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Localization of a C-Terminal Region of λ2 Protein in Reovirus Cores

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The 144-kDa λ2 protein is a structural component of mammalian reovirus particles and contains the guanylyltransferase activity involved in adding 5′ caps to reovirus mRNAs. After incubation of reovirus T3D core particles at 52°C, the λ2 protein became sensitive to partial protease degradation. Sequential treatments with heat and chymotrypsin caused degradation of a C-terminal portion of λ2, leaving a 120K core-associated fragment. The four other proteins in cores—λ1, λ3, μ2, and σ2—were not affected by the treatment. Purified cores with cleaved λ2 were subjected to transmission cryoelectron microscopy and image reconstruction. Reconstruction analysis demonstrated that a distinctive outer region of λ2 was missing from the modified cores. The degraded region of λ2 corresponded to the one that contacts the base of the σ1 protein fiber in reovirus virions and infectious subvirion particles, suggesting that the σ1-binding region of λ2 is near its C terminus. Cores with cleaved λ2 were shown to retain all activities required to transcribe and cap reovirus mRNAs, indicating that the C-terminal region of λ2 is dispensable for those functions.

Much remains to be learned about the relationship between reovirus protein sequences and the structural details of reovirus particles, such as those that are observed following transmission cryoelectron microscopy (cryoTEM) and three-dimensional (3-D) image reconstruction (12). Advances in this area are expected to come from studies with X-ray crystallography (8, 27); however, the success of those studies at generating atomic-scale models of reovirus particles remains uncertain. In the meantime, biochemical and genetic manipulations to alter specific proteins or protein regions, coupled with cryoTEM and 3-D image reconstruction to reveal the associated structural changes at resolutions of ≤30 Å, provide a way to determine sequence-structure relationships. The present study applies this approach to core particles of reovirus type 3 Dearing (T3D).

Reoviruses are nonenveloped viruses whose virions comprise two concentric, icosahedral protein shells surrounding a 10-segment, double-stranded RNA genome. Sequential loss of subsets of the outer-shell proteins generates two types of subvirion particles, infectious subvirion particles (ISVPs) and cores, which play essential roles in infection (see reference 22 for a review). Cores are noninfectious but contain all the enzymatic activities required for the synthesis and release of full-length viral mRNAs, which contain a 5′ cap 1 structure but no 3′ poly(A) sequences (see reference 30 for a review). Cores are composed of five proteins: λ1 and σ2, which define the icosahedral lattice of the inner capsid (36, 37); λ3 and μ2, which are present at low copy numbers and are probably located at the 12 fivefold axes of the inner capsid (11, 38); and λ2, which forms pentameric turrets that project high above the inner capsid around each fivefold axis (12, 36). The roles of the different core proteins in transcription, capping, and export of the reovirus mRNAs continue to be defined. λ3 is the catalytic subunit of the reovirus RNA polymerase (33), for which μ2 may be a cofactor (38). Genetic analyses indicate that λ1 and μ2 influence core nucleoside triphosphatase activities, possibly representing an RNA helicase required for transcription (24, 25). λ2 plays one or more roles in mRNA capping as described below.

This study focuses on sequence-structure-function relationships of the reovirus λ2 protein. λ2 is an important structural component of mammalian reovirus particles, making contacts with at least five of the seven other proteins in virions (12, 33), including the cell attachment protein σ1 (12, 19). It has been proposed that λ2 plays a role in assembling the outer capsid (17, 21). Early in cell infection, an outer region of the λ2 protein, including the part that contacts the σ1 protein in virions, undergoes a substantial rearrangement such that a channel is opened at each of the icosahedral fivefold axes (12). This conformational change is thought to permit egress of the viral mRNAs as they are synthesized by the RNA polymerase located at the base of the channel (3, 11, 12). The λ2 protein mediates the guanylyltransferase activity involved in adding 5′ caps to these mRNAs (6, 13, 20, 30) and may also mediate the methyltransferase activities that generate the final cap 1 structure (18, 28). The current study attributes a C-terminal region of λ2 sequence to the portion of λ2 that contacts σ1 and is translocated to open the fivefold channels prior to transcription. This C-terminal region of λ2 is dispensable for transcription and capping by reovirus cores.

