Loss of Activities for mRNA Synthesis Accompanies Loss of λ2 Spikes from Reovirus Cores: An Effect of λ2 on λ1 Shell Structure

Cindy L. Luongo,*†‡ Xing Zhang,‡ Stephen B. Walker,‡ Ya Chen,§ Teresa J. Broering,*†§ Diane L. Farsetta,†|| Valerie D. Bowman,‡ Timothy S. Baker,‡ and Max L. Nibert*†‡§||

*Department of Biochemistry, †Institute for Molecular Virology, ‡Integrated Microscopy Resource, and §Program in Cellular and Molecular Biology, University of Wisconsin–Madison, Madison, Wisconsin 53706; ‡Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907; and §Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115

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INTRODUCTION

Mammalian orthoreovirus (reovirus), a member of the family Reoviridae, replicates in the cytoplasm of its host cells. Theicosahedrally symmetric core particle, which is an assembly intermediate (Fields et al., 1971; Morgan and Zweerink, 1974) and partial uncoating product (Mayor and Jordan, 1968; Shatkin and Sipe, 1968; Smith et al., 1969) of the intact virion, is composed of five proteins and 10 segments of genomic double-stranded RNA (dsRNA). The main core shell, which surrounds the genome, consists of 120 copies of the λ1 core protein in a T = 1 arrangement with two copies of λ1 per asymmetric unit (Reinisch et al., 2000). The shell is stabilized by 150 nodule-like monomers of the σ2 core protein, which bind to the outer surface of λ1 and appear to serve a clamp-like function (Dryden et al., 1993; Reinisch et al., 2000; Xu et al., 1993). Sixty copies of the λ2 core protein form 12 pentameric “spikes” that protrude around each of the icosahedral fivefold axes of the core (Dryden et al., 1993; Reinisch et al., 2000; White and Zweerink, 1976). Each of these λ2 pentamers binds to the outer surface of the λ1 shell and also makes contact with five adjacent copies of σ2 (Reinisch et al., 2000). The λ3 and μ2 core proteins, which are present in 12 and 24 copies per core, respectively (Coombs, 1998), are thought to contribute to an inwardly projecting structure that is bound to the underside of the λ1 shell near each fivefold axis (Dryden et al., 1998). Thus, although in solution λ2 can associate with both λ1 and λ3 (Starnes and Joklik, 1993), in the core λ2 interacts with λ1 and σ2 but appears not to interact with the more internally located λ3 and μ2 proteins (Dryden et al., 1998; Reinisch et al., 2000).

Reovirus cores contain all of the enzymes necessary to synthesize full-length plus-strand RNA copies from each of the 10 dsRNA genome segments and to add a eukaryotic cap 1 structure (m7N[GpppG]n[O]O) to the 5′ ends of those transcripts (Shatkin and Kozak, 1983). The transcripts are not 3′-polyadenylated. The λ3 protein is the reovirus RNA-dependent RNA polymerase (Morozov, 1989; Starnes and Joklik, 1993), and the λ1 protein exhibits a nucleoside triphosphate phosphohydrolase (NTPase)/helicase activity that may be responsible for...
unwinding or reannealing dsRNA during RNA synthesis (Bisaillon et al., 1997; Noble and Nibert, 1997a). The A1 protein also exhibits an RNA 5’ triphosphate phosphohydrolase (triphasmatase) activity (Bisaillon and Lemay, 1997). This enzymatic activity represents the first step in RNA capping, which yields a 5’-diphosphorylated plus-strand RNA as substrate for the capping RNA guanylyltransferase (GTase) (Furuichi et al., 1976) (see next paragraph). Genetic analysis has indicated that μ2 also influences the NTPase activity of cores (Noble and Nibert, 1997b), but its specific role in transcription or capping remains unknown. The inwardly projecting structures which underlie the λ2 spikes in cores are thought to be composed of λ3, μ2, and an N-terminal region of A1 have been proposed to constitute the enzyme complexes that mediate both plus-strand RNA synthesis and the capping RNA 5’ triphosphatase activity (Dryden et al., 1998). These complexes may also mediate the minus-strand replicase activity that generates the genomic dsRNA segments from plus-strand templates during reovirus assembly.

The λ2 protein in reovirus cores, which contains 1289 amino acids and has a mass of 144 kDa, is implicated in catalyzing the last three reactions in viral plus-strand RNA capping. An N-terminal 42-kDa domain of λ2 is the RNA GTase, which adds a GMP moiety to the 5’ end of the nascent capped transcripts (Bartlett et al., 1974; Luongo et al., 1976). Whether this loss of activity accompanied by a large decrease in transcription activity (White and Zweerink, 1976) resulted from λ2 removal or some other effect of the treatment was undetermined. Nevertheless, the correlation between loss of λ2 and decrease in transcription activity suggested that λ2 is essential for function of either the RNA polymerase or an accessory protein involved in transcription (White and Zweerink, 1976). To investigate the possible role of λ2 in transcription, we attempted to remove λ2 from cores by high pH treatment as in the previous study but were unsuccessful (M. L. Nibert, unpublished data). Efforts in another laboratory have demonstrated selective loss of the λ2 spikes upon exposure of cores to 65°C (K. M. Coombs, personal communication).

Particles that lack the 24K C-terminal portion of λ2 can be produced when cores of reovirus type 3 Dearing (T3D) are heated to 52°C and then digested with chymotrypsin (CHT) (Luongo et al., 1997). Analysis of these heat-and-CHT-treated cores (HC-cores) showed that they retain a 120,000-Mr (120K) N-terminal cleavage product of λ2 and normal amounts of the other core proteins and the dsRNA genome. These HC-cores possess levels of transcription and RNA capping similar to those of untreated cores (Luongo et al., 1997). Thus, heat treatment and subsequent proteolytic removal of the 24K C-terminal region of λ2 do not inhibit core functions. Another new type of reovirus particle can be produced when virions are digested with CHT in the presence of the ionic detergents sodium dodecyl sulfate (SDS) or sodium tetradecyl sulfate (STS) (Chandran and Nibert, 1998). The particles obtained with STS remain fully infectious (Chandran and Nibert, 1998). Hence, this detergent treatment appears not to inhibit the particle-associated transcription and capping enzymes, whose functions are essential for infection. For the current study we hypothesized that, by adding STS to the protocol for generating HC-cores, we might cause additional conformational changes in the N-terminal portion of λ2, making it subject to selective degradation and providing a distinct approach to generate particles with reduced λ2 content. By obtaining and characterizing such λ2-deficient particles, we aimed to gain further information about the roles of λ2 in core structure and function.

