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Studies of the Major Reovirus Core Protein σ2: Reversion of the Assembly-Defective Mutant *ts*C447 Is an Intragenic Process and Involves Back Mutation of Asp-383 to Asn

KEVIN M. COOMBS,* SIN-CHI MAK, AND LYDIA D. PETRYCKY-COX

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

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The reovirus group C temperature-sensitive mutant *ts*C447, whose defect maps to the S2 gene, which encodes the major core protein σ2, fails to assemble core particles at the nonpermissive temperature. To identify other proteins that may interact with σ2 during assembly, we generated and examined 10 independent revertants of the mutant. To determine which gene(s) carried a compensatory suppressor mutation(s), we generated intertypic reassortants between wild-type reovirus serotype 1 Lang and each revertant and determined the temperature sensitivities of the reassortants by efficiency-of-plating assays. Results of the efficiency-of-plating analyses indicated that reversion of the *ts*C447 defect was an intragenic process in all revertants. To identify the region(s) of σ2 that had reverted, we determined the nucleotide sequences of the S2 genes. In all revertant sequences examined, the G at nucleotide position 1166 in *ts*C447 had reverted to the A present in the wild-type sequence. This reversion leads to the restoration of a wild-type asparagine (in place of a mutant aspartic acid) at amino acid 383 in the σ2 sequence. These results collectively indicate that the functional lesion in *ts*C447 is Asp-383 and that this lesion cannot be corrected by alterations in other core proteins. These observations suggest that this region of σ2, which may be important in mediating assembly of the core particle, does not interact significantly with other reovirus proteins.

The assembly of a virus particle is an important late step in its replication. Assembly involves the extremely specific and reproducible stepwise condensation of multiple proteins and nucleic acids into a highly organized and functionally active macromolecular complex. Details of this complicated process have been elucidated for some bacteriophages (2, 45) and plant viruses (3) but remain poorly understood for animal viruses. The availability of conditionally lethal assembly-defective bacteriophage mutants aided elucidation of their assembly. Such mutants have been described for the mammalian reoviruses (16) and may prove useful in understanding molecular signals that direct assembly of an animal virus.

The reoviruses have a genome of 10 double-stranded RNA segments which are enclosed in a double shell of protein (for reviews see references 43 and 55). After entry into a host cell the virus is proteolytically uncoated to yield the inner shell (core), the transcriptionally active form of the virus. The core is a multienzyme complex composed of three major proteins (λ1, λ2, and σ2) and two minor proteins (λ3 and μ2). Cores contain all necessary components for transcription, capping, and methylation of progeny mRNA (4, 19). The genomic RNA almost completely fills the interior of the core (23) and remains inside during transcription (4). Signals that direct assembly of the core from the five different proteins and 10 different RNA segments remain unknown. Therefore, to better understand, mechanistically, how various enzymatic functions can be carried out on the densely organized genomic RNA during transcription and to elucidate assembly of the particle, more detailed information is needed about core structure, functions of the various proteins, and protein-protein, protein-RNA, and RNA-RNA interactions.

The λ2 proteins, organized as pentameric spikes at each icosahedral vertex (32, 46), possess guanylyltransferase activity (5), have GTP-binding sequences (57), and undergo major conformational alterations as the virus is uncoated to the core (13). Proteins λ1 and λ3 also appear to play roles in transcription (1, 12, 39, 40). However, the precise locations of λ1, λ3, μ2, and σ2 remain unknown. Cores have been crystallized (7) and examined by cryoelectron microscopy (13, 38). However, available crystals diffract only to 8 Å (0.8 nm) resolution and current cryoelectronmicroscopic reconstructions do not allow definitive identification of the other structural proteins. In addition, little is known about the protein and RNA interactions responsible for maintaining the core structure and those involved in early stages of particle assembly. Assembly of the protein shell does not require the presence of RNA; empty virion particles (called top component) which lack all or most of the RNA are naturally produced in an infection (60), and expression of the major capsid proteins of the closely related bluetongue virus and rotavirus leads to production of capsid-like structures (18, 65). In addition, the ability to remove the λ2 spikes, apparently without disturbing the structure of the underlying protein shell (68), suggests that most of the information required for assembly of the core protein shell resides in one or both of the major proteins λ1 and σ2. Furthermore, a conditionally lethal temperature-sensitive (*ts*) mutant (*ts*C447), whose defect has been mapped to the major core protein σ2 (49), fails to generate core particles at the nonpermissive temperature (17). These collective observations suggest that protein σ2 plays a pivotal role in assembly of the core protein shell. Thus, better understanding of σ2, its interactions with other core proteins, and the nature of the defect in *ts*C447 should aid in understanding the mechanism of core particle assembly.

* Corresponding author. Mailing address: Department of Medical Microbiology and Infectious Diseases, University of Manitoba, Basic Sciences Building, 730 William Ave., Winnipeg, Manitoba, Canada R3E 0W3. Phone: (204) 789-3309. Fax: (204) 783-5255. Electronic mail address: coombs@bldghsc.lan1.umanitoba.ca.

Little is known about protein σ2. It binds double-stranded RNA (11) and is presumed to occupy an internal position in the core (68). Biochemical attempts to cross-link σ2 to other core proteins with various cross-linking agents have failed to demonstrate interactions between σ2 and the other proteins (6). Therefore, we decided to use a genetic approach to examine form-determining, structure-function, and assembly interactions of σ2. Genetic studies of conditional-lethal mutations and of their compensatory suppressor mutations have proven useful in understanding assembly pathways of various bacteriophages (2, 45), and of protein-protein interactions in a large number of viruses, bacteria, and eukaryotic cells (reviewed in reference 22). Suppression by an extragenic mutation implies that the two genes (or, more commonly, their protein products) interact with each other at either a structural or functional level or both. Identification of a number of such extragenic suppressor mutations has been useful in describing protein interactions in such diverse viruses as bacteriophage P22 (25), foot-and-mouth disease virus (27), influenza virus (64), and the outer capsid proteins of reovirus (37). Therefore, we attempted to identify both intragenic and extragenic suppressor mutations of σ2 in order to identify other proteins with which σ2 interacts and to identify regions of σ2 that may interact with the region(s) that contains the *ts* lesion(s). Our results, presented below, indicate that reversion of the mutant S2 gene of *ts*C447 was an intragenic process, involving a true reversion of the G at position 1166 in the *ts*C447 S2 sequence to a wild-type A, which resulted in restoration of an asparagine residue at amino acid 383 in the σ2 protein. These findings have important implications for understanding how σ2 may be involved in assembly of the particle.