Proteolytic truncation of core-bound λ2 protein by sequential treatments with heat and chymotrypsin. In an effort to localize portions of proteins within reovirus cores, we heated cores to different temperatures, cooled them, and added pro-

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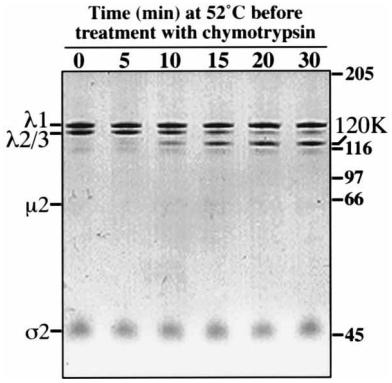

FIG. 1. SDS-PAGE of reovirus cores following heat-and-chymotrypsin treatment: time course of heat treatment. Cores of reovirus T3D in virion buffer (10 mM Tris [pH 7.5], 150 mM NaCl, 10 mM $MgCl_2$) were heated to 52°C for the times indicated, cooled to 37°C, and treated with 200 μg of α-chymotrypsin (Sigma, St. Louis, Mo.) per ml for 10 min. Following treatment, the chymotrypsin was inactivated with 1 mM phenylmethylsulfonyl fluoride (Sigma). Samples were then mixed in a 1:1 (vol/vol) ratio with 2× sample buffer (250 mM Tris [pH 8.0], 20% sucrose, 2% SDS, 4% β-mercaptoethanol, 0.02% bromophenol blue) and boiled for 2 min. Equivalent amounts of samples were loaded on a 1.5-mm-thick 7.5% phosphate-urea-SDS polyacrylamide gel (31, 40) in a minigel format (Bio-Rad, Hercules, Calif.) and subjected to electrophoresis at 15-V constant voltage for ~16 h. Proteins were visualized by staining with Coomassie brilliant blue R-250 (Sigma). Positions of reovirus proteins are indicated at left (note that the minor core protein μ2 was poorly visualized in this experiment). Positions of marker proteins (Sigma) are indicated at right (numbers are molecular weights, in thousands).

tease to determine if any protein was newly sensitized to partial degradation, as a result of heat-induced conformational changes. Initial studies revealed that when cores of reovirus T3D were heated to temperatures near 50°C, significant amounts of a fragment with a molecular weight of 120,000 (120K) were generated after cooling and incubation with chymotrypsin or trypsin (data not shown). This fragment was presumably derived from one or more of the three λ proteins (λ1, λ2, and λ3), which range in size from 137 to 144 kDa but comigrate in the standard Tris-glycine-sodium dodecyl sulfate (SDS) gels used in these experiments. Since λ1 can be separated from the similarly sized λ2 and λ3 proteins in phosphate-urea-SDS gels (31, 40), an experiment was next performed in which T3D cores were incubated at 52°C for different times, cooled to 37°C, and treated with 200 μg of chymotrypsin per ml for 10 min, after which the proteins were resolved on a phosphate-urea-SDS gel (Fig. 1). Under these conditions, the 120K fragment was observed with increasing intensity from 10 to 30 min of heat treatment. In concert with fragment accumulation, the intensity of the λ2/3 protein band decreased to low levels. Because λ2 outnumbers λ3 by a 5:1 ratio in cores (60 copies of λ2 to 12 copies of λ3), these findings indicated that the 120K fragment was derived from λ2.

A proteolytic treatment that generates a 120K fragment from the 144-kDa λ2 protein might yield a 24K fragment as well, if the fragments were generated by a single cleavage. However, when cores treated with heat and chymotrypsin were analyzed on gradient gels that resolve proteins of that size, no such small fragments were seen (data not shown). The most likely explanation is that the 24K region of λ2 was extensively degraded, possibly due to heat-induced denaturation of that region.

Other observations suggested that the heat-and-chymotrypsin protocol had selective effects on the λ2 protein and would be useful for generating modified cores for structural studies. The reovirus λ1 and σ2 proteins constitute the icosahedral shell which surrounds the viral genome and from which the turrets of λ2 protein project outward around the axes of five-fold symmetry in reovirus cores (12). Since the λ1 and σ2 protein bands are not detectably altered following heat and chymotrypsin treatment (Fig. 1), the core shell seemed likely to have survived the treatment protocol. In addition, similar amounts of the 120K fragment of λ2 were generated despite variations in the protocol, including 52°C treatment for times from 20 to 60 min (Fig. 1; data not shown for later times) and chymotrypsin treatment for times from 5 to 120 min (data not shown; see Fig. 6 for similar data). The latter findings suggested that the 120K fragment was substantially resistant to further degradation and may represent a more stable portion of λ2 that may be localized in structural studies of the modified cores.

