African Swine Fever Virus NP868R Capping Enzyme Promotes Reovirus Rescue during Reverse Genetics by Promoting Reovirus Protein Expression, Virion Assembly, and RNA Incorporation into Infectious Virions

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ABSTRACT Reoviruses, like many eukaryotic viruses, contain an inverted 7-methylguanosine (m7G) cap linked to the 5' nucleotide of mRNA. The traditional functions of capping are to promote mRNA stability, protein translation, and concealment from cellular proteins that recognize foreign RNA. To address the role of mRNA capping during reovirus replication, we assessed the benefits of adding the African swine fever virus NP868R capping enzyme during reovirus rescue. C3P3, a fusion protein containing T7 RNA polymerase and NP868R, was found to increase protein expression 5- to 10-fold compared to T7 RNA polymerase alone while enhancing reovirus rescue from the current reverse genetics system by 100-fold. Surprisingly, RNA stability was not increased by C3P3, suggesting a direct effect on protein translation. A time course analysis revealed that C3P3 increased protein synthesis within the first 2 days of a reverse genetics transfection. This analysis also revealed that C3P3 enhanced processing of outer capsid μ 1 protein to μ 1C, a previously described hallmark of reovirus assembly. Finally, to determine the rate of infectious-RNA incorporation into new virions, we developed a new recombinant reovirus S1 gene that expressed the fluorescent protein UnaG. Following transfection of cells with UnaG and infection with wild-type virus, passage of UnaG through progeny was significantly enhanced by C3P3. These data suggest that capping provides nontraditional functions to reovirus, such as promoting assembly and infectious-RNA incorporation.

IMPORTANCE Our findings expand our understanding of how viruses utilize capping, suggesting that capping provides nontraditional functions to reovirus, such as promoting assembly and infectious-RNA incorporation, in addition to enhancing protein translation. Beyond providing mechanistic insight into reovirus replication, our findings also show that reovirus reverse genetics rescue is enhanced 100-fold by the NP868R capping enzyme. Since reovirus shows promise as a cancer therapy, efficient reovirus reverse genetics rescue will accelerate production of recombinant reoviruses as candidates to enhance therapeutic potency. NP868R-assisted reovirus rescue will also expedite production of recombinant reovirus for mechanistic insights into reovirus protein function and structure.

KEYWORDS capping, reovirus, reverse genetics, NP868R, C3P3

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The mRNAs of eukaryotes and many eukaryotic viruses contain an inverted 7-methylguanosine (m⁷G) cap linked to the 5' nucleotide by a 5'-5' triphosphate linkage. The cap serves several biological functions (1–4). For RNA transcribed in the nucleus, the cap supports mRNA splicing and nuclear export (5). The cap also promotes mRNA stability by obstructing 5'-to-3' exonuclease-mediated degradation (6). To be recycled, mRNAs are decapped within processing bodies (P bodies), which then permits access to 5' exonucleases. Caps also promote protein translation by engaging eukaryotic translation initiation factor 4E (eIF4E), which subsequently forms a preinitiation complex and recruits ribosomes to mRNA (7, 8). Finally, the cap helps cells distinguish between self and foreign RNAs (9, 10). Uncapped RNAs are recognized by cellular cytoplasmic pattern recognition receptors, such as retinoic acid-inducible gene I (RIG-I), which subsequently induce expression of a cohort of innate immune response cytokines (i.e., interferons [IFNs]) and antiviral effectors.

Given the importance of the methylguanosine cap, it is not surprising that many viruses have evolved strategies to cap their RNAs. In fact, the discovery of RNA capping came from studies on mammalian orthoreovirus (reovirus) (11–13). Reoviruses are nonenveloped, segmented, double-stranded RNA (dsRNA) viruses that replicate in the cytoplasm. Though reoviruses are nonpathogenic, other members of the family *Reoviridae*, such as bluetongue virus and rotavirus, are pathogens in livestock and humans, respectively. The relatively benign nature of reoviruses, along with the fact that they are preferentially cytotoxic to transformed cells, has led them to become promising contenders for cancer therapy (14, 15). They have exhibited antitumor activity in a wide range of preclinical mouse tumor models (16–20). Furthermore, when administered to patients intratumorally and intravenously, the virus appears to have limited side effects and to possess antitumor activity (21–28). A deep molecular understanding of reovirus replication, including the mechanisms and consequences of capping, is still being sought.

Uniquely among mammalian viruses, reoviruses do not fully uncoat when establishing infection. Instead, reoviruses shed their outer capsids to produce transcriptionally active core particles that synthesize and secrete capped positive-sense RNA (+RNA) into the cytoplasm. Reoviruses encode their own capping enzymes that recapitulate the activities of the host capping machinery. An RNA 5' triphosphatase (RTPase) first hydrolyzes the γ -phosphate of the 5' mRNA nucleotide to produce diphosphate RNA (ppNp-RNA). A guanylyltransferase (GTase) transfers an inverted GMP to the RNA 5' end, forming GpppNp-RNA. The guanosine moiety is then methylated at position N7 by a methyltransferase (N7-MTase), producing the cap-0 basic structure (m⁷GpppNp). Subsequent 2'-O methylation of the first, or first and second, RNA 5' nucleotide by nucleoside-2'-O-methyltransferases (2'OMTases) generates cap-1 (m⁷GpppNm_{2'-O}) and cap-2 (m⁷GpppNm_{2'-O}Nm_{2'-O}) structures, respectively. In order to avoid recognition by RIG-I and induction of antiviral signaling, mRNA must possess at least the cap-1 structure (29). By recapitulating the host capping structure, reovirus RNA may acquire advantages, such as enhanced stability, efficient translation, and diminished antiviral signaling. However, it remains unclear to what extent mRNA capping is important for each of these processes and whether RNA capping confers additional roles and benefits during reovirus replication.