MATERIALS AND METHODS

Cells and viruses. Reovirus type 1 Lang (T1L), type 3 Dearing (T3D), and the group C (S2 gene, σ2 protein) temperature-sensitive mutant *ts*C447 derived from T3D are laboratory stocks obtained from B. Fields. Virus stocks 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 agammaglobulin-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.

Virus titer determinations and EOP determinations. Serial 10-fold dilutions of viral lysate stocks were made in gel saline (137 mM NaCl, 0.2 mM $CaCl_2$, 0.8 mM $MgCl_2$, 19 mM HBO_3, 0.1 mM $Na_2B_4O_7$, 0.3% [wt/vol] gelatin), and their titers were determined in duplicate on L cell monolayers in six-well cluster dishes. After viral attachment (1 h at room temperature), cells were overlaid with 3 ml per well of a 50:50 (vol/vol) mixture of 2% Bacto Agar (Difco Laboratories, Detroit, Mich.) and completed 2× medium 199 (medium 199 [GIBCO], 2.5% FCS, 2.5% VSP, 4 mM glutamine, 200 U of penicillin per ml, 200 μg of streptomycin sulfate per ml, 3 μg of amphotericin B per ml). Cells were incubated at 32°C (the permissive temperature) or 39°C (the nonpermissive temperature) essentially as described previously (16). Monolayers were overlaid a second time with 2 ml of completed agar-199 per well at 2 days after inoculation for 39°C plates and at 6 days after inoculation for 32°C plates. A final overlay with medium 199 which contained 1% agar and 0.04% neutral red was performed 5 days after inoculation for 39°C plates and 14 days after inoculation for 32°C plates. Plaques were counted 18 to 24 h after neutral red overlay. Efficiency-of-plating (EOP) values were determined by dividing the apparent titer at the nonpermissive temperature by the apparent titer at the permissive temperature (16). EOP values of ≥0.1 were considered to be wild type, and EOP values of <0.05 were considered to be *ts* (16).

Generation of *ts*C447 revertants. Aliquots (50 μl) of 10 different undiluted second-passage stocks of *ts*C447 were plated in 100-mm tissue culture dishes, adsorbed for 1 h, overlaid with agar-medium 199, and incubated at the nonpermissive temperature of 39°C for 5 days, and stained with neutral red as described above. Three to five well-separated plaques were picked from each of the 10 stock plates (total of 40 clones) into 1 ml of gel saline. Dilutions of each plaque were serially replated twice at the nonpermissive temperature. The final triply purified plaques were amplified through two passages at 37°C to obtain viral lysate stocks.

Electron microscopy. Ultrastructural examination of L929 cells infected with T3D, *ts*C447, and various revertants at the nonpermissive temperature was carried out essentially as previously described (17). Briefly, cells were concentrated to 2×10^7/ml and infected at a multiplicity of infection of 10 PFU per cell. After 30 min of adsorption at 37°C with periodic swirling, cells were diluted to 5×10^5/ml in completed minimal essential medium that was maintained at 39°C. Cells were incubated in suspension, and at various times aliquots of infected cells were withdrawn, fixed by the addition of 1/25 volume of 50% glutaraldehyde, and chilled to 4°C. After fixation for at least 1 h, cells were rinsed in 0.1 M sodium cacodylate buffer (pH 7.2), postfixed with 1% osmium tetroxide, and pelleted in 1.5% low-temperature-gelling agarose, essentially as described previously (70). Specimens were dehydrated by a graded acetone series and embedded in DER 332-732 plastic (EM Sciences, Fort Washington, Pa.). Embedded cells were sectioned with an LKB ultrotome III ultramicrotome, mounted on uncoated 300-mesh copper grids, poststained with saturated ethanolic uranyl acetate and 0.25% aqueous lead citrate (61, 66), and examined under a Phillips 201 electron microscope operating at an accelerating voltage of 60 kV.

Construction of intertypic reassortants and electropherotyping of gene segments. Mixed infections and subsequent plaque purifications and reassortant amplifications were incubated at 32°C so as not to select against either *ts* or wild-type-like (ts^+) clones. Subconfluent L cell monolayers in 24-well dishes were infected at a multiplicity of infection of 10 PFU per cell with T1L and either *ts*C447 or each revertant (5 PFU per cell for each parent). Mixed infections were incubated for 36 h to generate reassortants and then freeze-thawed three times. Serial threefold dilutions of the cell lysates were plated in 60-mm tissue culture dishes, overlaid with agar-medium 199, incubated for 17 days, and stained with neutral red. Individual well-separated plaques were picked, and each plaque was amplified to obtain second-passage stocks. Cytoplasmic extracts were prepared from each second-passage stock as described previously (59). Briefly, L cell monolayers in 60-mm dishes were infected with each reassortant clone, infected cells were harvested when they showed greater than 75% cytopathic effect and were lysed in 0.5% Nonidet P-40, nuclei were removed, and the cytosolic fractions were extracted once with phenol-chloroform. RNA was precipitated overnight at −20°C in ethanol and resuspended in electrophoresis sample buffer (2% sodium dodecyl sulfate [SDS], 100 mM dithiothreitol, 50 mM Tris [pH 6.8]), and gene segments were resolved in SDS-polyacrylamide gel electrophoresis slab gels (28) (10% polyacrylamide; 16.0 by 16.0 by 0.15 cm) at 18 mA per slab for 45 h. The gels were stained with ethidium bromide, double-stranded RNA was visualized under UV irradiation, and the