RESULTS

Removal of λ2 protein from cores by treatments with heat, protease, and detergent

Purified cores were heated to temperatures between 52 and 60°C to induce conformational changes that render the C-terminal 24K portion of λ2 sensitive to proteolysis (Luongo et al., 1997). Particles were then cooled and treated with CHT in the presence of STS. Analysis by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) revealed that the band containing the three λ proteins had lower intensity than the same band obtained from untreated samples, consistent with the ex-
Particles generated with the sequential protocol formed a distinct band in a CsCl gradient at a density of 1.46 g/cm³ (cores = 1.43 g/cm³). Autoguanylylation followed by SDS–PAGE and phosphorimager analysis revealed these particles to contain only 3.6% (±0.9%) of the amount of full-length λ2 in cores (Fig. 2A). No λ2 cleavage products were detected by immunoblot analysis (data not shown) or autoguanylylation analyses (Fig. 2A). Through other immunoblots, the particles were shown to contain approximately full levels of the other core proteins, including the transcriptase-associated proteins λ3 and μ2 (Fig. 2B). In addition, these particles retained high levels of the 10 genomic dsRNA segments (Fig. 2C). From these experiments we concluded that the sequential treatment protocol is the one of choice for selective removal of λ2 from cores.

Transmission cryo-electron microscopy and three-dimensional image reconstruction of cores treated with heat, protease, and detergent show loss of λ2 spikes and subtle changes in the A1 shell

To determine the structural effects of λ2 removal, cores and λ2-deficient cores generated by the sequential treatment protocol were embedded in vitreous ice and subjected to transmission cryo-electron microscopy (cryo-TEM) and three-dimensional (3-D) image reconstruction. Comparison of surface-shaded and cross-sectional representations of the 3-D reconstructions for cores and λ2-deficient cores showed the most striking

![FIG. 1. Analysis of core particles treated with heat, CHT, and STS. Purified reovirus cores (C) or treated cores (S, for “spikeless”) were analyzed for protein content (A) and λ2 autoguanylylation activity (B). Equivalent amounts of each particle type were resolved by SDS–PAGE. (A) Proteins were stained with Coomassie brilliant blue. The apparent molecular weights of protein standards (X 10⁻³) are indicated at left. (B) Phosphorimager analysis was used to detect [³²P]GMP-labeled λ2 in the dried gel.](Image 88x608 to 234x721)

![FIG. 2. Analysis of heat-and-CHT-treated cores (HC-cores) after further treatment with heat, CHT, and STS. Reovirus cores (C) or treated HC-cores (S, for spikeless) were analyzed for autoguanylylation activity (A), protein content (B), and dsRNA content (C). Equivalent amounts of each particle type were resolved by SDS–PAGE. (A) Phosphorimager analysis was used to detect [³²P]GMP-labeled λ2 in the dried gel. (B) Following transfer of sample pairs in duplicate to nitrocellulose, the membrane was divided into three equivalent strips, which were then probed with polyclonal anti-core, polyclonal anti-λ3, or polyclonal anti-μ2 sera and visualized colorimetrically. The predominant reactivity of the anti-core serum is against the A1, λ2, and α2 proteins. (C) Proteins and dsRNA were stained, respectively, by sequential incubation with Coomassie brilliant blue and methylene blue.](Image 306x189 to 544x342)
differences around the 12 fivefold axes, where the outwardly protruding \( \Lambda_2 \) spikes in cores (Figs. 3C and 3D) were missing from the sequentially treated particles (Figs. 3A and 3B). This dramatic difference is the basis for naming these particles "spikeless cores" (a term introduced by Hazelton and Coombs (1999) to describe a \( \Lambda_2 \)-deficient core-like particle that accumulates at non-permissive temperatures in cells infected with reovirus mutant tsA279). In contrast, no major differences were noted in the arrangement or relative density of either the underlying \( \Lambda_1 \) protein shell or the 150 surface nodules of \( \sigma_2 \) protein, which bind atop \( \Lambda_1 \) at positions distinct from those occupied by \( \Lambda_2 \) in cores. In cross-sectional views of the core and spikeless-core reconstructions (Figs. 3B and 3D), interior features attributable to the genomic dsRNA (arrowheads) and transcriptase complexes (arrows) also appeared very similar in the two particles, consistent with the presence of unaltered levels of these components by gel and immunoblot. Moreover, the similarities in all features below a particle radius of 290 Å were evident in radially averaged electron-density plots of the two reconstructions (Fig. 4). Particularly notable were the series of small peaks ascribed to the concentric rings of genomic dsRNA at radii below 250 Å, which were present in the same numbers (eight), and with almost identical intensities and spacings, in the two particles (Fig. 4). Differences above a radius of 290 Å in the radial density plots of the two particles are attributable to the presence or absence of \( \Lambda_2 \).