To determine the roles of RNA capping during reovirus infection, we took advantage of the existing cap-independent reverse genetics system for producing reovirus (30–36) and characterized the impact of adding a cytosolic capping enzyme. The reverse genetics system consists of plasmids that carry all 10 reovirus genes under the control of a T7 promoter (T7p). Synthesis of reovirus RNA in the cytoplasm is mediated by a T7 RNA polymerase (T7RNAP) that is either stably integrated into baby hamster kidney (BHK) cells or cotransfected alongside the reovirus plasmids. The system is presumed to generate uncapped reovirus +RNAs that function as templates for synthesis of all reovirus proteins. The following steps are then presumed to recapitulate normal reovirus infection (37). The 10 genomic +RNAs are assembled into progeny reovirus cores comprised of the λ 3 RNA polymerase, μ 2 polymerase cofactor, λ 1 and σ 2 inner

capsid proteins, and $\lambda 2$ reovirus capping enzyme. Progeny cores are transcriptionally active and synthesize negative-sense RNA (–RNA) within the core to generate dsRNA genomes. Moreover, dsRNA then serves as a template for transcription, capping, and secretion of *de novo* reovirus capped RNA (m7G' +RNA) into the cytoplasm, thereby causing intracellular amplification of reovirus replication. Finally, reovirus cores are coated with outer capsid proteins $\mu 1C$, $\sigma 3$, and $\sigma 1$ to produce fully assembled infectious virions. Given the presumed importance of RNA capping for viruses and hosts, it was surprising that the reovirus reverse genetics system was effective despite being deficient in RNA capping.

To introduce cytoplasmic reovirus +RNA capping capacity into the reverse genetics system, we exploited the African swine fever virus (ASFV) capping enzyme. ASFV is a large double-stranded DNA virus in the family *Asfarviridae* that replicates in the cytoplasm and therefore cannot depend on cellular nuclear capping enzymes. Similar to other cytoplasmic large DNA viruses, like poxviruses, ASFV encodes its own capping enzyme, called NP868R. Bioinformatics annotation and empirical analysis previously showed that the single-subunit protein NP868R has all three enzymatic activities required for cap-0 synthesis, including RTPase, GTase, and N7-MTase, but not 2'OMTase activity for cap-1 or cap-2 production (38). We confirmed the overall capping enzymatic activity of recombinant NP868R protein using a nonradioactive enzymatic assay and found NP868R to be $85.2\% \pm 1.2\%$ efficient at capping mRNAs relative to the well-studied vaccinia virus capping system (reference 39 and unpublished results), as well as the GTase and N7-MTase activities of NP868R, using standard biochemical assays (unpublished results). Accordingly, NP868R and T7RNAP were cloned into eukaryotic expression plasmids either alone or in various combined configurations. For example, NP868R and T7RNAP were expressed as independent proteins, as a fusion protein, or with leucine zipper dimerization domains. The effects of capping on +RNA stability, protein expression, and subsequent steps of reovirus replication were then examined.

Our studies showed that when expressed with T7RNAP, NP868R increased the protein expression levels of T7p-driven genes by 6 to 12 times relative to T7RNAP alone. The T7RNAP-NP868R fusion protein, abbreviated C3P3 (chimeric cytoplasmic capping-prone phage polymerase), also increased reovirus titers 100-fold when incorporated into the reverse genetics system. To determine which step(s) of reovirus production benefits from RNA capping and to decipher the kinetics of the reverse genetics system, a time course analysis was conducted for 1 to 5 days after transfection of reovirus reverse genetics components in the presence of T7RNAP alone or C3P3. Neutralization of extracellular virions using reovirus antibodies was done to establish the time point of primary versus subsequent rounds of infection and showed that reverse genetics-driven virion production became saturated by day 2 or 3, with subsequent days serving to amplify reovirus rescue through cell-cell dissemination. Quantitation of RNA and protein levels, genomic double-stranded RNA production, and reovirus titers revealed that by day 2 posttransfection, C3P3 promotes reovirus protein expression by 2 to 4 times. The outer capsid $\mu 1$ was also processed to $\mu 1C$ at a higher rate, suggesting more efficient reovirus assembly. However, production of infectious virions was increased by ~ 60 times, suggesting that RNA capping confers advantages beyond enhancing protein expression and protein assembly. An approach was developed to directly test if capped RNA affects genome incorporation in progeny. The results suggested that the inclusion of C3P3 promotes ~ 6 -fold-higher efficiency of RNA incorporation into progeny virions than T7RNAP alone. These studies present new roles for capping during virus replication and a new strategy to increase the efficiency of reovirus reverse engineering.

RESULTS

The African swine fever virus capping enzyme NP868R promotes expression of the green fluorescent protein (GFP) reporter gene from a T7 RNA polymerase-driven plasmid. The reovirus reverse genetics system employs plasmids carrying all 10 reovirus genes under the control of a T7 promoter. T7RNAP is the prototypical

Autographivirinae bacteriophage polymerase and provides the advantage of a defined 5' sequence, which is critical for conserving the authentic sequence of reovirus +RNA. To generate authentic 3' ends, a ribozyme sequence was incorporated downstream of all reovirus genes. The original reverse genetics system consisted of 10 individual plasmids, each carrying one reovirus gene (36). The reverse genetics system then underwent several iterations, including the convergence of the 10 reovirus genes into 4 plasmids (35) and the plasmid-encoded expression of T7RNAP (34). Given that T7RNAP produces RNA devoid of a methylguanosine cap (40), we sought to determine the impact of a cytosolic capping enzyme on reovirus replication and the efficiency of reverse engineering.

Both T7RNAP and the ASFV NP868R capping enzyme were cloned individually into eukaryotic cytomegalovirus (CMV) promoter-driven plasmids (Fig. 1A). NP868R was also cloned upstream of T7RNAP as a fusion protein with a flexible (G₄S)₄ linker (C3P3). As a negative control, C3P3 was modified to contain a lysine-to-asparagine mutation at amino acid 282, which is predicted to suppress GTase activity based on homologous domains found in the vaccinia virus capping enzyme D1 (41). To easily monitor the effects of these plasmids on T7 promoter-driven proteins, we also cloned enhanced green fluorescent protein (EGFP) into the reverse genetics plasmid that normally carries the reovirus S4 gene, specifically replacing nucleotides 112 to 769 of the 1,196-nucleotide S4 gene with EGFP. The final T7p-GFP plasmid was dependent upon T7RNAP for transcription. Transcripts contained authentic reovirus 5' and 3' untranslated regions (UTR) and the hepatitis delta virus ribozyme to generate authentic reovirus 3' ends devoid of a poly(A) tail. Translation produced EGFP fused to the first 29 amino acids of the S4-encoded σ 3 protein at the N terminus. Finally, EGFP under the control of a CMV promoter was used as a positive control for nuclear transcription and capping (pEGFP). Plasmids were transfected into BHK-T7, BHK-21 (the parental BHK cell line devoid of T7RNAP), human embryonic kidney (HEK293T) cells, or monkey kidney (COS-7) cells using Mirus TransIT-LT1 transfection reagent (Mirus, Madison, WI) and were monitored for green fluorescence at 48 h posttransfection (hpt) by microscopy or flow cytometry.