Binding of MAb 7F4 to a C-terminal portion of λ2, which is degraded upon heat-and-chymotrypsin treatment. Monoclonal antibody (MAb) 7F4 binds to the λ2 proteins of many reovirus isolates, including T3D (34). To identify which region of λ2 is lost in generating the 120K fragment, T3D cores before and after heat-and-chymotrypsin treatment were probed with 7F4. Using Western blots (Fig. 2A), we showed that 7F4 binds to intact λ2 from cores but not to the 120K λ2 fragment from cores treated with heat and chymotrypsin. The 120K fragment was detected by a polyvalent antireovirus serum, which is known to recognize the λ2 protein. These findings indicate that the epitope bound by 7F4 is in the region of λ2 that was denatured at 52°C and then degraded by chymotrypsin.

As shown by 7F4 reactivity in Western blots (Fig. 2A), a small amount of intact λ2 remained present in cores that had been heated to 52°C for 30 min before chymotrypsin treatment. Cores heated to 55°C for 30 min before chymotrypsin treatment retained only slightly less intact λ2 than cores heated to 52°C (data not shown). Since the majority of the λ2 was cleaved after heating to 52°C, we were concerned that resorting to more extreme temperatures would introduce other effects on cores. As a consequence, 52°C was used to generate cleaved λ2 in all subsequent experiments.

Given the size and extended conformation of λ2 protein in reovirus cores (12), the portion of λ2 removed in generating the 120K fragment seemed likely to include sequences from only one of the protein's two ends. Based on limited data and predictions about the orientation of λ2 in cores (12, 13), we hypothesized that sequences at the C terminus of λ2 were most likely to have been lost during treatment. To test this hypothesis, we transferred the previously cloned L2 gene of reovirus T3D (5) (provided as an insert in pBluescript [Stratagene, La Jolla, Calif.] by K. M. Reinisch and S. C. Harrison, Harvard University) into the pET21a plasmid (Novagen, Madison, Wis.) to permit the λ2 protein to be expressed in *Escherichia coli*. Both full-length λ2 protein (1,289 amino acids) and a C-terminal truncation (1,202 amino acids) generated using a unique *Xho*I site in the L2 gene were expressed. Western blots of the expressed proteins showed that full-length λ2 was bound by MAb 7F4 while the C-terminally truncated λ2 was not bound (Fig. 2B). The truncated λ2 was detected by the polyvalent antireovirus serum. These results indicate that an essential portion of the 7F4 epitope is located within the C-terminal 87 amino acids of the T3D λ2 protein. Additionally, the results map the sequences missing from the 120K fragment of T3D λ2 to the C terminus of λ2.

To determine whether sequences were lost from the N terminus of λ2 as well, we transferred the 120K λ2 fragment from an SDS-polyacrylamide gel to polyvinylidene fluoride paper (Applied Biosystems, Foster City, Calif.) and submitted it for N-terminal sequencing (University of Michigan Medical