To assist in identifying other changes in the structure of spikeless cores, a difference map between the core and the spikeless core reconstructions was generated (Figs. 3E and 3F). The only significant features in the difference map were 12 structures representing the 12 pentameric spikes of \( \Lambda_2 \) that had been removed from cores during the sequential treatments. The absence of other significant features in the difference map suggested again that there are limited changes in the \( \Lambda_1 \) shell and \( \sigma_2 \) surface nodules in spikeless cores compared with untreated cores. We placed the crystal-derived structure of the core-bound \( \Lambda_2 \) pentamer (Reinisch et al., 2000) into one of the 12 difference-map features (see Materials and Methods). All regions of \( \Lambda_2 \) were found to coincide closely in this analysis (Fig. 5). The coincident features included those at the base of the \( \Lambda_2 \) spike, near its sites of interaction with \( \Lambda_1 \) and \( \sigma_2 \). Thus, we found no evidence that changes in the \( \Lambda_1 \) shell or \( \sigma_2 \) nodules contribute features to the difference map.
In a final effort to detect changes in the λ1 shell that may accompany the loss of λ2, we displayed the core and spikeless-core reconstructions over a range of density contours to reveal regions that may differ in electron density between the two particles. Most parts of λ2 and α2 were removed from these images by radial cropping to permit unobstructed views of the shell. A pore through the shell at the icosahedral fivefold axis, visible in the core crystal structure (Reinisch et al., 2000), was not seen in either particle (Fig. 6). The absence of this pore may reflect an artifact of the averaging used to obtain the reconstructions, and as a result the relative openness of this pore in the two particles could not be addressed. In contrast, pores through the shell beneath the λ2 GTase domains, which are also evident in the core crystal structure (Reinisch et al., 2000; see Fig. 10), became visible when the contour level was raised by only a small amount for T3D cores (Fig. 6F), as previously described for type 1 Lang (T1L) cores (Spencer et al., 1997). These pores were not visible in the T3D spikeless cores displayed at even higher contour levels (Figs. 6B–6D), suggesting a conformational change in λ1. These results provide evidence that changes in the A1 shell do indeed accompany the loss of λ2. The basis and significance of these changes, including the elimination of pores through the shell, are addressed in the Discussion.

Scanning cryo-electron microscopy shows rare residual spike structures in spikeless cores

Since the 3-D reconstruction of spikeless cores represents an average of data from multiple particles (Baker et al., 1999), structural features unique to individual particles were lost. As a result, the structure of the residual
low levels of full-length λ2 in spikeless cores could not be addressed by that approach. Therefore, scanning cryo-electron microscopy (cryo-SEM) was performed to visualize the surface features of individual spikeless cores (Centonze et al., 1995). The majority (94%) of spikeless cores lacked any detectable λ2 spike structures. Of the remaining particles, 2% contained one and 4% contained two evident spikes (Fig. 7). Because only 5 or 6 of the 12 icosahedral fivefold axes can be visualized in any single particle by cryo-SEM, λ2 spike structures were calculated to have been detected at only 1.6 to 1.9% of the observed fivefold axes. This frequency of spikes is similar to the amount of full-length λ2 protein in spikeless cores as determined in the autoguanylylation assay (3.6%). Hence, this result suggests that much of the residual λ2 protein remained particle-associated in the form of intact, pentameric spike structures. This result indicates that the residual spikes are stable oligomeric structures because all five of their λ2 subunits remained refractory to disruption during the treatments used to generate spikeless cores. The mechanism by which this subset of λ2 spikes resisted disruption remains unknown.

Another feature of spikeless cores observed by cryo-SEM was the formation of clumps of particles (Fig. 7). These clumps ranged in size from 4 to greater than 50 particles (data not shown). Individual spikeless cores were rarely observed. In contrast, unaggregated core particles are more commonly observed with this technique, and small clumps of cores occur infrequently and range in size from only 2 to 10 particles (Centonze et al., 1995). This difference in particle behavior suggests that removal of λ2 unmasks regions of λ1 or σ2 that promote interparticle associations. Consistent with this idea, core-like particles generated from recombinant λ1 and σ2 proteins in the absence of λ2 show a greater tendency to aggregate than those containing λ2 (J. Kim and M. L. Nibert, unpublished data).

Spikeless cores lack RNA nucleoside-7N-methyltransferase activity

The pentameric form of λ2 in reovirus cores is implicated in mediating both the RNA nucleoside-7N- and the
RNA nucleoside-2’O-methyltransferase activities required for cap 1 formation on reovirus transcripts (Reinisch et al., 2000). The availability of spikeless core particles provided a means to test the role of 2 in these activities. The capacity of cores to transfer the methyl group from SAM to externally supplied GpppRNA (Furui-chi et al., 1977; Mao and Joklik, 1991; Morgan and Kingsbury, 1981) was extended to assay for RNA methyltransferase activity by spikeless cores. Reovirus GpppRNA and [3H-methyl]SAM were incubated with cores, HC-cores, spikeless cores, and a control sample containing no particles. The amount of 3H incorporated into acid-precipitable RNA was quantitated by scintillation counting. In contrast to cores and HC-cores, spikeless cores lacked detectable activity in this assay (Fig. 8). This lack of activity by spikeless cores is consistent with a requirement for 2 in at least the nucleoside-7N-methyltransferase function in RNA capping. Since the product of the nucleoside-7N-methyltransferase reaction, 6GpppRNA (cap 0), is the substrate for the nucleoside-2’O-methyltransferase (Furui-chi et al., 1976), the role of 2 in the latter activity could not be addressed in these experiments.

λ2 spike loss correlates with loss of transcription and other core activities

Having demonstrated loss of 2 from spikeless cores, but retention of high levels of the other core components and integrity of other aspects of particle morphology, we investigated whether the reovirus transcription enzymes remained functional in spikeless cores. Measurement of [α-32P]CTP incorporation into acid-precipitable RNA demonstrated that transcription activity in spikeless cores was less than 2% of that in cores or HC-cores (Fig.

![FIG. 8. Quantitative analysis of RNA cap methylation by spikeless cores. Cores (C), HC-cores (HC), and spikeless cores (S) were analyzed. Cap methylation was analyzed by the incorporation of [3H]methyl from [3H-methyl]SAM into transcripts. Transcripts were precipitated with TCA and the extent of 3H incorporation was quantitated by scintillation counting. A no-particle control reaction containing storage buffer alone was also analyzed (No). Reactions were performed in triplicate to give the means and standard deviations (error bars) shown on the graph.](image)

