changes in secondary structure, whereas the second amino acid alteration, a relatively nonconservative change from proline to histidine at amino acid 414, results in significant predicted changes in secondary structure, with conversion of a region of the β-strand between amino acids Pro-408 and Leu-413 to an α-helix motif (data not shown). The μ2 protein has no significant similarity with other proteins in GenBank (86), and little is known about its function(s). Reassortant mapping experiments suggest that μ2 plays a role in determining the severity of cytopathic effect in cultured cells (52) and the level of virus growth in cardiac cells (48) and endothelial cells (47). It is also involved in myocarditis (78), in organ-specific virulence in SCID mice (32), and in in vitro transcription of ssRNA (90). Such observations have led to the speculation that μ2 may play a role in RNA metabolism (47, 48, 78, 90). The results presented in this paper suggest that μ2 also is involved in producing dsRNA from mRNA.

The reovirus core is released when outer capsid proteins are removed during virus entry into a host cell (7; for a review, see reference 59). This structure, as described in the introduction, is responsible for transcribing mRNA from each of the 10 enclosed segments of dsRNA. Negative-stain electron microscopy (4) and image reconstruction of electron cryomicroscopy images (89) of actively transcribing core particles suggest that mRNA is extruded through the central channels of the λ2 spikes. Nascent viral mRNA is used initially to make progeny viral proteins (for reviews, see references 76 and 91). It is thought that as viral proteins are synthesized, some associate with the mRNA, forming a ribonucleoprotein complex. As additional proteins, including nonstructural ones, and mRNA corresponding to each of the 10 genes are recruited into the complex, it undergoes conformational changes that allow the synthesis of the second RNA strands (2, 40, 41). This process results in the eventual generation of a full complement of 10 progeny dsRNA genes in a precore-like structure (2, 40, 41). This structure also is used to make late transcripts, which are used for protein synthesis and account for the dramatic increase in viral protein synthesis seen at late times in infection (76, 83). The ability of a *ts* mutant with a mutation in the μ2 protein to produce ssRNA and proteins early in infection and the concommitant inability of the mutant to make dsRNA or protein late in a restrictive infection (this study) are consistent with such a process and suggest that minor core protein μ2 plays a role in the conversion of ssRNA to dsRNA. Thus, *ts*H11.2 represents a new dsRNA$^-$ mutant. Recently, a recombinant cell line that constitutively expresses wild-type μ2 has been shown to be capable of complementing the defect in *ts*H11.2 at the nonpermissive temperature (93).

Relationship of *ts*H11.2 to other reovirus *ts* mutants. There are several possible reasons why some of our prototypic *ts* mutants did not behave as expected in the recombination assay (Table 1). Previous reassortant mapping experiments with *ts*H26/8 used two parental *ts* viruses, examined primarily the panel of ts$^+$ reassortants, and implicated several genes (65). Clone *ts*26/8 was assigned to the M1 gene because it appeared to be capable of recombining with other mutants (1). However, recent analyses of numerous *ts* and ts$^+$ reassortants derived from crosses between wild-type T1L and the *ts*26/8 clone used in the present study indicated that our clone of *ts*26/8 has its *ts* lesion in the L2 gene (35). Alternatively, our clone of *ts*26/8 may not be the same as that originally used more than 20 years ago. Our clones of *ts*F556, *ts*I138, and *ts*J128 appeared incapable of recombining with many other prototypic *ts* mutants. One reason for this may be the high EOP values of some of these clones. For example, our clone of *ts*J128 had an EOP of 0.1, which is nearly indistinguishable from the EOP of wild-type T1L or T3D. In addition, numerous reassortant analyses of several J128 clones failed to map a *ts* defect to any gene (80). Furthermore, the lesion in *ts*F556 has not been mapped. Finally, clone *ts*I138 grows poorly, even at the permissive temperature, which may have led to low recombination values. Reassortant analyses of numerous *ts* and ts$^+$ reassortants derived from crosses between T1L and *ts*I138 confirmed that the *ts* lesion in *ts*I138 resides in the L3 gene (35), as previously reported (65).

Several other dsRNA$^-$ *ts* mutants have been identified and partially characterized (17, 39, 66). These include mutants with mutations in core protein σ2 (*ts*C447), a protein that binds dsRNA (73) and is required for capsid assembly (15, 88); in core protein λ3 (*ts*D357), the RNA polymerase (20, 54, 82); and in nonstructural protein σ_{NS} (*ts*E320), a protein that binds ssRNA (36). Ramig and his group have generated rotavirus *ts* mutants (61, 62), mapped many of them to gene segments (30,