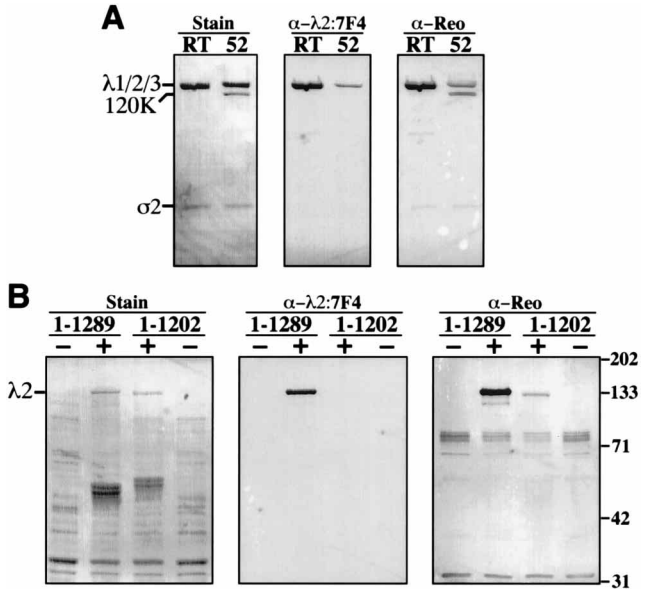

FIG. 2. Western blots with MAb 7F4, which binds reovirus protein λ2, and a polyvalent antireovirus serum. (A) Cores of reovirus T3D in virion buffer were incubated at either room temperature (RT) or 52°C (52) for 30 min, cooled to 37°C, and treated with 200 μg of α-chymotrypsin per ml for 10 min. Following treatment, the chymotrypsin was inactivated with 1 mM PMSF. Samples were mixed in a 1:1 (vol/vol) ratio with 2× sample buffer and boiled for 2 min. Equivalent amounts of samples were loaded onto a 0.8-mm-thick 8% Tris-glycine-SDS minigel and subjected to electrophoresis until the dye front approached the bottom of the gel. Proteins in the gel were then transferred to nitrocellulose by electroblotting at 100 V for 1 h at 4°C with a Tris-glycine blotting buffer (34) in a minigel transfer apparatus (Bio-Rad). Immediately after electroblotting to nitrocellulose, proteins on the blot were visualized with a reversible protein detection kit (Sigma), and an image was captured by high-resolution scanning. The membrane was probed with a 1/1,000 dilution of purified MAb 7F4 (courtesy of K. L. Tyler and H. W. Virgin IV). After the 7F4 antibody was bound with an alkaline phosphatase-conjugated goat anti-mouse immunoglobulin secondary antibody, signal was visualized colorimetrically with Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolylphosphate (Bio-Rad), and a second image was captured by scanning. After the membrane was blocked again, it was probed with a 1/2,000 dilution of polyvalent serum against reovirus T3D (courtesy of K. Tyler). The antireovirus antibodies were bound by goat anti-rabbit antibodies conjugated with alkaline phosphatase (Bio-Rad) and visualized colorimetrically as before. This third image was also scanned. (B) Proteins expressed in *E. coli* that were visualized as described for panel A. 1-1289, full-length λ2; 1-1202, λ2 truncated at amino acid 1202 (*Xho*I site of the L2 gene); +, lysate from BL21DE3 *E. coli* cells (Novagen) expressing the protein of interest; −, lysate from BL21 *E. coli* cells (Novagen) not expressing the protein of interest due to the absence of T7 RNA polymerase. The 1-1289 and 1-1202 forms of λ2 exhibited more similar reactivities when probed directly with the polyvalent antireovirus serum, that is, without being previously visualized with 7F4 as in this experiment. Molecular weights of protein markers are on the right, in thousands.

School, Ann Arbor). The results suggested that the fragment's N terminus was covalently blocked (data not shown), similar to full-length λ2 (26). Although these findings are not definitive, we conclude that no amino acids were removed from the N terminus of λ2 during the heat-and-chymotrypsin protocol.

Purification of modified cores and analysis by cryoTEM and image reconstruction reveals loss of an outer region of the λ2 turret. Following heat-and-chymotrypsin treatment, samples containing the particles were loaded onto a preformed CsCl gradient (ρ = 1.30 to 1.55 g/cm^3) and subjected to equilibrium centrifugation. Afterwards, the particles formed a well-visualized band at a density of ~1.45 g/cm^3 (data not shown). The particles were dialyzed into core buffer (8) and remained in suspension. Phosphate-urea-SDS gels stained with Coomassie blue revealed that the 120K fragment of λ2, as well as proteins

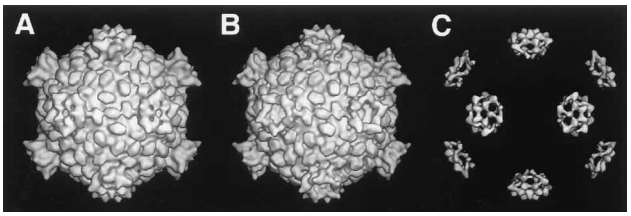

FIG. 3. Image reconstructions of cryoTEM images of reovirus T3D cores and T3D cores with cleaved λ2: surface views of whole particles and difference map. Surface views of T3D cores (A) and T3D cores with cleaved λ2 (B) were generated from the averaged data sets for each. The contour level was set at 5 standard deviations above the average noise level for generating each image. A difference map (12) (C) was obtained by subtracting the data set for T3D cores with truncated λ2 from that for unmodified T3D cores. Densities appearing in the difference map at radii of <300 Å (near the tops of the core nodules) were removed from these images so that differences in the regions of the λ2 turrets were seen more clearly. For this comparison of the two particle forms and for that in Fig. 4, the data set for T3D cores was scaled so that the density peaks for the core shells in the two particle forms were approximately matched in both radius and intensity.

λ1 and σ2, remained particle bound at approximately pretreatment levels (data not shown). Similarly, gels stained with methylene blue showed that these particles still contained approximately pretreatment levels of the 10 genomic double-stranded RNA segments (data not shown). The behavior of these particles, as well as their protein and RNA contents, indicated that they were good candidates for structural studies.