As anticipated, transfection of pEGFP into BHK-T7 cells resulted in an abundance of green fluorescent cells (Fig. 1B). When T7p-GFP was transfected with pcDNA3, very few fluorescent cells were visible, suggesting that the stably expressed T7RNAP was minimally effective at EGFP expression. Transfection of T7p-GFP with either T7RNAP or NP868R alone caused a notable increase in EGFP expression, albeit still lower than that of nuclear CMV-driven pEGFP. Strikingly, expression of EGFP from the T7p-driven plasmid was drastically increased by cotransfection of C3P3. Our *in vitro* analysis showed that a K282N mutation in the C3P3 enzymatic domain prevents C3P3 capping activity (unpublished data). The C3P3 construct with a K282N capping inactivation mutation failed to promote EGFP expression, demonstrating that the contribution of NP868R was capping dependent. These findings suggest that both elevated transcription and mRNA capping are important for high cytoplasmic T7-driven expression of EGFP.

To quantitatively assess the effects of transcription and capping on levels of T7p-driven EGFP expression, we used flow cytometric analysis for green fluorescence following transfection of T7p-EGFP with T7RNAP, NP868R, C3P3, or inactive C3P3 (Fig. 1C). The proportion of green-fluorescing cells (GFP⁺) is presented relative to C3P3 (set to 100%). The intensity of fluorescence per cell was also quantified as mean fluorescence intensity (MFI) and presented relative to C3P3 (set to 100%). T7RNAP and NP868R both increased the proportion of GFP⁺ cells, as well as the MFI, when transfected with T7p-GFP relative to a pcDNA3 negative control (Fig. 1D). C3P3 produced the largest increase of GFP expression and was dependent on functional capping activity, as demonstrated by loss of expression by the inactive (K282N) C3P3. The experiments were then repeated in BHK21 (Fig. 1E), HEK293T, and COS-7 (Fig. 1F) cells, all of which are devoid of stably expressed T7RNAP. Not surprisingly, NP868R capping enzyme did not promote GFP expression in the absence of T7RNAP. T7RNAP alone did marginally

FIG 1 The African swine fever virus capping enzyme, NP868R, promotes expression from T7 RNA polymerase-driven plasmids. (A) Diagram of constructs used for panels B to F. (B) BHK-T7 cells were either untransfected or cotransfected with two plasmids, as indicated. The cells were assessed for green fluorescence (GFP) by microscopy at 48 hpt. Similarity in cell density and health was confirmed by DIC microscopy. (C to F) Cells were cotransfected with T7p-GFP and a secondary plasmid as indicated. Live cells were assessed by flow cytometry at 48 hpt. The proportion of GFP⁺ cells was measured with a marker set to generate 1 to 2% GFP⁺ cells for the pcDNA3 negative control. MFI was calculated based on a marker that spanned the entire spectrum. The proportion of GFP⁺ cells and the MFI were graphed relative to C3P3-transfected cells, which were set to 100% to standardize among experiments. Experiments were conducted in BHK-T7 (C and D), BHK21 (E), and HEK293T and COS-7 (F) cells. (D and E) One-way analysis of variance (ANOVA) was used with Bonferroni's multiple-comparison test relative to C3P3. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significant; $n = 3$. The error bars indicate standard deviations (SD).

increase GFP expression. However, in all cases, the combination of T7RNAP and NP868R as the C3P3 fusion protein produced the greatest expression of GFP. Together, these results demonstrate that inclusion of cytoplasmic mRNA capping promotes T7 promoter-driven gene expression.

The African swine fever virus capping enzyme NP868R is associated with reduced mRNA levels but a 10-fold increase in protein expression from T7 RNA polymerase-driven plasmids. Having found that NP868R capping enzyme can promote expression of the T7 promoter-driven GFP reporter gene, we next sought to determine if cytoplasmic mRNA capping would also promote expression of reovirus proteins from T7p-driven reverse genetics plasmids. Moreover, since reverse engineering of reovirus requires transfection and expression from multiple plasmids, we assessed the effects of capping on simultaneous expression of GFP, reovirus $\sigma 3$ outer capsid protein encoded by the S4 gene, and reovirus $\mu 1$ outer capsid protein encoded by the M2 gene. Either pcDNA3, T7RNAP, or C3P3 was cotransfected into BHK-T7 cells alongside the three T7p-driven genes. DNA extraction and quantitative PCR (qPCR) showed similar transfection levels of T7-driven plasmids in all cases (Fig. 2A). Western blot analysis showed that transfection of T7RNAP marginally increased levels of GFP, $\sigma 3$, and $\mu 1$ proteins relative to pcDNA3 (Fig. 2B). Addition of the T7RNAP-NP868R fusion protein C3P3 resulted in a notable increase of GFP, $\sigma 3$, and $\mu 1$ proteins relative to T7RNAP alone.

In addition to promoting protein expression, capping of mRNA has been associated with extended mRNA stability. We therefore assessed whether C3P3 increased the levels of both mRNA and proteins using quantitative assays over 3 or 4 independent experiments. Inclusion of C3P3 produced 10.1 ± 4.8 , 6.5 ± 1.2 , and 11.3 ± 2.4 times more $\sigma 3$, $\mu 1$, and GFP proteins, respectively, than T7RNAP (Fig. 2B and C). Surprisingly, levels of S4, M2, and GFP mRNAs were decreased in C3P3-expressing cells relative to T7RNAP-transfected cells (Fig. 2D). The finding that mRNA levels are higher in T7RNAP- than in C3P3-transfected cells might reflect a decreased processivity of transcription by T7RNAP when fused to the NP868R capping enzyme. Given that C3P3 is a fusion protein between T7RNAP and NP868R, a possible explanation for our observation is that NP868R interferes with T7RNAP transcription in the fusion protein construct. As for why we did not see mRNA accumulation associated with C3P3, as would be expected if mRNA stability was promoted by capping, there are multiple possibilities, which are discussed below in the Discussion section. Nevertheless, the results clearly demonstrate a consistent increase in protein expression levels associated with C3P3-mediated capping.

The African swine fever virus capping enzyme NP868R promotes reovirus production from the T7 RNA polymerase-driven reverse genetics system 100-fold.