![FIG. 9. Analysis of transcription, abortive oligonucleotide synthesis, and ATPase activities in spikeless cores. The following particles were analyzed in particular experiments: cores (C), HC-cores (HC), spikeless cores (S), HC-cores additionally treated with heat alone (+h), HC-cores additionally treated with heat and the protease CHT (+hp), and HC-cores additionally treated with the protease CHT and the detergent STS (+pd). A no-particle control reaction containing storage buffer alone was also analyzed (No). (A) Transcription. Transcription activity was quantitated by liquid scintillation counting from the amount of 32P incorporated from [α-32P]CTP into TCA-precipitable material. Reactions were performed in triplicate to give the means and standard deviations (error bars) shown on the graph. (B) Abortive oligonucleotide synthesis. Abortive transcription activity was demonstrated by phosphorimagery analysis from the amount of 32P incorporated from [α-32P]CTP into acid-precipitable RNA. Reactions were performed in triplicate to give the means and standard deviations (error bars) shown on the graph. (C) ATP hydrolysis. ATPase activity was quantitated by colorimetric assay for free phosphate ion. Samples containing ATP alone were included to permit correction for background attributable to phosphate release by nonenzymatic hydrolysis of ATP. Reactions were performed in triplicate to give the means and standard deviations (error bars) shown on the graph.](image)
9A). Though spikeless cores showed a greatly reduced capacity to produce elongated, acid-precipitable RNAs, they might still be capable of initiating transcription and producing shorter transcripts. Reovirus cores are known to generate two- to four-nucleotide abortive transcripts at a rate 5- to 100-fold higher than full-length transcripts (Yamakawa et al., 1981). Detection of these products provides a sensitive assay for transcription initiation. We therefore assayed for incorporation of \([\alpha-^32P]ATP\) into GC and GCU, the primary abortive transcripts (Farsetta et al., 2000; Yamakawa et al., 1981), and found that spikeless cores retained only 6.6% (±2.5%) of the level of transcription initiation by cores (Fig. 9B). Thus, spikeless cores obtained with our sequential treatment protocol exhibit severely reduced transcription initiation activity, consistent with the low transcription activity of spikeless cores obtained by high pH treatment (White and Zweerink, 1976).

Reovirus cores show NTPase activity indicative of the putative RNA helicase or RNA 5’ triphosphatase roles of the \(\lambda1\) protein (Bisaillon et al., 1997; Bisaillon and Lemay, 1997; Noble and Nibert, 1997a). The \(\mu2\) protein also contributes to the NTPase activity of cores (Noble and Nibert, 1997b). Since \(\lambda1\) and \(\mu2\) are present at similar levels in spikeless cores and cores, our expectation was that both particles would mediate similar levels of ATPase activity. The activity was analyzed using a sensitive colorimetric assay, which measures the amount of phosphate released upon ATP hydrolysis (Noble and Nibert, 1997a). ATPase activities in cores and spikeless cores were measured at pH 8.5 and 35°C as well as at pH 6.5 and 55°C to discriminate the contributions of \(\lambda1\) and \(\mu2\) (Noble and Nibert, 1997b). At pH 8.5 and 35°C, spikeless cores exhibited less than 4% of the ATPase activity of cores (Fig. 9C). At pH 6.5 and 55°C their ATPase activity was less than 2% that of cores (data not shown). Thus, the absence of \(\lambda2\) in our spikeless cores also correlates with a severe reduction in ATPase activity.

Relative roles of heat, protease, and detergent in loss of transcription activity from spikeless cores

Because HC-cores retain full transcription activity (Luongo et al., 1997) (Fig. 9A), it was unlikely that the second exposure to heat or the second exposure to CHT by itself caused spikeless cores to lose transcription activity. To identify the specific effects of each of these treatments, we subjected HC-cores to heat alone or to heat followed by CHT digestion. Particles were recovered by centrifugation in a CsCl gradient and assayed for transcription activity. Both particle types retained full transcription activity (Fig. 9A). Both particle types also retained large amounts of the 120K N-terminal fragment of \(\lambda2\) (data not shown). However, incubation of HC-cores with CHT and STS without prior heat treatment led to recovery of particles after equilibrium centrifugation in which transcription activity was greatly reduced (Fig. 9A). These particles lacked the 120K \(\lambda2\) fragment (data not shown) and thus demonstrated that the second round of heat treatment is not necessary for removing this fragment. The effect of STS treatment alone was explored by heating HC-cores and then exposing them to 0.5 mM detergent. These particles did not form bands after centrifugation in a CsCl gradient, rendering further analysis impossible. Particles were alternatively treated with 0.5 mM STS (above the critical micellar concentration for this detergent) and then diluted to 0.1 mM STS (below its critical micellar concentration) (Chandran and Nibert, 1998) by addition to a transcription reaction. Cores or HC-cores treated in this manner retained only 3 or 0.4%, respectively, of the transcription activity of the respective particles exposed to only 0.1 mM STS (data not shown). For both particles, the \(\lambda2\) protein and/or 120K N-terminal fragment remained particle-bound after centrifugation through sucrose (data not shown), indicating that \(\lambda2\) loss is not strictly required for loss of transcription activity. However, since exposure to micellar concentrations of STS is known to alter the \(\lambda2\) conformation, as indicated by the degradation of \(\lambda2\) once CHT is added, conformational change in \(\lambda2\) remains correlated with the loss of transcription.

Binding recombinant \(\lambda2\) protein to spikeless cores

A hypothesized use for spikeless cores was the development of a molecular-genetic approach for analyzing \(\lambda2\) functions in core-like particles, similar to strategies developed for outer-capsid proteins \(\mu1\), \(\sigma3\), and \(\sigma1\) in virion-like particles (Chandran et al., 1999, 2001; Jané-Valbuena et al., 1999). In such “recoating” experiments we envisioned removing \(\lambda2\) from cores to generate spikeless cores that retain the viral genome but lack activities in transcription and capping. Subsequent binding of recombinant \(\lambda2\) to these particles in vitro might be expected to allow recovery of one or more of the missing activities. The use of specific mutant forms of recombinant \(\lambda2\) could then be used to identify important \(\lambda2\) sequence determinants for these activities. The use of spikeless cores as a substrate for \(\lambda2\) binding was tested by incubating such particles with lysate from insect cells infected with a recombinant baculovirus that expressed wild-type \(\lambda2\) protein (Luongo et al., 1998). Though analyses of the particles purified from such reactions showed greater binding of recombinant \(\lambda2\) protein to spikeless cores than to cores, the bound \(\lambda2\) exhibited negligible autoguanlylation activity and did not form spike-like structures visible by cryo-SEM (data not shown). Also, these putatively recoated particles showed negligible transcription activity (data not shown). Hence, recombinant \(\lambda2\) protein appears to bind to spikeless cores in a manner that does not permit recovery of the
missing core activities, and this type of particle preparation is apparently not suitable for the anticipated molecular-genetic studies of \( \lambda 2 \).