In an effort to determine what portion of the λ2 turret was lost due to λ2 cleavage, T3D cores and modified T3D cores that had been gradient purified after heat-and-chymotrypsin treatment were embedded in vitreous ice and subjected to cryoTEM (2) and 3-D image reconstruction. Images were recorded under conditions of minimal irradiation (10 electrons/Å^2), at similar levels of defocus for the two particle types (0.8 to 1.0 μM), and at magnifications of ×30,000 for T3D cores and ×36,000 for T3D cores with cleaved λ2. In the micrographs, the modified cores were seen to be generally well isolated and to have intact capsids that possessed features similar to those of untreated cores (data not shown). Image reconstruction was performed as described elsewhere (1, 11, 15), using a previously determined reconstruction for reovirus type 1 Lang (T1L) cores as the initial reference model. After several iterative steps of refinement, no improvement in resolution beyond 28 Å could be obtained with either data set, as determined by phase residuals (14) and correlation coefficients (7). Final reconstructions were calculated from 41 and 58 particles for the T3D cores and modified T3D cores, respectively. The particle orientations were sufficiently random to satisfy sampling requirements for Fourier inversion (9).

Surface-shaded views of the 3-D image reconstructions for T3D cores (Fig. 3A) and T3D cores with cleaved λ2 (Fig. 3B) were generated for comparison. Differences in the λ2 turrets of the two particle forms were evident, particularly in their outermost, fivefold-axis-proximal regions (Fig. 3C). Otherwise, the two particle forms appeared to be very similar. These same conclusions were reached when the contour level for displayed electron density was adjusted over a wide range of values, demonstrating that the apparent differences in the λ2 turrets, and general lack of differences outside the turrets, were not artifacts of the contouring and display procedures.

Differences in the outermost regions of λ2 turrets were better visualized when turrets from cores (Fig. 4A and B) and cores with cleaved λ2 (Fig. 4C and D) were viewed close-up. The outer regions of the turrets in the cores include five symmetrically equivalent elements that surround the fivefold axis

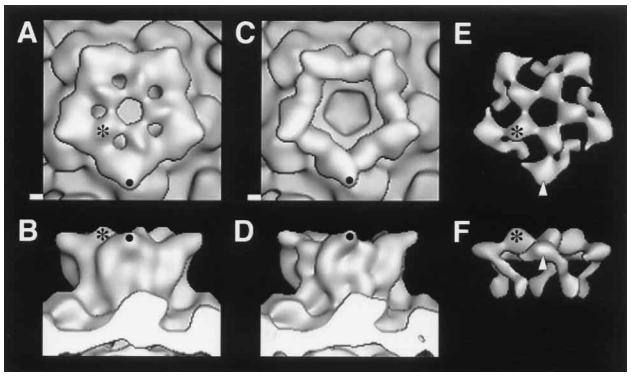

FIG. 4. Image reconstructions of cryoTEM images of reovirus T3D cores and T3D cores with cleaved λ2: close-up views of the λ2 turret. Surface views of T3D cores (A and B), T3D cores with truncated λ2 (C and D), and the difference map (Fig. 3) between them (E and F) are shown in close-up for one representative λ2 turret. In panels B and D, each display has been sectioned to permit the λ2 turret to be viewed in profile as would be seen by looking toward the turret from the bottom of the view in panels A and C, respectively (hatch marks). Asterisks in panels A, B, E, and F indicate the fivefold-axis-proximal elements that are present in normal cores but missing from cores with cleaved λ2. Black dots in panels A to D represent corresponding regions near the points of the λ2 penton (as viewed from above), which are present in both cores and modified cores. Arrowheads in panels E and F indicate elements in the difference map that are interpreted to result from rearrangement of the protein regions near these points.

(Fig. 4A and B) but are missing from cores with cleaved λ2. In virions, these elements approach the fivefold axis more closely and appear to contact the base of the σ1 fiber (12), which has been lost from cores. Thus, we interpret these findings to indicate that the σ1-binding region of each λ2 subunit was specifically degraded during the heat-and-chymotrypsin protocol.

Besides the clear loss of mass from each λ2 turret in modified cores, other changes in the turrets suggest that regions of λ2 that were not degraded during the heat-and-chymotrypsin protocol were induced to undergo shifts in position. For example, the C-shaped ridge extending down toward the core shell from each point of the λ2 penton (Fig. 4A to D) has undergone a counterclockwise (side view) rotation in cores with cleaved λ2 so that each point extends to a slightly higher radius in the modified cores than in the unmodified cores (Fig. 4B and D). Close inspection of the turret sides in Fig. 4B and D reveals other differences consistent with overall maintenance of structure accompanied by subtle rearrangements within the turret.