The existing reverse genetics system for reovirus does not include a capping enzyme, so we next assessed if cytoplasmic mRNA capping could promote production of reovirus particles in the reverse genetics approach. In 2007, Kobayashi et al. (36) developed the plasmid-based reverse genetics system for reovirus, which consisted of the 10 reovirus gene sequences cloned between a 5' T7RNAP promoter and a 3' ribozyme sequence. Then, in 2010, Kobayashi et al. (35) advanced the efficiency of virus rescue by combining the 10 reovirus genes into 4 plasmids, thereby increasing the probability of cotransfection. In 2014, Komoto et al. (34) showed that increased expression of T7RNAP using an exogenously transfected expression plasmid could further enhance production of reovirus. Accordingly, we used the four-plasmid reovirus reverse genetics system, transfected into BHK-T7 cells, and assessed the impacts of exogenously transfected plasmids expressing variations of T7RNAP and NP868R in various permutations on the titers of rescued virus.

Relative to the addition of excess T7RNAP alone, transfection of NP868R led to a 10-fold increase in reovirus rescue efficiency (Fig. 3). The benefits of NP868R were dose dependent (i.e., increased titers with increased levels of NP868R plasmid relative to constant levels of reovirus genome plasmids) but were saturated under the conditions used for Fig. 3 and subsequent experiments (unpublished data). The combination of T7RNAP with NP868R, when transfected either as individual plasmids (T7RNAP plus

A. Plasmid DNA Levels

B. Protein Levels

C. Protein Levels

D. RNA Levels

FIG 2 The African swine fever virus capping enzyme, NP868R, is associated with reduced mRNA levels but a 10-fold increase in protein expression from T7 RNA polymerase-driven plasmids. BHK-T7 cells were left untransfected (UT); transfected with pcDNA3 only; or transfected with T7p-GFP, T7p-S4, and T7p-M2 in combination with either T7RNAP, C3P3, or pcDNA3, as indicated. (A) Levels of M2, S4, or GFP plasmids were measured by quantitative PCR and are presented relative to T7RNAP-cotransfected conditions. No pairs were significantly different by two-way ANOVA and Tukey's multiple-comparison test ($n = 3$). (B) Western blot analysis for levels of GFP, $\sigma 3$, $\mu 1$, and β -actin proteins. (C) Densitometric analysis for intensities of GFP, $\sigma 3$, and $\mu 1$ proteins standardized to β -actin levels and normalized to T7RNAP-transfected conditions (set to 1). P values relative to T7RNAP were calculated by one-way ANOVA with Dunnett's multiple-comparison test ($n = 4$). ns, $P > 0.05$. (D) Levels of M2, S4, and GFP mRNA were assessed by qRT-PCR. Statistics as for panel C ($n = 3$). Horizontal lines indicate means.

FIG 3 The African swine fever virus capping enzyme, NP868R, promotes reovirus production from the T7 RNA polymerase-driven reverse genetics system 100-fold. BHK-T7 cells were transfected with the four reovirus reverse genetics plasmids that together express all 10 reovirus genes flanked by T7 polymerase and ribozyme sequences. The cells were cotransfected with various combinations of T7 RNA polymerase, NP868R, LZip-T7RNAP, LZip-NP868R, and C3P3, as indicated. Lysates of transfected BHK-T7 cells were then assessed for titers of rescued reovirus by standard plaque assay on L929 mouse fibroblasts. One-way ANOVA and Bonferroni's multiple-comparison test were used to compare conditions for 3 to 8 independent experiments. The error bars indicate SD.

NP868R) or as a fusion protein (C3P3), produced a 100-fold increase in reovirus titers relative to titers with T7RNAP alone. These findings suggest that T7RNAP levels are limiting in BHK-T7. The findings also suggest that when excess T7RNAP is provided through exogenous transfection, NP868R capping enzyme can produce a 100-fold increase in infectious-reovirus production. Since we previously saw a small decrease in mRNA levels when T7RNAP was directly fused to NP868R, we used an alternative strategy to encourage dimerization of T7RNAP and NP868R. Furthermore, each enzyme was cloned with 5' heterodimerization leucine zipper sequences containing four pairs of attractive electrostatic interactions (42). T7RNAP contained the acidic EE1234L sequence (LZIP-T7RNAP), and NP868R contained the basic RR1234L sequence (LZIP-NP868R). Addition of the leucine zipper did not affect the enzyme activities of T7RNAP or NP868R, as indicated by relatively consistent titers with or without the leucine zippers (compare T7RNAP to LZip-T7RNAP). Combining LZIP-T7RNAP and LZIP-NP868R brought about a modest but significant increase in reovirus titers relative to C3P3 or cotransfection of T7RNAP and NP868R on independent plasmids. Specifically, the combination of two zippered enzymes produced 2.5- and 2.6-fold-higher titers than the zipper-free T7RNAP and NP868R enzymes or C3P3 fusion protein, respectively.

During reovirus rescue, the capping enzyme NP868R promotes modest accumulation of reovirus RNA and proteins but greatly enhances the levels of infectious progeny reovirus production. Monitoring of reovirus protein expression in the

presence or absence of NP868R was done at 1 to 2 days posttransfection (Fig. 2), while assessment of titers from reverse genetics was completed at 5 days posttransfection (Fig. 3). It was therefore unclear whether the 10-fold increase in protein production was concomitant with the 100-fold increase in titers. During reovirus reverse genetics, only the initial T7RNAP-transcribed RNAs are uncapped in the absence of a cytoplasmic capping enzyme. Once progeny reovirus cores are assembled, they can produce capped mRNA using the inherent capping activities of reovirus $\lambda 2$ turret proteins (29, 43–46). Furthermore, completely assembled progeny that spread to neighboring cells can also produce capped RNA. Accordingly, it was anticipated that NP868R should contribute to enhancement of reovirus rescue only at the initial steps of first-round progeny core production. However, since the kinetics of reovirus rescue have not yet been described, it was unclear whether NP868R indeed enhanced reovirus progeny at initial stages or also during subsequent rounds of virus amplification. We therefore conducted a time course analysis of reovirus macromolecular synthesis and progeny virus titers over a 5-day time course of reovirus rescue by reverse genetics to assess which stage(s) was promoted by NP868R.