TABLE 1. Electropherotypes and EOPs of T1L-*ts*C447 reassortants

Clone	Gene segment[a]										EOP[b]
	L1	L2	L3	M1	M2	M3	S1	S2	S3	S4	
*ts*C447	C	C	C	C	C	C	C	**C**	C	C	0.00002
LC 3	1	C	1	1	1	1	1	**C**	1	1	0.00001
LC 18	C	1	1	C	C	C	C	**C**	C	C	0.00005
LC 30	1	C	C	1	C	C	C	**C**	C	C	0.00001
LC 31	1	1	1	1	1	1	1	**C**	1	C	0.00038
LC 32	C	C	1	C	C	1	1	**C**	C	C	0.00010
T1L	1	1	1	1	1	1	1	**1**	1	1	1.04
LC 15	C	1	C	C	C	C	C	**1**	C	C	1.08
LC 19	C	C	C	1	C	1	1	**1**	C	1	1.20
LC 24	C	1	1	C	C	C	C	**1**	1	1	0.74
LC 42	C	C	1	C	C	1	1	**1**	C	C	0.63
LC 43	C	1	C	C	C	C	1	**1**	1	C	0.44

[a] Symbols in table denote parental origin of gene. 1, T1L; C, *ts*C447.
[b] EOP is expressed as titer at 39°C/titer at 32°C.

gels were photographed. Gene segment identities were determined from their electrophoretic mobilities as described previously (49, 58).

Cloning and sequencing the revertant S2 genes. The S2 genes of T3D, *ts*C447, and various revertants were cloned and sequenced by standard procedures (53). Briefly, virus was grown in suspension cultures at 32°C for 66 h, freon extracted, purified in cesium chloride gradients, and dialyzed, all as described previously (13, 60). Genomic double-stranded RNA was phenol-chloroform extracted from purified virions (53). A cDNA copy of the S2 genes of each virus was constructed by using oligonucleotide primers and reverse transcriptase (Boehringer Mannheim Biochemicals, Indianapolis, Ind.). The forward-sense primer 5′-CCGAATTCATGGCTCGCGCTG CG-3′ and the reverse-sense primer 5′-GAATTCGGATCCT CACTCCAAGACGATC-3′ were used to introduce *Eco*RI (underlined) and *Bam*HI (double-underlined) sites near both ends of the S2 sequence. The cDNA was amplified by the polymerase chain reaction (52), digested with proteinase K and phenol-chloroform-extracted (9), digested with *Eco*RI and *Bam*HI, and cloned into pBTac1 (10) that had been previously digested with *Eco*RI and *Bam*HI. Recombinant plasmids were identified and isolated by restriction digest mapping. Sequences of the complete σ2 open reading frames were determined in both directions by the dideoxynucleotide method (54) with the engineered primers and available internal S2 primers (11).

RESULTS

The *ts* lesion in *ts*C447 is not suppressed by T1L genes. We first confirmed the identity of our *ts*C447 clone and tested whether the presence of T1L genes would suppress the temperature sensitivity of the *ts*C447 S2 gene by generating reassortants between T1L and *ts*C447 and using them to map temperature sensitivity (Table 1). The *ts*C447 parent had an EOP value of 2×10^{-5} whereas the T1L parent had an EOP value of approximately 1.0. Every reassortant with an EOP value similar to that of parental *ts*C447 had the S2 gene derived from the mutant. Every reassortant with an EOP value similar to that of parental T1L had a T1L S2 gene. Furthermore, every other gene was randomly associated with respect to temperature sensitivity. The combination of these three observations clearly confirms the prior mapping of temperature sensitivity of *ts*C447 to the S2 gene (49). In addition, within the panel of *ts* reassortants, every non-S2 gene was replaced at least once with the homologous T1L gene. The observation that each of these reassortants are more *ts*-like than wild-type-like (ts^+) (by at least 3 orders of magnitude) indicated that no single wild-type T1L gene served as a suppressor of the *ts*C447 defect. Furthermore, the background of multiple T1L genes in reassortants LC3 and LC31 indicates that virtually every potential T1L multigenic suppressor also can be ruled out. These observations indicated that T1L genes would not interfere with the ability to use reassortants to map the gene(s) responsible for *ts*C447 reversion.

Isolation and phenotypic characterization of *ts*C447 revertants. The EOP value of 2×10^{-5} for *ts*C447 indicated that approximately 1 of every 50,000 PFU that can grow at the permissive temperature also can grow at the nonpermissive temperature and may represent a revertant clone. We selected 40 independent revertants of *ts*C447 as detailed in Materials and Methods and evaluated the EOP values of second-passage stocks of each. Every revertant clone had an EOP value between 0.068 and 2.37 (data not shown), values that are more similar to that of wild-type T3D than to that of the parent *ts*C447, indicating that each of the new clones represented a revertant of the *ts*C447 lesion(s). Ultrastructural analyses of cells infected with *ts*C447 at the nonpermissive temperature of 39°C revealed small viral inclusions that contained predominantly hollow outer shell particles (Fig. 1B, arrowhead), as previously reported (17). In contrast, revertant clones grown under identical conditions successfully assembled both empty double-shelled (top component) particles (Fig. 1C, arrow) and intact virus particles, indicating that the temperature sensitivity of these clones and their ability to assemble the core capsid are tightly linked.