Differences in the λ2 turrets were also revealed by subtracting the data set for modified cores from the data set for unmodified cores and displaying the difference map for the radii spanned by the turrets (Fig. 3C and 4E and F). The outer, fivefold-axis-proximal elements that are missing from modified cores are evident in the difference map (Fig. 4E and F), but additional changes are also seen. While these additional elements may represent regions of the λ2 sequence that were degraded during the heat-and-chymotrypsin protocol, we interpret the major portion of them to reflect subtle conformational rearrangements (as discussed above). For example, a major portion of the difference elements corresponding to regions near the points of the λ2 penton (Fig. 4E and F) appears likely to reflect the shift in position of the protein regions that form the upper part of the C-shaped ridge in cores and modified cores (see above). We favor a similar explanation for the difference elements that extend down toward the core shell, primarily because the different local regions suggested by the surface relief of the λ2 turret in cores appear to remain present in modified cores (Fig. 4B and D), inconsistent with any large loss of mass from those regions.

It should be noted that the upper regions of the λ2 turrets observed in the reconstruction of T3D cores have morphological features distinct from those observed in a previous reconstruction for T1L cores (12). These differences appear to reflect a difference in the degree to which an outer region of λ2 has rotated up and away from the fivefold axis in the transition from ISVPs to cores (12). More extensive structural analysis of these differences and analysis of their genetic basis using T1L× T3D reassortant cores are being completed (35).

Cores with cleaved λ2 possess intact enzymatic activities associated with transcription and capping. Having observed the changes in core structure that result from the heat-and-chymotrypsin treatment, we wished to determine whether the changes affect core transcription and capping activities. One aim was to determine if the C-terminal region of λ2 removed by this protocol plays any role in the known (guanylyltransferase [6, 13, 20, 30]) or suspected (methyltransferase [18, 28]) activities of λ2 in mRNA capping. By using incorporation of [α-^{32}P]CTP as a measure of transcription in both time course (data not shown) and end point (Fig. 5A) experiments, we found that the transcriptase activity of treated cores was unchanged from that of untreated cores. Given the similar transcriptase activities of the two particle types, we were able to utilize incorporation of *S*-adenosyl-L-[*methyl*-^{3}H]methionine during transcription as a measure of capped and methylated transcript production. Using this assay in both time course (data not shown) and end point (Fig. 5B) experiments, we found that the capping activity of modified cores was reduced only slightly from that of unmodified cores. These results suggest that the structural changes in reovirus particles caused by the heat-and-chymotrypsin protocol have little or no effect on their transcription and capping activities and in particular that the degraded C-terminal region of λ2 plays no significant role in capped mRNA production.

Since GMP becomes covalently bound to λ2 in the first step of the guanylyltransferase reaction (6, 13, 20, 29), we also performed experiments to determine whether GMP becomes bound to the 120K fragment of λ2. T3D cores or T3D cores with cleaved λ2 were incubated under appropriate conditions for guanylyltransferase activity in the presence of [α-^{32}P]GTP. The particles were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE), Coomassie blue staining, and phosphorimaging (Fig. 5C). Both the intact λ2 protein in cores and the 120K fragment in treated cores were covalently labeled with [^{32}P]GMP, indicating that the 120K fragment retained the capacity to mediate autoguanylylation. Quantitative analysis of the ^{32}P-labeled bands in a similar experiment revealed that the autoguanylylation capacities of cores and modified cores were indistinguishable (data not shown).

No effects of heat-and-chymotrypsin treatment on core proteins λ3 and μ2. The evidence suggests that structural changes in cores treated with heat and chymotrypsin correlate with λ2 cleavage and not with any effects on the core-shell proteins λ1 and σ2. Two minor core proteins, λ3 and μ2, are also present in an estimated 12 copies each per particle (36). To ascertain whether cleavage of λ3 or μ2 might also contribute to changes in the turret structure observed in treated cores, we performed experiments to examine those proteins specifically.

To confirm that λ3 was not cleaved during the heat-and-chymotrypsin protocol, we used Tris-glycine-SDS gels formed with half the normal concentration of bisacrylamide (reduced-bis gel), in which the λ3 protein of reovirus T3D is well separated from λ1 and λ2 (6, 11, 23). An experiment in which T3D cores were heated to 52°C for 30 min, cooled to 37°C, and