**DISCUSSION**

**Reovirus core structure**

The structure of our spikeless T3D cores determined by cryo-electron microscopy is consistent with the core structure determined by X-ray crystallography (Reinisch et al., 2000). The core crystal structure indicates that the \( \lambda 2 \) pentamers bind to the outer surface of the \( \lambda 1 \) shell, suggesting that the \( \lambda 2 \) spikes might be detached from this shell without destroying the basic integrity of the particle. The current study confirms that \( \lambda 2 \) is not required for the integrity of the core shell in previously assembled particles, in that removal of \( \lambda 2 \) did not affect the basic appearance of the shell at 28-Å resolution or the gel-defined abundance of the other particle components including the genomic dsRNA. Other findings indicate that \( \lambda 2 \) is not allowed for assembly of the core shell containing \( \lambda 1 \) and \( \sigma 2 \) (Xu et al., 1993; J. Kim and M. L. Nibert, manuscript in preparation).

The findings in this study corroborate a view of the organization of proteins in reovirus cores indicated by the crystal structure (Reinisch et al., 2000). In particular, the recent data show that the core shell per se is formed by \( \lambda 1 \) alone, with \( \lambda 2 \) and \( \sigma 2 \) binding to its outer surface and making no direct contributions to the \( T = 1 \) lattice. Thus, the \( \sigma 2 \) nodules and the similarly nodule-like N-terminal GTase domain of \( \lambda 2 \) (Reinisch et al., 2000) (seen tangential to the shell in cross sections (Figs. 3D and 3F)) can be considered to form an incomplete middle layer of protein in reovirus virions, between the \( T = 1 \) inner layer formed by \( \lambda 1 \) and the \( T = 13 \) outer layer formed by \( \mu 1 \) (Dryden et al., 1993). The more C-terminal regions of \( \lambda 2 \) project into the outer layer and make direct contributions to the \( T = 13 \) lattice at its peripental positions (Dryden et al., 1993) such that \( \lambda 2 \) can be considered an outer capsid component as well. Unlike \( \lambda 2 \), \( \sigma 2 \) appears to be required for assembly of a stable core shell (Xu et al., 1993; J. Kim and M. L. Nibert, manuscript in preparation). Whether \( \sigma 2 \) can be removed from the previously assembled shell without destroying its basic integrity, as can \( \lambda 2 \), remains to be determined.

The cross-sectional views of the 3-D reconstructions of both cores and spikeless cores show density in concentric rings within the central region occupied by the genomic dsRNA (Figs. 3B, 3D, and 4). In each particle type, eight rings of putative RNA density are distinguishable, indicative of similar separations between rings within the two particles and consistent with the fact that spikeless cores appear by gel to have retained approximately core-like levels of the 10 dsRNA segments (Fig. 2C). The average center-to-center distance between adjacent rings in each reconstruction is 26–28 Å. This finding is reminiscent of results from low-angle X-ray diffraction studies of reovirus particles (Harvey et al., 1981), close-to focus cryo-electron micrographs of reovirus cores (Dryden et al., 1993), and the reovirus core crystal structure (Reinisch et al., 2000), all showing densities separated by 25–27 Å within the RNA region and indicative of RNA packing with statistical order to fill the available space (Reinisch et al., 2000). The cross-sectional views of both cores and spikeless cores also show evidence of the transcriptase complexes anchored to the \( \lambda 1 \) shell at or near each fivefold axis (Dryden et al., 1998).

**Potential role of \( \lambda 2 \) in reovirus transcriptase activity and its structural basis**

As expected for particles lacking the enzymatically active \( \lambda 2 \) protein (Cleveland et al., 1986; Luongo et al., 1998, 2000; Mao and Joklik, 1991; Reinisch et al., 2000; Shatkin et al., 1983), spikeless cores were shown in this study to lack the RNA GTase (Figs. 1 and 2A) and RNA nucleoside-7-MN-methyltransferase (Fig. 8) activities that are required for formation of a cap 1 structure on viral transcripts. The effects of \( \lambda 2 \) removal on transcription and ATP hydrolysis by spikeless cores are not as easily explained, however, because these activities are ascribed to core proteins \( \lambda 1 \), \( \lambda 3 \), and \( \mu 2 \) (Bisaiillon et al., 1997; Bisaiillon and Lemay, 1997; Morozov, 1989; Noble and Nibert, 1997a, b; Starnes and Joklik, 1993; Yin et al., 1996), which are found at normal levels in spikeless cores (Fig. 2B). Heat and CHT treatment, which removes only a 24K C-terminal portion of \( \lambda 2 \) (Luongo et al., 1997), has no effect on transcription (Luongo et al., 1997) (Fig. 9A) or ATP hydrolysis (T. J. Broering and M. L. Nibert, unpublished data). These observations suggest that effects on more N-terminal regions of \( \lambda 2 \) are required for loss of transcription activity.

Contacts of \( \lambda 2 \) with \( \lambda 1 \) and \( \sigma 2 \) in cores are made exclusively through the 42-kDa N-terminal GTase domain of \( \lambda 2 \) at the base of the \( \lambda 2 \) spike (Luongo et al., 2000; Reinisch et al., 2000; Breun et al., 2001) (Fig. 5; also see Fig. 10). Loss of transcription and ATPase activities observed in the present study may thus be specifically attributable to changes in other core proteins resulting from loss or alteration of their interactions with this specific \( \lambda 2 \) region. Though the \( \lambda 3 \) and \( \mu 2 \) core proteins were not resolved in the core crystal structure (Reinisch et al., 2000), it seems unlikely that either makes direct contacts with \( \lambda 2 \) in cores because they are thought to constitute the bulk of the putative transcriptase complexes that are bound beneath the \( \lambda 1 \) shell near the icosahedral fivefold axes (Dryden et al., 1998). Thus, \( \lambda 3 \) and \( \mu 2 \) are likely separated from \( \lambda 2 \) by the thickness of the \( \lambda 1 \) shell (Reinisch et al., 2000). Loss of transcription and ATPase activities in spikeless cores may nonetheless be explained by local alterations in the \( \lambda 1 \) shell that result when the interactions of \( \lambda 2 \) with \( \lambda 1 \) and/or \( \sigma 2 \) are
spikeless cores, including abortive transcript production, disengagement of the dsRNA genome segments from the RNA polymerase protein A3 in spikeless cores is a possibility worth investigating in future studies. According to this proposed explanation, A2 may play an important role in reovirus transcription by stabilizing the transcriptase complexes in a functional conformation through protein–protein and/or protein–RNA interactions within the core.