Identification of the gene(s) that contains the compensatory suppressor mutation. We selected 10 revertant clones for more detailed analyses. Clones were chosen such that one from each of the initial 10 plates (see Materials and Methods) was used to ensure that they represented independent clones. The rationale for using reassortants to segregate original mutations from their extragenic suppressor mutations, rescue temperature sensitivity, and identify the gene that contains the suppressor mutation has been described (37). To determine which gene(s) was responsible for reversion in each revertant, we constructed intertypic reassortants between each and T1L. Initially, we picked 48 to 76 plaques from each cross. This initial screen provided enough reassortants to confidently test

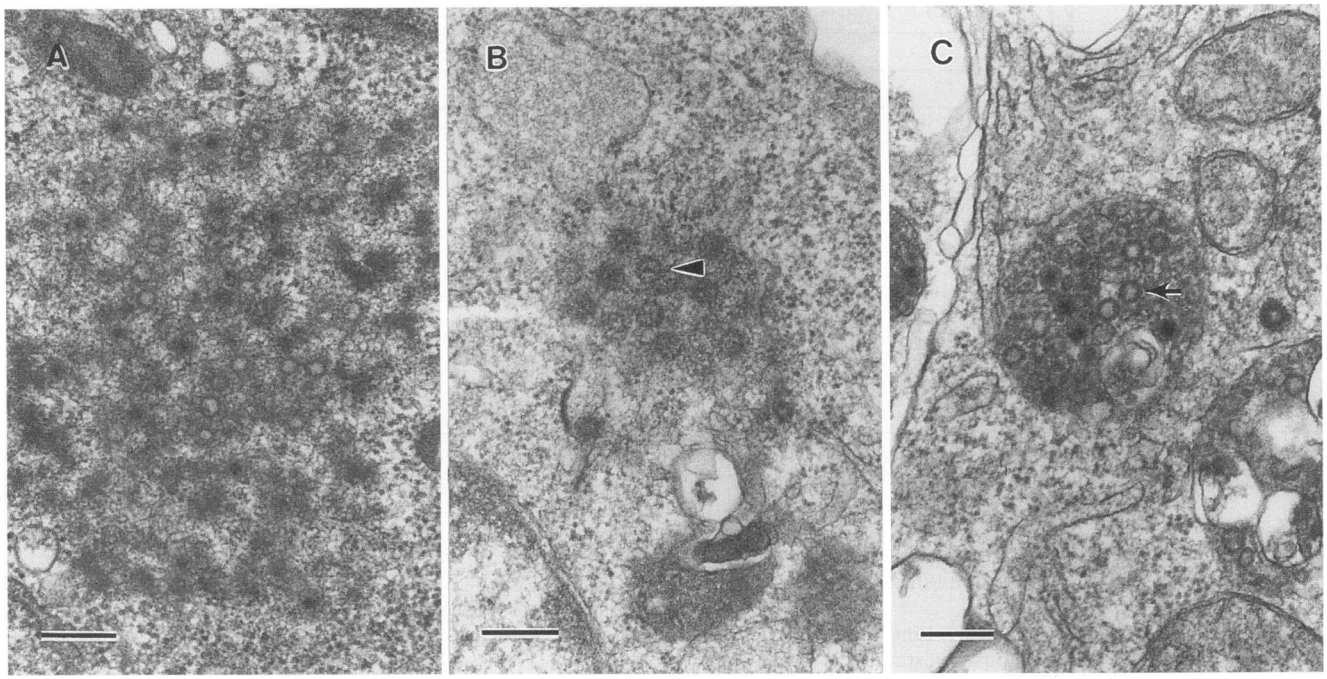

FIG. 1. Electron micrographs of thin-sectioned L929 cells infected with serotype 3 Dearing (A), *ts*C447 (B), or revertant *ts*Cr1 (C) and grown at 39°C for 48 h. The large viral inclusion in panel A contains cores, empty double-shelled (top component) particles, and intact virus. The smaller viral "factory" in panel B contains predominantly empty outer capsid structures (large arrowhead), and that in panel C contains top component (smaller arrow) and intact virus. Magnification, ×50,000; bar, 200 nm.

for the presence of all possible extragenic suppressors in only two of the crosses. For example, we screened 66 progeny plaques after crossing T1L with *ts*Cr11 (Table 2). Of the 66 clones, 23 had parental electropherotypes, 34 were reassortants, and 9 were either too faint to identify all genes or were mixed infections. There were enough unique reassortants that contained a revertant S2 gene to allow us to test every non-S2 gene for extragenic suppressors at least four times (Table 2, rightmost column; Table 3, bottom row). We similarly identified sufficient progeny reassortants from every cross to test every non-S2 gene in every revertant at least twice (Table 2, rightmost column). The reassortment efficiency ranged from a low of 20% (32 reassortants among 159 screened progeny from the T1L × *ts*Cr28 cross) to a high of almost 52% (34 of 66 from the T1L × *ts*Cr11 cross). The distribution of parental electropherotypes also was variable. Most of the parental electropherotypes recovered were the respective revertants, from a low of 23% (15 of 66 from the T1L × *ts*Cr11 cross) to a high of 66% (128 of 193 from the T1L × *ts*Cr6 cross). The T1L parent was infrequently recovered, between 1.5% (3 of 200 from *ts*Cr23) and 15% (15 of 106 from *ts*Cr16) of the time.

TABLE 2. Summary of clones derived from crosses between T1L and indicated revertants

Revertant clone	No. of progeny screened[a]	No. with parental electropherotype[b]		No. of reassortants[c]	No. of gene tests[d]
		Revertant	T1L		
*ts*Cr1	98	57	7	24	2
*ts*Cr6	193	128	9	41	3
*ts*Cr11	66	15	8	34	4
*ts*Cr16	106	41	15	31	2
*ts*Cr18	158	93	3	46	2
*ts*Cr23	200	129	3	48	3
*ts*Cr28	159	90	7	32	3
*ts*Cr31	136	80	7	34	2
*ts*Cr36	206	112	10	43	2
*ts*Cr39	68	40	6	18	2

[a] Number of progeny plaques picked, amplified, and double-stranded RNA profiles screened.
[b] Number of screened clones with either parental revertant, or parental T1L, electropherotype.
[c] Number of screened clones that represented reassortants.
[d] Minimum number of times that each non-S2 gene could be tested for presence of an extragenic suppressor mutation within a corresponding panel of unique reassortants with a revertant-S2 gene.