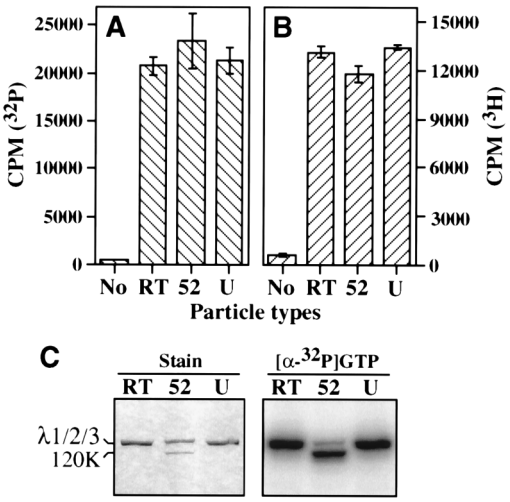

FIG. 5. Quantitative analysis of transcription and cap methylation and qualitative analysis of λ2 autoguanylylation. Types of particles included T3D cores incubated at room temperature (RT) or heated to 52°C for 30 min (52) prior to chymotrypsin treatment and untreated T3D cores (U). Chymotrypsin treatments (200 μg/ml in virion buffer) were performed for 30 min at 37°C. (A) Transcription was analyzed in vitro as described previously (10), with the following modifications: reaction mixtures (30 μl) included 10 U of RNasin (Promega, Madison, Wis.) in place of bentonite and 2.3 μCi of [α-^{32}P]CTP (3,000 Ci/mmol; DuPont NEN, Wilmington, Del.). Following addition of 6×10^{10} particles in 12 μl of virion buffer (or virion buffer alone for the no-core control [No]), reaction mixtures were incubated at 42°C for 1 h, and reactions were terminated by cooling on ice. Each reaction volume was spotted onto a 3-mm-diameter cellulose paper circle (Whatman, Maidstone, United Kingdom), and ^{32}P-labeled transcripts were detected by trichloroacetic acid precipitation onto the filter and liquid scintillation counting. Core reactions were performed in triplicate to give the means and standard deviations (error bars) shown. (B) Cap methylation was analyzed in vitro with a concurrent transcription and capping reaction supplemented with 1.1 μCi of *S*-[*methyl*-^{3}H]adenosyl-L-methionine (55 Ci/mmol; DuPont NEN) (16). Following addition of 9×10^{9} cores in 1 μl of virion buffer (or virion buffer alone for the no-core control [No]), reaction mixtures (20 μl) were incubated at 45°C for 1 h, and reactions were terminated by addition of 5 μl of 250 mM EDTA. One microliter of 10-mg/ml bovine serum albumin, 2 μl of 10-mg/ml *E. coli* tRNA, and 60 μl of 10% trichloroacetic acid were then added to each reaction mixture. Precipitated material was washed with 100 μl of 10% trichloroacetic acid and 200 μl of 55% ethanol and resuspended in 50 mM Tris (pH 8.0)–50 mM KCl by incubation at 65°C overnight. ^{3}H-labeled transcripts in the precipitates were detected by liquid scintillation counting. Each reaction was performed in triplicate to give the means and standard deviations (error bars) shown on the graph. (C) Autoguanylylation of λ2 was analyzed in vitro as described previously (20) with the following modifications: reaction mixtures (20 μl) contained 9×10^{10} untreated or treated cores (added to the reaction mixture in 10 μl of virion buffer), 50 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 2 mM dithiothreitol, and 5.1 μCi of [α-^{32}P]GTP (3,000 Ci/mmol; DuPont NEN). Reactions were stopped by addition of sample buffer, and the resulting mixtures were boiled for 2 min. Equal volumes of samples were then subjected to electrophoresis in an SDS–8% polyacrylamide gel, which does not separate the λ bands. Proteins were visualized by staining with Coomassie brilliant blue R-250. ^{32}P-labeled proteins were detected in the dried gel by using a PhosphorImager (Molecular Dynamics, Sunnyvale, Calif.).