To conduct the time course analysis, BHK-T7 cells were transfected with the four plasmids expressing all 10 reovirus genes flanked by T7p and ribozyme sequences, along with either T7RNAP or C3P3. We also transfected C3P3 with only the S4 and M2 reovirus genes that encode $\sigma 3$ and $\mu 1$, respectively, to establish the baseline levels of virus macromolecule synthesis in the absence of amplification by progeny cores and disseminated virions. Levels of plasmid DNA were determined by quantitative PCR. Viral RNA was quantified following two DNase treatments, reverse transcription, and quantitative PCR. The levels of reovirus proteins were tested by Western blot analysis, and infectious virions were quantified by plaque assay. The levels of S4 plasmid DNA decreased steadily over time (Fig. 4A, left) but remained similar between T7RNAP- and C3P3-transfected cells (Fig. 4A, right). The reovirus structural protein $\mu 1$ was then monitored by Western blot analysis. As $\mu 1$ assembles into mature virions, it undergoes processing to $\mu 1C$ (47–52). To establish the time point at which rescued reovirions disseminate to neighboring cells, we monitored $\mu 1$ plus $\mu 1C$ levels at 1 to 5 days posttransfection in the presence or absence of rabbit polyclonal anti-reovirus antibodies previously demonstrated to neutralize reovirus (53). In the absence of neutralization, $\mu 1$ and $\mu 1C$ levels rose drastically at 4 to 5 days posttransfection relative to samples devoid of neutralizing antibodies (Fig. 4B). These results therefore suggest that limited dissemination occurs within the first 3 days of reovirus rescue.

The levels of synthesis of reovirus proteins in T7RNAP- and C3P3-cotransfected cells were then compared (Fig. 4C). When only S4 and M2 reovirus genes were transfected with C3P3, expression of $\mu 1$ fell drastically at day 3 (Fig. 4C, i). Furthermore, $\mu 1$ was not processed to $\mu 1C$, suggesting the absence of progeny virion assembly, as expected. Transfection of T7RNAP with all reovirus genes looked similar to transfection with only S4 and M2 (Fig. 4C, ii, left), except that $\mu 1$ was effectively processed to $\mu 1C$, indicative of virus assembly (Fig. 4C, iii, left). Transfection of C3P3 with all reovirus genes resulted in increasing levels of $\mu 1$ (Fig. 4C, ii, left), as well as processing of $\mu 1$ to $\mu 1C$ (Fig. 4C, iii, left).

Over four independent experiments done in duplicate, there was variation in the levels of $\mu 1$ and processing to $\mu 1C$, but the trends and relative differences among samples expressing C3P3 versus T7RNAP seemed consistent. With regard to total virus protein expression, C3P3 brought about 1.3 ± 0.4 -, 2.5 ± 1.5 -, and 4.2 ± 0.4 -fold-higher $\mu 1/\mu 1C$ levels than T7RNAP at days 1, 2, and 3, respectively (Fig. 4C, ii, right), with significance reached only at day 3 ($P < 0.0001$). The increased protein expression by C3P3 was especially noteworthy, considering that reovirus mRNA levels were approximately 4-fold lower in C3P3-transfected cells than in T7RNAP-transfected cells (Fig. 4D), as was observed and discussed for Fig. 2. The inability to detect increasing reovirus transcripts until 4 to 5 days posttransfection likely reflects the high abundance of transcripts made from DNA plasmids relative to the small and slowly escalating transcripts made from progeny reovirus. However, taking into consideration the relative

A. Reovirus Plasmid DNA

B. Cell-Cell Amplification

C. Reovirus Protein

D. Reovirus mRNA

E. Reovirus Titers

FIG 4 NP868R capping enzyme promotes modest accumulation of reovirus RNA and proteins but greatly enhances the levels of progeny reovirus production. BHK-T7 cells were transfected with T7 RNA polymerase (T7) or C3P3, along with the four reverse genetics plasmids that carry all 10 reovirus genes. As a control for macromolecular synthesis in the absence of virus rescue, C3P3 was transfected with only the S4 and M2 reovirus genes, with pCDNA3 used to equalize relative plasmid ratios. Lysates were collected at 1 through 5 days posttransfection (D.P.T.). All plots of absolute levels (A and C, left, and D and E, top) are plotted as means $\pm$ SD for 3 independent experiments. All plots for relative levels between T7 or C3P3 conditions (A and C, right, and D and E, bottom) show means and *P* values above each time point as analyzed by column statistics and one-sample *t* tests relative to the hypothetical value of 1. Similarly filled or open symbols represent duplicates of 3 independent experiments. (A) Levels of S4 plasmid were quantified by qRT-PCR and plotted as means and SD for each condition (left) or as relative plasmid levels between T7 and C3P3 conditions (right). (B) Reovirus rescue with C3P3 and all reovirus genes was conducted in the presence or absence of neutralizing antibodies in the media of BHK-T7 cells. Cell lysates collected at 1 to 5 days were subjected to Western blot analysis with reovirus polyclonal and β -actin-specific antibodies. The locations of reovirus structural proteins μ 1 and μ 1 cleavage to produce μ 1C (encoded by M2), σ 3 (encoded by S4), and β -actin loading control are indicated. (C) Levels of reovirus proteins μ 1, μ 1C, and σ 3 were also evaluated, in the absence of neutralizing antibodies, among the three conditions (T7 plus all reovirus segments, C3P3 plus all reovirus segments, and C3P3 plus S4 and M2). (i) Western blot analysis as for panel B showing one duplicate each from two independent experiments.

(Continued on next page)

levels of mRNA, our results suggest that capping by C3P3 promotes protein synthesis approximately 5- to 10-fold within the first 2 days posttransfection.

With regard to $\mu 1$ processing to $\mu 1C$, C3P3 brought about 1.0 ± 0.5 , 1.6 ± 0.3 , and 1.2 ± 0.1 -fold-higher processing than T7RNAP at days 1, 2, and 3, respectively (Fig. 4C, iii, right). Differences in $\mu 1$ -to- $\mu 1C$ processing were small but significant at days 2 and 3. These results suggest that capping by C3P3 also improves the probability of virion assembly.