We determined the EOP of various reassortants to map reversion—of reassortants that contained an S2 gene derived from *ts*Cr11 to identify the location of any potential extragenic reversion events and of reassortants that contained a T1L S2 gene, in case the extragenic suppressor, in the absence of the S2 gene that the suppressor would normally correct, was itself *ts*. Every tested reassortant that contained an S2 gene derived from *ts*Cr11 had a ts^+ EOP, with values between 0.153 and 2.225 (Table 3). Because every gene that could contain an extragenic suppressor had been replaced with a T1L gene at least four times and none of the reassortants had a *ts*-like EOP, reversion in *ts*Cr11 cannot be attributed to a compensatory suppressor mutation in any single non-S2 gene. In addition, the ts^+ EOP of r11.50 (in which every non-S2 gene was replaced by corresponding T1L genes) indicated that reversion of *ts*Cr11 cannot be a multiextragenic suppressor event. Therefore, reversion of the *ts*Cr11 S2 gene was intragenic. Two reassortants from this cross contained a T1L S2 gene and had *ts* EOPs (see below). However, because prior results indicated that reversion was intragenic, the temperature sensitivity of these two reassortants probably represented spontaneous mutations.

TABLE 3. Electropherotypes and EOPs of T1L-*ts*Cr11 reassortants with revertant S2 genes

Clone	Gene segment[a]										EOP[b]
	L1	L2	L3	M1	M2	M3	S1	S2	S3	S4	
T1L	1	1	1	1	1	1	1	1	1	1	0.816
*ts*Cr11	R	R	R	R	R	R	R	R	R	R	0.579
*ts*C447	C	C	C	C	C	C	C	C	C	C	0.00004
r11.50	1	1	1	1	1	1	1	R	1	1	0.153
r11.44	1	R	1	R	R	R	1	R	R	R	0.343
r11.38	1	1	R	R	R	1	1	R	R	R	0.473
r11.72	R	R	R	R	R	R	1	R	R	1	0.524
r11.17	R	1	1	1	R	1	1	R	1	1	0.564
r11.5	1	R	1	R	R	R	R	R	R	R	0.617
r11.63	1	R	1	1	1	1	R	R	R	R	0.772
r11.27	1	R	1	R	1	R	1	R	R	R	0.905
r11.6	R	1	1	R	R	R	R	R	1	1	0.990
r11.18	1	R	1	1	R	1	R	R	R	R	1.374
r11.35	R	1	R	1	1	1	R	R	1	R	1.627
r11.36	1	1	R	R	1	R	1	R	R	R	2.225
No. of T1L genes[c]	8	6	8	5	5	6	7		4	4	

[a] Symbols in table denote parental origin of gene. 1, T1L; C, *ts*C447; R, *ts*Cr11.
[b] EOP is calculated as described in Table 1, footnote *b*. Values represent the average obtained from three different experiments. The sample variance was less than 0.5 $\log_{10}$ unit.
[c] Number of times each non-S2 gene was ruled out as an extragenic suppressor.

For many revertants, it was possible to identify a panel of reassortants in which every possible extragenic suppressor was replaced by a corresponding T1L gene, and, similar to what was found for *ts*Cr11, none of the reassortants that contained revertant S2 genes had *ts* EOPs, indicating that reversion in each of these revertant clones also was intragenic. However, some reassortants that contained revertant S2 genes, generated by crosses between T1L and other revertants, did possess *ts* EOPs (Table 4). The M3 gene of r28.22 was replaced by a T1L gene, and this reassortant had a *ts* EOP of 0.0006. However, three other reassortants (r28.73, r28.78, and r28.102) also had the M3 gene replaced but had ts^+ EOPs of 0.164, 0.551, and 0.149, respectively, which suggests that the *ts* EOP of r28.22 was caused by a spontaneous mutation unrelated to the reversion phenomenon and that reversion of *ts*Cr28 also was intragenic.

The *ts* lesion in r28.22 resides in its S4 gene. To test the possibility that the *ts* EOP of r28.22 was caused by a spontaneous mutation event unrelated to reversion and to determine the identity of the gene(s) responsible for the temperature sensitivity of r28.22, we chose a ts^+ clone with a complementary electropherotype (r1.13, which has a revertant M3 gene in a background of T1L genes) to cross with r28.22 to make additional reassortants. EOP determinations of the resulting

TABLE 4. Electropherotypes and EOPs of T1L-*ts*Cr28 reassortants with revertant S2 genes

Clone	Gene segment[a]										EOP[b]
	L1	L2	L3	M1	M2	M3	S1	S2	S3	S4	
T1L	1	1	1	1	1	1	1	1	1	1	1.60
*ts*Cr28	R	R	R	R	R	R	R	R	R	R	0.621
*ts*C447	C	C	C	C	C	C	C	C	C	C	0.00005
*ts*Cr28.22	R	R	R	R	R	1	R	R	R	R	0.0006
*ts*Cr28.27	R	R	1	R	R	R	R	R	R	R	0.137
*ts*Cr28.134	R	R	R	1	R	R	R	R	1	R	0.140
*ts*Cr28.102	1	R	1	1	R	1	1	R	R	1	0.149
*ts*Cr28.73	R	1	R	R	R	1	1	R	R	1	0.164
*ts*Cr28.160	R	R	R	R	R	R	R	R	1	1	0.171
*ts*Cr28.148	1	R	R	R	R	R	R	R	R	R	0.238
*ts*Cr28.78	R	R	R	R	1	1	R	R	1	R	0.551
*ts*Cr28.127	1	1	R	R	1	R	1	R	R	R	0.579
*ts*Cr28.45	1	R	R	R	R	R	1	R	1	R	0.716
*ts*Cr28.59	R	R	R	1	1	R	R	R	R	R	0.863
*ts*Cr28.81 B	R	1	1	R	1	R	1	R	R	1	1.86
No. of T1L genes[c]	4	3	3	3	4	3	5		4	4	

[a] Symbols in table denote parental origin of gene. 1, T1L; C, *ts*C447; R, *ts*Cr28.
[b] EOP is calculated as described in Tables 1 and 3, footnotes *b*.
[c] Number of times each non-S2 gene ruled out as an extragenic suppressor.