The analysis of cores and spikeless cores at different density contours identified some changes in the A1 shell associated with A2 loss (Fig. 6), consistent with one or more of the preceding possibilities to explain the loss of transcriptase and NTPase activities by spikeless cores. The finding that pores through the A1 shell beneath the A2 GTase domains are less open in spikeless cores suggests that plugging of these pores may explain at least some of those particles’ lost functions. The pores in question are evident in the core crystal structure (Reinisch et al., 2000). Each GTase domain in the A2 pentamer mediates major contacts with two adjacent fivefold-proximal subunits of A1 (Figs. 10A and 10B) and a minor contact with the interdigitating threefold-proximal subunits of A1 (Fig. 10A) in the A1 decamer unit (Reinisch et al., 2000). The footprint of each A2 GTase domain (Fig. 10A, black ovals) overlies the intersection of these three copies of A1. Thirty-one different A1 residues (Fig. 10A) are likely involved in these contacts, as judged by their approach to within 4.0 Å of 32 different residues in each respective A2 GTase domain (Breun et al., 2001) (Fig. 10B). By having removed A2 from these contacts in spikeless cores, small shifts in the positions of the A1 subunits may occur and result in further changes inside the shell. The contacts of each A2 GTase domain with the fivefold-proximal A1 subunit adjacent to the one on which it primarily sits (Figs. 10A and 10B) are mediated through two projecting regions of A1 structure: a loop (residues 661–667) and a bent α-helix (residues 686–698) (Fig. 10B, arrows). Upon removal of A2 from these contacts, these projecting regions of A1 may collapse. Interestingly, these regions of A1 in cores project beside a pore (Fig. 10A, peripheral white circles) that opens into the core interior and corresponds to the pores observed in cores, but not spikeless cores, in cryo-TEM reconstructions (Fig. 6). Collapse of these regions of A1 may therefore plug the top of this pore. The relative roles of this pore and the one at each fivefold axis (Fig. 10A, central white circle) in substrate (nucleoside triphosphate; NTP) entry and product (plus-strand RNA) and by-product (pyrophosphate, phosphate, and NDP) release during reovirus mRNA synthesis remain to be addressed (Reinisch et al., 2000; Spencer et al., 1997), but these pores may be key targets for regulation of core functions. One possible explanation is that the pores that open beneath each A2 GTase domain in cores may be essential for NTP entry to the core interior and, by being plugged upon A2 release...
from spikeless cores, may cause all NTP-dependent activities to cease.

Recent data indicate that addition of reovirus outer-capsid proteins \( \mu_1 \) and \( \sigma_3 \) to cores to produce recoated, virion-like particles blocks the transition from initiation to elongation during transcription (Farsetta et al., 2000). That study therefore provides other evidence that reovirus transcription activity can be affected across the intervening \( \Lambda_1 \) shell, possibly as translated through either \( \Lambda_2 \) or \( \sigma_2 \), which are both believed to contact the \( \mu_1 \) outer-capsid protein in virion-like particles (Dryden et al., 1993; Reinisch et al., 2000). This regulation of core activities by \( \mu_1 \) and \( \sigma_3 \) could conceivably also involve the opening and closing of specific pores through the \( \Lambda_1 \) shell.

The 3-D reconstruction of reovirus T3D cores in this study is notable in that no pore is evident through the topmost “flaps” of the \( \Lambda_2 \) spike (Fig. 3D). This was also noted for T3D cores in a previous cryo-TEM reconstruction (Luongo et al., 1998) and contrasts with the more open conformation of the spike first noted with T1L cores (Dryden et al., 1993). The crystal structure of reovirus cores shows the outer portion of \( \Lambda_2 \) in the closed conformation, which may be characteristic of the T3D \( \Lambda_2 \) protein in cores since the crystal structure was determined for cores of T1L \( \times \) T3D reassortant virus F18, which contain T3D-derived \( \Lambda_2 \) (Reinisch et al., 2000). The basis for the conformational variability of this outer region of \( \Lambda_2 \) structure and its significance for the release of nascent capped transcripts through the top of the \( \Lambda_2 \) spike (Bartlett et al., 1974; Yeager et al., 1996) remain important questions.

Assembly of \( \Lambda_2 \) and molecular-genetic studies of \( \Lambda_2 \) functions in particles

Our failure to reconstitute enzymatically active particles by recoating spikeless cores with recombinant \( \Lambda_2 \) is disappointing, but might be explained and perhaps corrected in several different ways. One possibility is that the binding sites for \( \Lambda_2 \) in spikeless cores were disrupted by detergent during preparation of these particles. In that case, a new protocol to generate genome-containing spikeless cores in the absence of detergent may yet allow the recoating approach to succeed. Another possibility is that \( \Lambda_2 \) requires another cellular or viral factor, absent from the recoating protocol, for binding to the core shell. For example, Hazelton and Coombs (1999) proposed that preformed complexes of \( \Lambda_2 \) pentamers and \( \sigma_1 \) trimers are the likely substrates for assembly onto the core shell within infected cells. Recombinant \( \Lambda_2 \) protein, including that used in this study, is known not to form pentamers on its own (Luongo et al., 2000; Mao and Joklik, 1991). Accordingly, we might find that addition of recombinant \( \sigma_1 \) protein to the recoating procedure might yet allow this approach to work. The generation of core-like particles from coexpressed \( \Lambda_1 \), \( \Lambda_2 \), and \( \sigma_2 \) core proteins (Xu et al., 1993; J. Kim and M. L. Nibert, manuscript in preparation), however, suggests that a specific requirement for \( \sigma_1 \) in \( \Lambda_2 \) assembly into cores is unlikely.