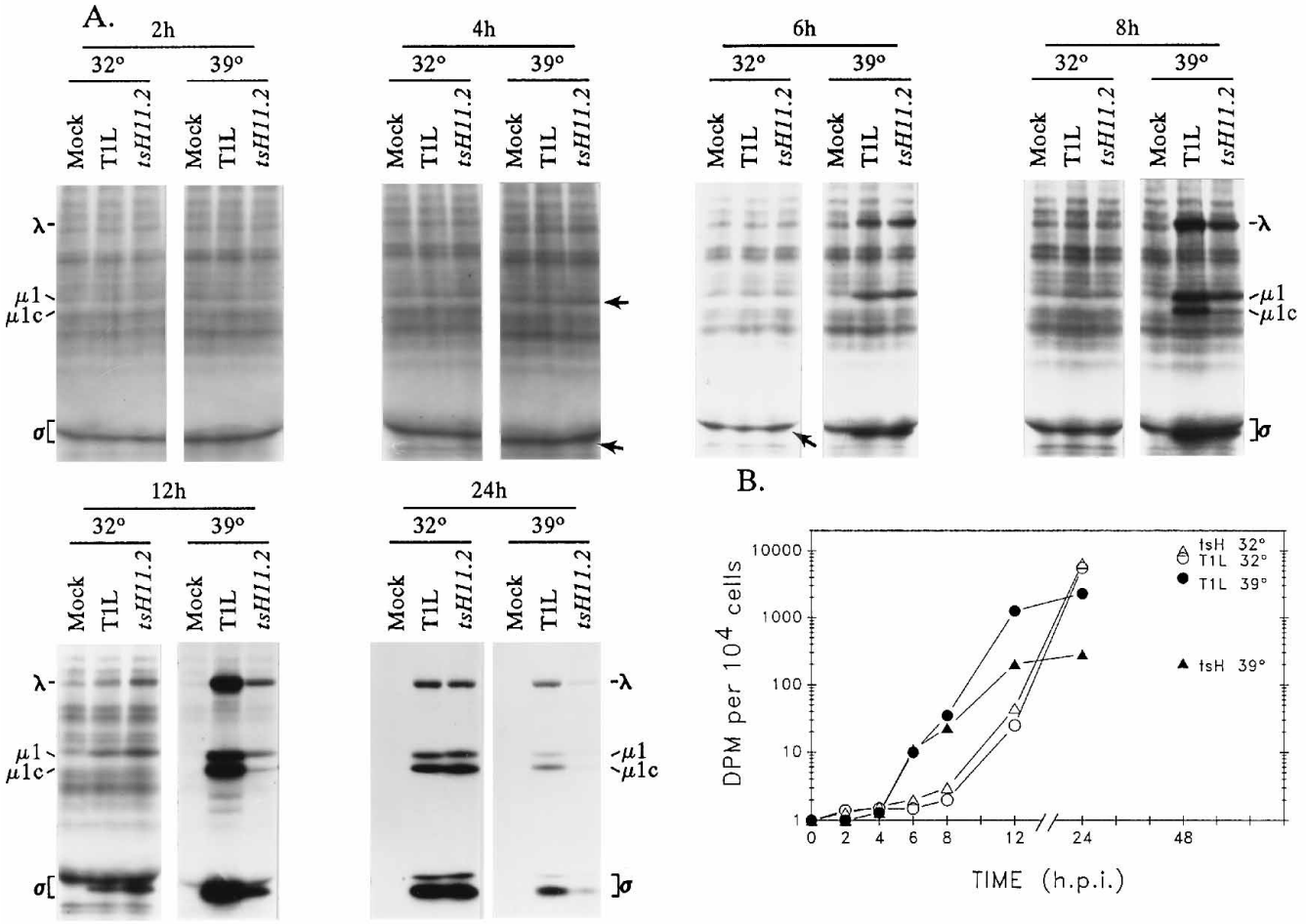

FIG. 5. Kinetics of viral protein production by the indicated virus clones at 32 and 39°C. Infection mixtures that had been pretreated with 1 μg of actinomycin D for 1 h were mock infected or infected with the indicated clones, labeled with [^{35}S]methionine-cysteine, incubated, and harvested at the indicated times. (A) Cytoplasmic extracts were prepared as detailed in Materials and Methods, and labeled peptides were resolved as described in the legend to Fig. 1B. Equivalent numbers of cell extracts were loaded within each time frame, but sample loadings and exposure times for each time frame were adjusted to optimally visualize virus proteins and differences between T1L and *ts*H11.2 (e.g., 2-h samples represent 2.5×10^6 cells exposed for 8 days, and 24-h samples represent a 4-h exposure of 3.8×10^5 cells). Barely detectable virus proteins (σ3 and μ1) at early time points are indicated by arrows. (B) The total amount of virus-specific protein produced by each clone at each temperature and time was determined by scaling densitometric scans of SDS-PAGE before and after immunoprecipitation to the total amount of TCA-precipitable material present in parallel aliquots. The symbols at 48 h (not connected by lines) represent the amounts of virus-specific protein produced at later times in parallel non-actinomycin D-treated infected cells (see Fig. 1B). h.p.i., hours postinfection.

31), and shown that a core protein mutant also is capable of making ssRNA and protein, but not dsRNA (10, 66). However, unlike the other dsRNA$^-$ reovirus mutants, in which ssRNA, dsRNA, and protein all are significantly reduced (17, 26, 72), *ts*H11.2 makes normal amounts of ssRNA and protein at early times and makes greatly reduced amounts of dsRNA and protein at late times at the nonpermissive temperature. Thus, *ts*H11.2 represents a novel reovirus *ts* mutant. Further study of this mutant may help elucidate the roles played by minor core protein μ2 in reovirus replication.

ACKNOWLEDGMENTS

I thank Elaine Freemont, Anne Georgi, and B. N. Fields for providing virus clones used in this study; Earl Brown, Terence Dermody, and Barbara Sherry for critical reviews of this study; Lydia Petrycky-Cox for stock cell maintenance and expert technical help; Paul Hazelton for electron microscopy, photography, and helpful discussion; and Mark Makarovsky and Peng Yin for helpful discussion.

This research was supported by grant MT-11630 from the Medical Research Council of Canada.

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