TABLE 5. Electropherotypes and EOPs of r1.13-r28.22 reassortants

Clone	Gene segment[a]										EOP at[b]:	
	L1	L2	L3	M1	M2	M3	S1	S2	S3	S4	39.0°C	40.0°C
13-22.70	○	●	●	●	○	●	○	●	●	●	0.249	0.000009
13-22.46	●	●	○	●	○	●	●	●	●	●	0.075	0.000013
13-22.33	●	○	●	○	○	●	○	●	○	●	0.124	0.00002
r28.22	●	●	●	●	●	●	●	●	●	●	0.0004	0.00016
13-22.62	●	●	●	●	○	○	●	●	●	●	0.571	0.00082
13-22.57	●	○	●	●	●	●	○	●	●	○	0.313	0.116
13-22.65	●	●	●	●	●	●	●	●	●	○	0.264	0.146
13-22.58	○	●	○	●	●	○	●	○	○	○	0.311	0.179
13-22.25	○	●	●	●	●	●	●	●	○	○	0.395	0.192
13-22.64	○	○	●	○	●	○	○	●	●	○	0.183	0.288
13-22.53	●	○	○	○	○	○	○	○	○	○	0.711	0.318
13-22.66	●	○	○	●	●	○	●	●	●	○	0.282	0.359
13-22.30	●	●	●	●	○	●	●	●	●	○	0.650	0.430
13-22.40	○	●	○	○	○	●	○	●	●	○	0.562	0.624
13-22.37	●	○	○	●	○	●	○	●	●	○	0.705	0.808
13-22.63	○	●	○	○	○	○	○	●	●	○	0.729	0.819
13-22.61	○	●	○	●	○	○	○	○	○	○	0.790	0.927
r1.13	○	○	○	○	○	○	○	○	○	○	0.585	1.18

[a] Symbols in table denote parental origin of gene. ●, r28.22; ○, r1.13.
[b] As described in Table 1, footnote *b*. Values represent the average of two separate experiments, with variance less than 0.5 $\log_{10}$ unit.

reassortants failed to map temperature sensitivity to any gene when carried out at 39°C. We have shown that the temperature sensitivity of various reovirus *ts* mutants can be more reliably determined when the nonpermissive temperature is increased to 40°C (24). Therefore, we tested each reassortant at a higher temperature. When the incubation temperature was increased to 40°C, the resulting EOP values indicated that the gene in r28.22 responsible for the phenotype of this mutant was the S4 gene (Table 5). No other gene (including S2) was correlated with temperature sensitivity by either chi square or Wilcoxon rank sum analyses. These results indicated that a spontaneous mutant, whose defect resides in the S4 gene, arose during the generation of reassortant r28.22.

Reversion of *ts*C447 was exclusively an intragenic process. While carrying out these screenings, we identified nine reassortants that were *ts* (Table 6). Three (r11.2, r11.31, and r31.13) had S2 genes derived from the T1L parent, whereas the other six had S2 genes derived from the appropriate parental revertant. However, generation of none of the latter six could be attributed to removal of an extragenic suppressor mutation during assortment. As described above (Tables 4 and 5), the *ts* phenotype of r28.22 was not caused by removal of an M3 extragenic suppressor mutation. Each of the other five *ts* clones with revertant S2 genes probably also resulted from spontaneous mutations. For example, r36.27 possessed a T1L L3 gene and was *ts*; however, three other reassortants (rightmost col-

TABLE 6. Summary of *ts* revertant reassortants recovered

Parental revertant	Number to map[a]	Reassortant clone	Gene segment[b]										EOP[c]	No. ruled out[d]
			L1	L2	L3	M1	M2	M3	S1	S2	S3	S4		
*ts*Cr1	18	NR[e]												NA
*ts*Cr6	23	NR												NA
*ts*Cr11	21	r11.2	R	1	1	1	R	R	1	1	1	1	0.0029	NA
		r11.31	1	R	1	R	R	1	1	1	R	R	0.013	NA
*ts*Cr16	20	NR												NA
*ts*Cr18	27	NR												NA
*ts*Cr23	16	r23.10	1	R	1	R	1	1	R	R	R	1	0.00003	4[f]
		r23.59	R	R	1	R	R	R	R	R	R	R	0.00009	13
		r23.66	R	1	R	R	R	R	R	R	R	R	0.00001	3
*ts*Cr28	23	r28.22	R	R	R	R	R	1	R	R	R	R	0.00011	3
*ts*Cr31	31	r31.13	R	R	R	R	R	R	R	1	R	R	0.00008	NA
		r31.25	1	R	1	R	R	1	R	R	R	R	0.0043	5[f]
*ts*Cr36	18	r36.27	R	R	1	R	R	R	R	R	R	R	0.00004	3
*ts*Cr39	15	NR												NA

[a] Number of reassortants with a T1L S2 or revertant S2 gene examined by EOP analyses for indicated parental revertant. Total number, 212. Number of *ts* reassortants, 9 (4.2%).
[b] Symbols in table denote parental origin of gene. 1, T1L; R, the indicated parental revertant.
[c] As defined in Tables 1 and 3, footnotes *b*.
[d] Number of times each suggested extragenic gene was ruled out by other members of the panel. NA, not applicable because no gene was suggested as an extragenic suppressor.
[e] NR, no *ts* reassortants recovered.
[f] Minimum number of times each potential single extragenic suppressor gene could be ruled out by other members of the panel.