C3P3 had the most drastic effects on reovirus titers relative to T7RNAP (Fig. 4E). During analysis, when titers were below the level of detection (1×10^2 PFU/ml), we conservatively set them to 1×10^2 PFU/ml to favor T7RNAP. Nevertheless, at 2 days posttransfection, despite only 2.5-fold-higher protein levels and 1.6-fold-higher $\mu 1$ to $\mu 1C$ processing, titers were 45-fold higher in C3P3- than in T7RNAP-transfected cells. At 3 to 5 days, when dissemination occurs (Fig. 4B), titers in both C3P3 and T7RNAP increased at a similar rate. These findings suggest that capping by C3P3 promotes production of the initial reovirus progeny. Given that the rescued initial progeny can then produce capped RNA, it is not surprising that once new virions are produced, the rate of amplification is similar.

Since uncapped RNA can also induce antiviral signaling, which would then affect protein synthesis and virus replication, we also measured mRNA levels for type I interferons and interferon-stimulated genes in reovirus-infected BHK-T7 cells, using primers designed based on orthologous antiviral proteins in the hamster genome. Although glyceraldehyde-3-phosphate dehydrogenase (GAPDH) could easily be detected by qPCR (cycle threshold, ~ 15), we could not detect beta interferon (IFN- β), IFIT3, RIG-I, IFITM, IFIT2, ISG15, and IFN- $\sigma 9$, and no change was found during infection for ISG20 and Cxcl (data not shown). These findings are in agreement with previous studies showing absence of antiviral signaling in BHK cells (54). It is therefore unlikely that interferon-mediated antiviral responses contribute to differences in reovirus rescue in BHK-T7 cells.

Capped RNAs have a higher probability of transfer through infectious virions.

Inclusion of NP868R capping enzyme increased infectious-reovirus production by over 10 times more than it increased expression of reovirus proteins. We considered two possible explanations for this discrepancy. First, it was possible that a small increase in virus protein levels could lead to a pronounced enhancement in progeny assembly, for example, through cooperation of proteins during assembly. This possibility is supported by the increased proportion of $\mu 1$ that was processed to $\mu 1C$ in C3P3-transfected cells, albeit the difference in $\mu 1$ -to- $\mu 1C$ processing was not large. The second possible explanation was that the capping of virus RNA had an additional advantage downstream of protein expression that promotes infectious-virus production. For example, capping could promote encapsidation of reovirus +RNA or transcription of positive-to-negative RNA within assembled virions.

To address the possibility that capping promoted capture of RNA into progeny or transcription to dsRNA, we developed an assay to monitor the frequency at which capped versus uncapped RNA was incorporated into infectious virions independently of protein levels. We constructed reporter plasmids that contained 3' and 5' regions from the reovirus S1 genome segment (Fig. 5A and B). The constructs comprised the sialic-acid binding N-terminal half of the $\sigma 1$ cell attachment protein ($\sigma 1$ -N), which was previously shown to be sufficient for producing reovirus that can replicate in cell culture (55, 56). $\sigma 1$ -N was followed by the small UnaG fluorescent protein (57), either

FIG 4 Legend (Continued)

(ii) Levels of total $\mu 1/\mu 1C$ quantified by densitometry and plotted as means $\pm$ SD for each condition (left) or as relative protein levels between T7 and C3P3 conditions (right). (iii) Levels of $\mu 1$ -to- $\mu 1C$ processing formulated as $100 \times [\mu 1C/(\mu 1 + \mu 1C)]$ and plotted as means $\pm$ SD for each condition (left) or as relative protein levels between T7 and C3P3 conditions (right). (D) Levels of S4 RNA quantified by qRT-PCR and plotted as means and SD for each condition (top) or as relative plasmid levels between T7 and C3P3 conditions (bottom). (E) Reovirus titers quantified by plaque assay on L929 cells and plotted as means and SD for each condition (top) or as relative plasmid levels between T7 and C3P3 conditions (bottom).

FIG 5 Reovirus RNAs are encapsidated more efficiently when coupled with NP868R-mediated capping. (A) Reovirus S1 genome segment modified to express the UnaG green fluorescent protein as a fusion protein with the N-terminal half of $\sigma 1$ (Ronin construct) or as an independent protein alongside $\sigma 1$ -N (UnaG construct). Both the Ronin and UnaG constructs were used to produce recombinant reovirus through reverse genetics, and the microscopy images show that the two recombinant viruses could produce green fluorescence. (B) Diagram of the experiment performed to generate the data for panels C and D. BHK-T7 cells were transfected with pcDNA3, T7RNAP, or C3P3 and either the UnaG or Ronin reporter plasmid for 1 to 2 days and were then infected with wild-type reovirus in the presence or absence of NOC. One, 2, or 3 days postinfection, the transfected/infected BHK-T7 lysates were used to infect Ras-transformed NIH 3T3 mouse fibroblasts (RasT) and were assessed for UnaG RNA and fluorescence. (C) (Top) Expression of UnaG in RasT cells was assessed using qRT-PCR for four independent experiments, each containing infection with wild-type reovirus and nocodazole, at 1 or 2 days (d) posttransfection, as indicated. (Bottom) Reovirus S4 transcript levels were monitored to determine if the UnaG or Ronin reporter construct affected reovirus replication. RNA levels under pcDNA3 versus T7RNAP and C3P3 versus T7RNAP conditions were compared using two-way ANOVA and Tukey's multiple-comparison posttest. (D) Silver staining confirmed that reovirus particles were successfully purified from BHK-T7 cells transfected with pcDNA3 (P), T7RNAP (T), or C3P3 (C) and either mock or reovirus infected, as indicated. One example lysate (E.g. Lysate) of C3P3-transfected and reovirus-infected BHK-T7 cells before purification is provided to demonstrate the extent of purification. The locations of reovirus major structural proteins $\mu 1$ and $\sigma 3$ are indicated. (E) RasT cells treated with purified virions from BHK-T7 cells transfected with pcDNA3, T7RNAP, or C3P3 and either mock or reovirus

(Continued on next page)

with a 2A self-cleaving peptide sequence to make σ 1-N and UnaG as separate proteins (UnaG) or as a σ 1-N–UnaG fusion protein (Ronin). Similar to the reverse genetics plasmids with reovirus genes, UnaG and Ronin were flanked by T7RNAP and ribozyme sequences to produce authentic reovirus 3' and 5' ends. We successfully used UnaG and Ronin to generate recombinant reoviruses by reverse genetics, which produces diffuse cytoplasmic UnaG staining or punctate staining, respectively (Fig. 5A). The ability of UnaG and Ronin to replace S1 and produce functional virions by reverse genetics shows that both UnaG and Ronin constructs are capable of incorporating into reovirus genomes.