Other interpretations of our failure to recoat spikeless cores with recombinant \( \Lambda_2 \) suggest less optimism for future success. For example, the effects of \( \Lambda_2 \) removal on the \( \Lambda_1 \) shell (Figs. 6 and 10) may not only explain the reductions in spikeless core transcription and NTPase activities but may also render the \( \Lambda_1 \) protein incompetent for subsequent rebinding of \( \Lambda_2 \). It may therefore be that \( \Lambda_2 \) can be assembled into cores only as the shell is being formed, i.e., before maturation of the final \( T = 1 \) structure. Other approaches, such as the generation of core- or virion-like particles wholly from recombinant proteins (Xu et al., 1993; J. Kim and M. L. Nibert, manuscript in preparation), may therefore prove more useful for molecular-genetic studies of at least some \( \Lambda_2 \) functions, including those in RNA capping.

MATERIALS AND METHODS

Reagents

All enzymes and chemicals were from Sigma Chemical Co. unless otherwise stated. The electrophoresis and immunoblot reagents and rigs were from Bio-Rad Laboratories. Nucleotides were obtained from Pharmacia Biotech. All radiolabeled reagents were from Dupont NEN.

Generation of cores, HC-cores, and spikeless cores

Virions of reovirus T3D were purified as described (Furlong et al., 1988), and their concentrations were determined from the relationship \( 1.0 \ A_{260} = 2.1 \times 10^{12} \) virions/ml (Smith et al., 1969). Cores of reovirus were prepared by digesting virions at a concentration of \( 3 \times 10^{12} \) particles/ml with \( 200 \mu g/ml \ \alpha\)-CHT for 1.5 h at 37°C in storage buffer (10 mM Tris, pH 7.5, 10 mM MgCl\(_2\), 150 mM NaCl) (Luongo et al., 1998). The CHT was inactivated by addition of 1 mM phenylmethylsulfonyl fluoride (PMSF). To purify cores, digests were loaded onto preformed CsCl gradients (\( \rho = 1.30 \) to 1.55 g/cm\(^3\) ) and subjected to equilibrium centrifugation. The particles were dialyzed into storage buffer and stored at 4°C. Core concentrations were determined from the relationship \( 1.0 \ A_{260} = 4.4 \times 10^{12} \) cores/ml (Coombs, 1998).

HC-cores were generated as described (Luongo et al., 1997). In brief, \( 1 \times 10^{13} \) cores in storage buffer were heated to 52°C for 30 min, cooled to 37°C, and treated with \( 200 \mu g/ml \) CHT for 10 min. Following treatment, the CHT was inactivated with 1 mM PMSF, and the HC-cores were isolated following equilibrium centrifugation as described for cores. The particles were dialyzed into storage buffer and stored at 4°C.

Spikeless cores were generated by heating \( 1 \times 10^{13} \) or
2 × 10^{13} cores or HC-cores, respectively, in storage buffer to 52°C for 30 min, cooling to 37°C, and treating with 200 μg/ml CHT and 0.5 mM STS for 10 min. Following treatment, the CHT was inactivated with 1 mM PMSF. To purify the particles, digests were loaded onto a CsCl gradient (ρ = 1.30 to 1.55 g/cm³) that had been overlaid with 20% (w/v) sucrose equaling one-third of the final gradient volume. After centrifugation at 55,000 rpm in a Beckman SW60Ti rotor at 5°C for greater than 2 h, the particles were recovered as an optically homogeneous band. The particles were dialyzed into storage buffer and stored at 4°C.

**SDS–PAGE**

Samples were prepared for SDS–PAGE by mixing in a 2:1 (v/v) ratio with 3X Laemmli sample buffer (3% SDS, 9% β-mercaptoethanol, 375 mM Tris, pH 8.0, 30% sucrose, and 0.004% bromophenol blue). Samples were incubated at 60°C for 3 min (for protein and dsRNA analysis) or boiled for 2 min (for protein analysis). Equal volumes of samples were then subjected to electrophoresis in an 8% polyacrylamide Tris–glycine–SDS gel. Proteins were stained with Coomassie brilliant blue R-250. After Coomassie staining in some cases, the dsRNA was stained with 2% methylene blue in 0.1% NaOH.

**Autoquanylylation analysis**

Reaction mixtures (10 μl) containing 1 × 10^{11} particles (added to the reaction mixture in 2 μl storage buffer), 50 mM Tris–HCl, pH 8.0, 10 mM MgCl₂, 2 mM dithiothreitol (DTT), and 5 μCi of [α-32P]GTP (3,000 Ci/mmol) were incubated for 30 min at room temperature. Reactions were terminated by the addition of 5 μl of 3X Laemmli sample buffer. The reactions were then subjected to SDS–PAGE. Radiolabeled protein was detected in the dried gel by phosphorimager analysis (Molecular Dynamics).

**Immunoblot analyses**

Equivalent amounts of particles in triplicate were subjected to SDS–PAGE. Proteins in the gel were transferred to nitrocellulose by electroblotting at 100 V for 1.5 h at 4°C with 25 mM Tris, 192 mM glycine, pH 8.3, buffer in a minigel transfer apparatus. The membrane was divided into three equivalent strips. Individual strips were probed with a 1/1000 dilution of a polyclonal anti-λ3 antiserum (D. L. Farsetta and M. L. Nibert, unpublished data), a polyclonal anti-core antiserum to detect λ1 and α2 (Chandran et al., 1999), or a polyclonal anti-μ2 antiserum (Zou and Brown, 1996). The polyclonal antibodies were bound by goat anti-rabbit antibodies conjugated with alkaline phosphatase and visualized colorimetrically with Nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate.

**Cryo-electron microscopy and 3-D image reconstructions**

For cryo-TEM, purified particles obtained from reovirus T3D were embedded in vitreous ice, and micrographs were recorded under conditions of minimal irradiation (≈6 and ≈20 electrons/A² for the core and spikeless core samples, respectively) in Philips CM200 and CM300 FEG microscopes at magnifications of ×27,500 and ×45,000. The micrograph of spikeless cores was recorded with minimal astigmatism at 3.1 μm under focus, whereas the micrograph of cores exhibited astigmatism with 2.0- and 3.3-μm defocus along orthogonal directions. The particle orientations in both micrographs were sufficiently random to satisfy sampling requirements for Fourier inversion: all inverse eigenvalues were <0.10 and more than 99% were <0.01. 3-D image reconstruction procedures were carried out as described (Dryden et al., 1998), with a previous 3-D reconstruction of the reovirus T1L core (Dryden et al., 1993) used as the initial reference for model-based refinement of particle origin and orientation parameters (Baker and Cheng, 1996). Several iterative steps of refinement were conducted until no improvement in resolution beyond 28 Å could be obtained with either data set as determined by various criteria, such as phase residuals and correlation coefficients (Baker et al., 1999). Corrections were applied to compensate for the effects of the microscope contrast transfer function, resulting in the minimization of the effects of the astigmatism in the micrograph of cores (Baker et al., 1999). Final reconstructions were generated at 28-Å resolution using 1454 and 1078 particle images of cores and spikeless cores, respectively. For cryo-SEM, sample preparation, image capture, and processing for publication were undertaken as described (Centonze et al., 1995). Figures 3 and 6 were assembled in Photoshop 5.5 (Adobe Systems).