TABLE 7. Summary of nucleotide and amino acid alterations in *ts*C447 and its revertants

Clone[a]	Nucleotide		Amino acid	
	Position	Change	Position	Change
*ts*C447	986	C→T	323	Ala→Val
	1166	A→G	383	Asn→Asp
*ts*Cr1	1166	G→A	383	Asp→Asn
*ts*Cr6	543	G→C	175	NC[b]
	1166	G→A	383	Asp→Asn
*ts*Cr11	787	T→C	257	Leu→Ser
	1166	G→A	383	Asp→Asn
*ts*Cr16	ND	ND	ND	ND
*ts*Cr18	403	G→A	129	Asp→Asn
	646	G→A	210	Ala→Thr
	1166	G→A	383	Asp→Asn
*ts*Cr23	1166	G→A	383	Asp→Asn
*ts*Cr28	1166	G→A	383	Asp→Asn
*ts*Cr31	1166	G→A	383	Asp→Asn
*ts*Cr36	1166	G→A	383	Asp→Asn
*ts*Cr39	411	A→G	131	NC
	1166	G→A	383	Asp→Asn

[a] Position numbers and changes for *ts*C447 represent alterations in *ts*C447 compared with parental T3D. Position numbers and changes for other clones represent alterations in each revertant compared with *ts*C447.
[b] NC, no change.
[c] ND, not determined.

umn) generated from *ts*Cr36 also had a T1L L3 gene but were ts$^+$. Similarly, reassortant analyses of every revertant we examined in detail indicated that in each, reversion was caused by an intragenic compensatory mutation.

Reversion of *ts*C447 involves back mutation of the aspartic acid at position 383 in σ2 to a wild-type asparagine residue. To locate the site of each reversion, we determined the nucleotide sequences of duplicate recombinant plasmids of the engineered S2 genes of T3D, *ts*C447, and various revertants (Table 7). Our *ts*C447 clone contained two of the three nucleotide and amino acid sequence changes previously identified (69). Some revertants contained a single nucleotide change compared with *ts*C447; the G at position 1166 had reverted to an A in *ts*Cr1, *ts*Cr23, *ts*Cr28, *ts*Cr31, and *ts*Cr36. This change resulted in the replacement of a mutant aspartic acid residue with a wild-type asparagine residue at amino acid 383 in the predicted σ2 sequence. In other revertant S2 genes there was more than a single nucleotide change; however, every revertant sequence had the G→A change at position 1166.

DISCUSSION

Reversion of the assembly-defective mutant *ts*C447 is an intragenic process. The segmented natures of the *Reoviridae* and *Orthomyxoviridae* genomes have important implications in using these viruses in genetic studies (for reviews, see references 41 and 50). Analyses of reassortant viruses in both systems has allowed mapping of particular biologic functions to individual proteins (for examples, see references 12, 34, 44, and 67), *ts* lesions to individual genes (33, 42, 47, 49), and compensatory suppressor mutations (37). The ability of wild-type genes to suppress *ts* lesions also has been reported (20, 56). Wild-type suppression would have made attempts to map *ts*C447 reversion in this study difficult to interpret. Therefore, we first tested whether the presence of wild-type genes would interfere with the *ts* phenotype of *ts*C447 by generating reassortants between T1L and the mutant. EOP results of the reassortants indicated that the temperature sensitivity of *ts*C447 resides in the S2 gene (Table 1), as previously determined (49). Our mapping was based on both the presence of a T1L S2 gene in every reassortant with a ts$^+$ EOP (as done previously by Ramig et al. [49]) and the presence of an S2 gene derived from the *ts*C447 parent in every *ts* reassortant. The second group of reassortants also was used to test whether any wild-type T1L genes could suppress the *ts* lesion in *ts*C447; the observation that none of these reassortants are ts$^+$ indicated that no wild-type T1L gene served as a suppressor of the defect in the *ts*C447 S2 gene and that the presence of T1L genes would not interfere with the ability to use reassortants to map the gene(s) responsible for *ts*C447 reversion.

The study of conditional-lethal mutations and of suppressor mutations that compensate for the original mutations has resulted in significant knowledge about a wealth of biologic processes (for a review, see reference 22). Such analyses have allowed elucidation of the assembly pathways of a number of bacteriophages (2, 45) and of protein-protein interactions in a large number of viruses, bacteria, and eukaryotic cells (22). Suppression by a mutation that lies in a gene other than the site of the original mutation (extragenic) has been interpreted to mean that the two gene products interact with each other at either a structural or functional level or both. Identification of a number of such extragenic suppressor mutations has been useful in describing protein interactions in a variety of viruses (25, 27, 37, 64). Extragenic suppression is the primary mechanism by which reovirus *ts* lesions are corrected (48). Genetic mapping of such extragenic reovirus suppressors suggested interactions between the outer capsid proteins μ1 and σ3 and between the core spike protein λ2 and the cell attachment protein σ1 (37). Interactions between μ1 and σ3, and between λ2 and σ1, had been previously demonstrated biochemically (29, 30), illustrating the validity of such genetic approaches. Therefore, we attempted to use this approach to identify other proteins that interact with σ2.

We generated and examined 10 independent revertants of the *ts* mutant *ts*C447. We constructed reassortants between each revertant and wild-type T1L to rescue temperature sensitivity. The ability to recover temperature sensitivity among a panel of reassortants, all of which had a gene(s) replaced by the corresponding T1L gene during assortment, would suggest that the replaced gene (or its corresponding protein) interacted with and corrected the mutation in *ts*C447. We were unable to identify any such panel of reassortants from any of the 10 groups of revertant reassortants, which strongly suggests that reversion of the *ts*C447 defect(s) proceeds primarily through an intragenic process. Intragenic reovirus revertants have been suspected (48), but, until now, none have been well characterized. This process has been found in a number of virus systems, including the bacteriophage P22 tailspike protein (15, 35), the bacteriophage ϕX174 prohead accessory protein (14), vaccinia virus (36), the influenza nucleocapsid protein PB2 (64), the influenza virus nonstructural gene (63), and the poliovirus 2C protease (31). Many of these proteins are involved in assembly of components of the respective viruses.