We next applied UnaG and Ronin as reporter constructs for packaging efficiency (Fig. 5B). First, we transfected either the UnaG or Ronin plasmid, along with a pcDNA3 control, C3P3, or T7RNAP, into BHK-T7 cells. BHK cells would therefore be primed with either capped (C3P3) or uncapped (T7RNAP or pcDNA3) UnaG or Ronin transcripts. Next, at 48 h posttransfection, BHK cells were infected with wild-type reovirus. The wild-type reovirus did not express UnaG but would produce all 10 reovirus transcripts in capped form and all reovirus proteins necessary to generate progeny. As wild-type reovirus replicated in the BHK-T7 cells, new progeny had the option of incorporating wild-type genomic segments, but also the capped or uncapped UnaG-containing transcripts. Note that with *Reoviridae*, transcription of positive- to negative-sense RNA (genome replication) occurs only within newly assembled progeny cores. Accordingly, UnaG/Ronin reporter RNAs would amplify only if they were packaged in progeny. We also performed the transfection/infection in the presence or absence of nocodazole (NOC), which has been previously shown to affect microtubules and virus factory formation (58). We reasoned that nocodazole might promote access of UnaG and Ronin transcripts to viral factories.

Finally, to determine if UnaG or Ronin mRNA was in fact incorporated into progeny, lysates of transfected/infected BHK-T7 cells (medium plus cells) were added to RasT cells that support high reovirus infection. Following 1 h at 37°C, the cells were washed extensively, incubated for 20 h, and assessed for expression of UnaG, by either quantitative reverse transcription (qRT)-PCR or flow cytometry. Higher levels of UnaG transcripts were detected under C3P3- versus T7RNAP-transfected conditions by qRT-PCR, regardless of the presence of NOC or the use of UnaG or Ronin or whether BHK-T7 lysates were collected at 1 day, 2 days, or 3 days postinfection (Fig. 5C, top). Importantly, UnaG and Ronin reporter constructs, whether expressed with T7RNAP or C3P3, did not have any positive or negative effect on reovirus replication, as assessed by monitoring expression of reovirus S4 transcript levels (Fig. 5C, bottom). To confirm that delivery of the UnaG gene to RasT cells was mediated by virions rather than other components of BHK lysates, we used our previously described method (59) to purify small quantities of reovirus (Fig. 5D). When added to RasT cells, virions produced in the presence of capped UnaG or Ronin transcripts led to green fluorescence that was easily visible by microscopy, so we conducted flow cytometric analysis (Fig. 5D). Consistently, RasT cells expressed UnaG when exposed to virions purified from C3P3- but not T7RNAP-transfected BHK-T7 cell lysates. Since C3P3 produced lower RNA levels than T7RNAP (Fig. 2D and 4D) and because protein levels of UnaG or Ronin did not affect infectious-reovirus production (Fig. 5C), these results support a role for RNA capping in the capture of RNA into progeny and/or transcription to dsRNA.

DISCUSSION

The contributions of RNA capping to mRNA stability, protein translation initiation, and antiviral signaling are well characterized. While capping was first discovered in reoviruses, the precise role of RNA capping during reovirus replication has not been

FIG 5 Legend (Continued)

infected (as indicated) were subjected to flow cytometric analysis to measure levels of UnaG protein fluorescence. FITC, fluorescein isothiocyanate. (F) Results from three independent experiments similar to that in panel E using either purified virions (black symbols) or unpurified lysates (red symbols).

FIG 6 Model in which RNA capping promotes reovirus protein expression and, most notably, encapsidation of reovirus RNA to produce infectious virions.

evaluated. To address which steps of reovirus infection benefit from RNA capping, we determined the levels of reovirus RNA, proteins, $\mu 1$ -to- $\mu 1C$ processing during virus assembly, and infectious-virion titers during reverse genetics reovirus rescue in the presence or absence of the NP868R cytoplasmic capping enzyme (Fig. 6). As anticipated, the results demonstrated that capping of reovirus mRNA promotes protein expression, but what was unanticipated was that inclusion of the cytoplasmic capping enzyme NP868R increased infectious-virion titers over 10 times more than it increased the levels of reovirus proteins. Both an indirect assessment of reovirus rescue kinetics and a more direct approach to monitor incorporation of exogenous UnaG-expressing mRNA into reovirus progeny suggested that capped RNAs are more successfully associated with infectious virions.

It was previously demonstrated that positive strands of reovirus genomes bear a 5' cap (60), but it was not known whether the capped status of genomes was a happenstance of having capped RNAs available to package or rather that capping was directly involved in genome production; our findings now suggest the latter. One explanation for our results is that capped mRNAs are more efficiently encapsidated. The reovirus polymerase protein $\lambda 3$ was previously demonstrated to have cap binding capacity (61), and therefore cap-mediated binding of reovirus RNA by $\lambda 3$ could facilitate assembly of RNA within progeny. Alternatively, it is also possible that reovirus incorporates RNA in a cap-independent manner but that the cap is required for synthesis of negative-sense RNA within progeny cores. This explanation is congruent with suggested template retention mechanisms of transcription by $\lambda 3$ (61). In either scenario, progeny viruses in the absence of NP868R would be less infectious because they would have fewer positive-sense RNA (first explanation) or fewer negative-sense RNA (second explanation) complements. The challenge in distinguishing between these possibilities is that reovirus undergoes secondary replication within infected cells, where newly assembled cores contribute capped RNAs that amplify replication. Therefore, it is hard to conduct direct biochemical assessments, such as measuring positive- and negative-sense RNAs in virions, without having dominance by-products of secondary replication. However, regardless of the explanation, this study suggests a new functional role for RNA capping in virus production.