**Placement of the λ2 crystal structure into the cryo-TEM difference map (Fig. 5)**

A single λ2 pentamer spike was cut out from the whole 3-D difference map and converted to proper format for use in the program O (Kjeldgaard et al., 1993). Using O, the reovirus core crystal structure (Reinisch et al., 2000) was then moved by eye to obtain an optimal fit within the difference map. Enhanced color images were prepared with the program Bobscript 2.4 (Esnouf, 1997) using the fitted structures output from O. The final figure was assembled in Photoshop 5.5 (Adobe Systems).

**Displays of λ1–λ2 interactions from the crystal structure (Fig. 10)**

The reovirus core crystal structure (Reinisch et al., 2000) was displayed using RasMac 2.7, including identification of the residues in λ1 and λ2 that approach within...
4 Å of the opposite subunit. The final figure was assembled in Illustrator 8.0 (Adobe Systems).

RNA cap methylation

Cap methylation was analyzed in vitro as described (Morgan and Kingsbury, 1981). Reovirus GpppRNA was generated from 6.7 × 10^12 T3D cores/ml in 100 mM Tris, pH 8.0, 12 mM MgCl2, a 2 mM concentration each of ATP, CTP, and UTP, 0.5 mM GTP, 0.5 mM S-adenosyl-L-homocysteine, and 0.02 U/µl inorganic pyrophosphatase (Roche Molecular Biochemicals) by incubation at 40°C for 1.5 h (Furuichi et al., 1976). The reovirus cores were then removed by centrifugation. The RNA was purified by addition of 1% SDS (final) before extraction with warm phenol and ethanol precipitation. The RNA was resuspended in deionized water and its concentration determined by spectrophotometry. For the methylation assay, 6 µg of reovirus GpppRNA was added per 10-µl reaction containing 50 mM Tris, pH 8.0, 10 mM magnesium acetate, 1 mM DTT, 1.5% PEG, 20 U RNasin (Promega), and 0.275 µCi [3H]SAM (80 Ci/µmol). After addition of 2.9 × 10^11 particles in 4.8 µl of storage buffer, the reaction mixtures were incubated at 40°C for 2 h. Reactions were terminated by the addition of 10 µg bovine serum albumin and 30 µl of 10% trichloroacetic acid (TCA). Precipitated material was washed with 100 µl of 10% TCA and 200 µl of 55% ethanol and resuspended in 50 mM Tris, pH 8.5, 50 mM KCl by incubation at 65°C overnight. 3H-labeled transcripts in the precipitates were detected by liquid scintillation counting.

Transcription assays

Transcription generating elongated transcripts was analyzed in vitro as described (Drayna and Fields, 1982). In brief, reaction mixtures (50 µl) contained 70 mM Tris, pH 8.0, 10 mM MgCl2, a 1 mM concentration each of ATP, CTP, and UTP, 3.5 mM GTP, 0.05 U inorganic pyrophosphatase, 10 mM phosphoenol pyruvate, 0.4 U pyruvate kinase, and 2.5 µCi of [α-32P]CTP (3000 Ci/µmol). Following the addition of 1 × 10^11 particles in 20 µl of storage buffer, reaction mixtures were incubated at 45°C for 1 h. Reactions were terminated by the addition of 10 µg bovine serum albumin, 20 µg tRNA (Roche Molecular Biochemicals), and 150 µl of 10% TCA. Precipitated material was washed with 100 µl of 10% TCA and 200 µl of 55% ethanol and resuspended in 50 mM Tris, pH 8.5, 50 mM KCl by incubation at 65°C. 32P-labeled transcripts in the precipitates were detected by liquid scintillation counting.

Transcription generating oligonucleotides was analyzed in vitro as described (Yamakawa et al., 1981). In brief, reaction mixtures (15 µl) contained 100 mM Tris, pH 8.3, 4 mM MnCl2, a 1 mM concentration each of ATP, CTP, UTP, and GTP, 3.3 mM phosphoenolpyruvate, 0.8 U pyruvate kinase, 6 U RNasin, 2.5 mM DTT, and 2.5 µCi of [α-32P]CTP. Following the addition of 3 × 10^11 particles in 3.2 µl storage buffer, reaction mixtures were incubated at 40°C for 1 h. Reactions were then treated with 20 U of calf intestinal alkaline phosphatase (New England Biolabs) at 37°C for 1 h to degrade the labeled CTP. The reaction mixtures were resolved by electrophoresis in a discontinuous 20% polyacrylamide Tris–borate–EDTA–urea gel. Labeled products were detected in the gel by phosphorimager analysis.

ATPase assay

ATPase activity of particles was analyzed in vitro as described (Noble and Nibert, 1997a). In brief, reaction mixtures (60 µl) contained 50 mM Tris–morpholinosulfonic acid, 7.5 mM NaCl, 5 mM MgCl2, 1 mM ATP, and 3.6 × 10^10 particles. Reactions were incubated for 30 min at pH 8.5 and 35°C or at pH 6.5 and 55°C. Reactions were terminated by the addition of 60 µl of 10% TCA. To measure the amount of free phosphate ion in each sample, 100 µl of the stopped reaction was mixed with 100 µl of 3:1:1 of 0.8% ammonium molybdate:6 N sulfuric acid:10% (w/v) ascorbic acid and incubated at 37°C for 30 min prior to determination of A_{250}. Samples containing ATP alone were included to permit correction for background attributable to phosphate release by nonenzymatic hydrolysis of ATP.

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