From these crosses we identified a few reassortants that had *ts* phenotypes. Many of these had single-gene replacements in a revertant background. However, these potential extragenic suppressor mutations could be ruled out because a large number of other reassortants generated from the same crosses had the same gene replaced but were not *ts*. This suggests that the temperature sensitivity of these few clones resulted from spontaneous mutations. Indeed, of the 212 clones that we

examined by EOP analyses, only nine were *ts*. This frequency of 4.2% is significantly higher than the rate of mutation among nonmutagenized reovirus stocks but comparable to the rate of mutation among mutagenized stocks (16). Isolation of spontaneous mutants at this frequency also illustrates the need to test each gene in *ts* mutant mapping experiments more than once. Two crosses generated larger proportions of *ts* clones; 3 of 16 reassortants (about 19%) in the r23 series and 2 of 31 (about 6%) r31 reassortants. The unusually high rate of mutation among the r23 reassortants suggests an altered polymerase. Some of the *ts* reassortants had numerous revertant genes replaced. For example, r31.25 had the revertant L1, L3, and M3 genes replaced by T1L genes. Each of these genes had been replaced numerous other times within the panel of *ts*Cr31 reassortants, and no other such reassortants had *ts*-like EOP values. Therefore, any of the potential single extragenic suppressors appear to have been ruled out. However, the possibility that suppression resulted from the combined effects of multiple genes may still exist. Such gene constellations have been proposed in the family *Orthomyxoviridae* (51, 62). Although this may be a possibility, a number of other *ts*Cr31 × T1L reassortants also had the same three genes replaced but had ts^+ EOPs. This implies that reversion was not caused by a multiextragenic event, that reversion of *ts*C447 was primarily intragenic, and that the few *ts* clones recovered represented spontaneous mutants.

The observation that all *ts*C447 revertants arose as a result of an intragenic event has a number of implications for how σ2 interacts with other virion proteins. Proteins σ2 and λ1 are believed to interact (26). However, our reversion mapping results indicate that no other reovirus proteins appear capable of correcting the defect present in the *ts*C447 σ2 protein. Furthermore, in contrast to conditionally lethal nonsense (*amber*) mutations, which generally generate truncated proteins of greatly perturbed structure and function, conditionally lethal *ts* (missense) mutations generally do not generate proteins that are significantly altered in overall conformation (8, 21). Therefore, the failure of *ts*C447 to assemble core particles suggests that the region(s) of σ2 that contains the *ts*C447 lesion(s) is directly responsible for interactions that lead to core shell assembly.

Reversion of the mutant aspartic acid at position 383 in protein σ2 to a wild-type asparagine restores core shell assembly. The sequence of the *ts*C447 S2 gene has been reported; it contains three nucleotide substitutions compared with parental T3D, each of which lead to changes in the predicted amino acid sequence of the 418-amino-acid σ2 protein (69). These changes are alanine-to-valine replacements at amino acid positions 188 and 323 and an asparagine-to-aspartic-acid change at position 383. The *ts*C447 clone that we used in this study contained only the last two alterations (Table 7). Our sequence determinations of other *ts*C447 clones have revealed that some contain all three lesions (11) and others contain only the last two lesions (data not shown). Clones that contain only the last two lesions are *ts* (Table 1) and fail to assemble cores (Fig. 1). These comparisons indicate that the alanine-to-valine substitution at position 188 is not responsible for either the *ts* phenotype or the inability of the mutant to assemble cores and suggests that these functional defects are conferred by either or both lesions at positions 323 and 383.

We determined the sequences of the revertant S2 genes to identify which altered residues were responsible for the functional defect(s) and to identify second-site intragenic alterations that could correct for the original mutation. To date, we have been unable to successfully clone the engineered *ts*Cr16 S2 gene; however, every revertant S2 gene that was sequenced contained a common alteration compared with *ts*C447 (Table 7). This change was replacement of a mutant guanine residue at nucleotide 1166 with a wild-type adenine. This change leads to replacement of a mutant aspartic acid residue at amino acid 383 with the wild-type asparagine residue. Some revertants (*ts*Cr1, *ts*Cr23, *ts*Cr28, *ts*Cr31, and *ts*Cr36) contained only this alteration. Such revertants not only lose their *ts* phenotype but also are capable of assembling core particles (Fig. 1). These results indicate that the defect in *ts*C447 that confers temperature sensitivity and prevents assembly of a core capsid is the aspartic acid at amino acid 383 in the σ2 protein.

Understanding reovirus core structure and assembly. The reovirus core serves multiple vitally important functions during the virus replication cycle. The core directs various distinct enzymatic reactions that are required for transcribing nascent mRNA early in infection, and it is involved in packaging the viral genome during virion assembly. An understanding of the structure, function, and assembly of this macromolecular complex would aid in understanding both types of functions. This study elucidates some of the molecular requirements for assembly of the protein capsid. Our results demonstrate that a region near the carboxy terminus of the σ2 protein is essential for assembly of the core particle. This region, which contains an asparagine residue at position 383 in the wild-type sequence, is predicted to be exposed on the surface of the protein (11), where it would be expected to be capable of interacting with other proteins. The introduction of a charged residue at this position in the amino acid sequence appears to prevent interaction of this protein with other proteins that would normally lead to capsid assembly. The primary way in which this alteration appears to be corrected is by true reversion; we were unable to identify extragenic suppressors or second-site mutations within σ2 that corrected for the *ts*C447 mutation. These results suggest that other core proteins do not interact with this region of σ2 and that σ2-σ2 interactions involving this region are important in assembly. Thus, determination of the structures of both wild-type and *ts*C447 σ2 proteins and elucidation of the supramolecular organization of this protein within the capsid shell are important for better understanding the molecular mechanisms of core assembly.

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