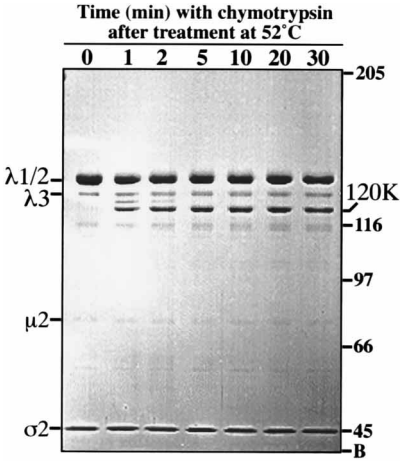

FIG. 6. SDS-PAGE of reovirus cores following heat-and-chymotrypsin treatment: time course of chymotrypsin treatment. Cores of reovirus T3D in virion buffer were heated to 52°C for 30 min, cooled to 37°C, and treated with 200 μg of α-chymotrypsin per ml for the times indicated. Following treatment, the chymotrypsin was inactivated with 1 mM phenylmethylsulfonyl fluoride. Samples were then mixed in a 1:1 (vol/vol) ratio with 2× sample buffer and boiled for 2 min. Equivalent amounts of samples were loaded on a 1.5-mm-thick 4-to-9% reduced-bis gradient polyacrylamide gel (23) in a standard format (Gibco-BRL, Grand Island, N.Y.) and subjected to electrophoresis for ~1.5 h at a 20-mA constant current through the stacking gel followed by ~2.5 h at a 50-mA constant current through the resolving gel. Proteins were visualized by staining with Coomassie brilliant blue R-250. Positions of reovirus proteins are indicated at left (note that the minor core protein μ2 was poorly visualized in this experiment). Positions of marker proteins (Sigma), as well as the bottom of the gel (B), are indicated at right (molecular weights are in thousands).

treated with chymotrypsin was performed. Samples taken at different times were examined on a reduced-bis gel (Fig. 6). The 120K λ2 fragment was observed with increasing intensity after 1 min of protease treatment. Since samples were overloaded for detection of λ3, the λ1 and λ2 bands were poorly separated in this gel. However, the intensity of the well-separated λ3 band did not vary throughout the time course, indicating that λ3 was not cleaved. Although poorly visualized in Fig. 6, the intensity of the μ2 band also remained similar throughout the time course in this and other (data not shown) stained gels, suggesting that μ2 also was not cleaved. To confirm these results, we performed Western blotting using polyclonal antibodies specific for the λ3 and μ2 proteins (4, 39). Purified cores and modified cores were found to have similar quantities of intact λ3 and μ2 and no new λ3- or μ2-derived fragments were seen (data not shown). These findings suggest that the λ3 and μ2 proteins are not affected by the heat-and-chymotrypsin treatment and are not involved in the structural changes observed in the treated cores.

Model for sequence-structure-function relationships in λ2 and the effects of heat-and-chymotrypsin treatment. The findings of this study suggest a simple model for the effects of heat and chymotrypsin on reovirus cores. Heating cores to 52°C causes denaturation of a limited region of the λ2 protein, which is degraded and removed from particles by subsequent treatment with protease at 37°C. Biochemical analyses indicate that the cleaved region of λ2 is formed by sequences at that protein's extreme C terminus, which include an essential portion of the epitope for MAb 7F4. CryoTEM and 3-D image reconstruction indicate that the sequences affected by heat and chymotrypsin treatment comprise portions of the λ2 turret at higher radii, including features that run from each point of the λ2 penton (as viewed from above) toward the fivefold axis. These features include a small globular element in each of the five λ2 subunits, which appears to contact the base of the σ1 protein fiber in virions and ISVPs and to move away from the fivefold axis at the ISVP-to-core transition (12). Thus, the data presented here suggest that the C-terminal sequences of λ2, which are degraded upon heat-and-chymotrypsin treatment, include the σ1-binding region of λ2. Although smaller rearrangements in structure occur in other regions of the λ2 turret upon heating to 52°C, those regions are not made susceptible to protease cleavage. The binding of MAb 7F4 to an outer region of λ2 structure is consistent with the fact that it binds to λ2 not only in cores but also in virions and ISVPs (34), in which only the outer regions of λ2 are well exposed (11).

Removing the C-terminal 24K portion of λ2 has no apparent

effect on either the guanylyltransferase or methyltransferase activities involved in mRNA capping. Therefore, these activities must be based in regions of λ2 and/or other core proteins located at lower radii. Guanylyltransferase activity is known to involve sequences near the N terminus of λ2, specifically, lysine 226, to which GMP becomes linked by a phosphoamide bond before GMP transfer to the RNA (13). There has been speculation that methyltransferase activity involves sequences near the middle of λ2, specifically, amino acids 827 to 831 (of 1,289 total), which are similar to part of a binding site for the substrate *S*-adenosyl-L-methionine (18). Thus, the findings in this study contribute to the following model of λ2 orientation in cores. N-terminal sequences of λ2 are found toward the base of the turret, near its site of contact with the core shell, whereas C-terminal sequences are found toward the top of the turret, near its site of contact with the base of the σ1 fiber in virions and ISVPs (12). According to this model, capping activities attributable to the more N-terminal, shell-proximal regions of λ2 are well positioned to modify the nascent reovirus mRNAs as they are newly synthesized and extruded from the reovirus transcriptase complex at the base of the λ2 turret (11, 32).

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