Addition of NP868R also increased the proportion of $\mu 1$ that was processed to $\mu 1C$. Reovirus cores are composed of $\sigma 2$ and $\lambda 1$ inner capsids, with $\lambda 2$ pentameric turrets at each vertex. The $\lambda 3$ polymerase and $\mu 2$ cofactor sit on the inner faces of the cores (37, 62–64). Cores are transcriptionally active, producing capped RNA from dsRNA tem-

plates and secreting the capped RNA through the $\lambda 2$ turrets into the cytoplasm. As cores transition to infectious complete virions, they are coated with outer capsid $\mu 1$ and $\sigma 3$ proteins. Numerous studies show that the 76.3-kDa $\mu 1$ is proteolytically processed to a 72.1-kDa $\mu 1$ C-terminal fragment ($\mu 1C$) and a myristoylated N-terminal fragment ($\mu 1N$) (47–52). The outer capsid proteins $\sigma 3$ and $\mu 1/\mu 1C$ then undergo further proteolysis during infection by gut or lysosomal proteases (47, 65–71), and products of $\mu 1C$ and $\mu 1N$ are involved in virus penetration into the cytoplasm (72, 73). Purified complete virions are predominantly composed of $\mu 1C$, although some $\mu 1$ remains. The finding that higher levels of reovirus protein expression by capped RNA correlates with more $\mu 1$ processing, interpreted here as more assembly, suggests that there might be a threshold of proteins required to form a virion. Considering the need for newly synthesized reovirus proteins to be localized in the same place for assembly, it might be that protein levels produced by T7RNAP are insufficient for efficient assembly and that, simply, 2- to 3-fold more viral proteins increase the probability of synergistic assembly more than 2- to 3-fold. Another possibility is that capped RNA, through an interaction with $\lambda 3$, directly promotes assembly and therefore $\mu 1$ to $\mu 1C$ processing.

Another surprising finding was that, despite an established role for capping in RNA stability (74–76), we did not see higher levels of RNA synthesized in the presence of NP868R capping enzyme. Although we did not follow RNA half-life directly, we did not see any indication of greater RNA accumulation, which is normally associated with extended RNA stability. There are many possible technical or mechanistic explanations for this. First, it is possible that reovirus RNAs have alternative mechanisms to prevent 5′-3′ RNA degradation. For example, reovirus RNAs are not polyadenylated (77, 78) yet are stable relative to traditional nonpolyadenylated mRNAs and are predicted to have alternative mechanisms, such as secondary structure, to promote stability (79). In frog oocytes, however, McCrae and Woodland did see an important contribution of capping for reovirus RNA stability (80). Therefore, another possibility is that BHK cells are defective in 5′-directed degradation. Using *in vitro* assays, BHK cells were previously found to degrade uncapped mRNA rapidly (81), although we cannot exclude the possibility that the BHK cells propagated in different laboratories exhibit distinct 5′ exonuclease abilities. It is also possible that the absence of accumulated RNA in NP868R-transfected cells is simply caused by incomplete capping. In other words, if NP868R capped only a small proportion of RNA, then our assays would reflect the fate of the dominant uncapped-RNA population. Unfortunately, methods for quantifying the efficiency of capping on specific mRNAs *in cellulo* have not been developed. We attempted to quantify the levels of capped versus uncapped reovirus RNAs in our transfected BHK-T7 cells using recombinant eIF4G or anti-cap antibodies (82), but like several colleagues, we were unable to achieve quantitative assessment of cap-dependent pulldown from cell lysates spiked with controls, such as *in vitro*-synthesized capped and uncapped reporter RNAs (unpublished data).

Given the large impact that capping had on reovirus rescue, it seems surprising that the cap-independent reverse genetics system is effective, albeit at 100-fold-reduced levels. The ability of the standard reverse genetics system to work might suggest that, although capping is important for efficient virus rescue, it is not absolutely necessary. For example, it is possible that capped RNAs simply have a much higher efficiency of incorporation into dsRNA genomes, as is supported by a low level of UnaG incorporation (Fig. 5). Such a preference is congruent with findings that purified reovirions possess capped positive-sense RNAs (60). Alternatively, perhaps T7RNAP-transcribed RNAs are modestly capped by cytoplasmic capping enzymes recently characterized in eukaryotic cells (82–85). It would be interesting to determine whether silencing or deletion of cellular cytoplasmic capping enzymes affects reovirus reverse genetics in the absence of exogenously transfected capping enzymes. Given that NP686R produces only cap-0 structures while reovirus produces cap-2 structures, it would also be interesting to determine if further methylation of the first two nucleosides by 2′OMTase

activities of reovirus $\lambda 2$ provides additional benefits to reovirus replication over the cap-0 moiety.

An intriguing observation was made by Zarbl et al. (86) that reovirions purified from cells at late time points of infection and subjected to *in vitro* transcription assay in the presence of capping substrate showed less incorporation of [β - 32 P]GTP on 5' termini of mRNAs. These findings might suggest that reovirus produces uncapped mRNAs late during infection, but such an interpretation has yet to be confirmed in cells. Our findings suggest that, regardless of whether reovirus produces capped or uncapped RNAs, capped RNAs are selectively packaged. This interpretation is supported by previous findings by Goubau et al. that genomic positive-sense RNAs are capped (60). It would be interesting to determine if reovirus does in fact produce uncapped RNAs in infected cells and, if so, whether uncapped RNAs modulate reovirus replication.

Overall, this study introduces new roles for RNA capping during reovirus replication and expands our understanding of how capping contributes to host and virus processes. The reverse genetics system for reovirus rescue has greatly advanced our ability to manipulate reoviruses and to address specific protein structure-function relationships. The discovery that C3P3 promotes reovirus rescue by over 100-fold will enable more efficient and successful production of reovirus recombinants. For example, we now obtain efficient reovirus rescue in 24-well plates rather than the 21-cm² dishes required for cap-independent rescue, which provides large reagent cost savings. Using C3P3, we were able to rescue reovirus expressing the UnaG green fluorescent protein as a nonstructural component that replaces the $\sigma 1$ head domain (UnaG), as was previously achieved with iLOV fluorescent protein (56). For the first time, we were also able to rescue reovirus containing UnaG as a structural component of the virion (Ronin). Inclusion of C3P3 is likely to promote discovery and optimization of reverse genetics systems for other viruses. Finally, reovirus is a promising contender for virus-mediated tumor oncolysis. We and others have found that genetic modification of reovirus can improve oncolytic activity *in vitro* and in animal cancer models (87–91). Reovirus also provides a potential vaccine vector (92, 93). Efficient production of reovirus recombinants will therefore expedite production and testing of genetically modified reovirus for oncolytic and vaccination purposes.

MATERIALS AND METHODS

Cell lines. BHK-T7 (a generous gift from Ursula Buchholz, NIH), HEK293T (American Type Culture Collection [ATCC], Manassas, VA), BHK21 (a generous gift from Deborah Burshtyn, University of Alberta), and COS-7 (a generous gift from Deborah Burshtyn) cells were cultured in high-glucose Dulbecco's modified Eagle medium (DMEM) (Sigma, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS) (Sigma), 2 mM L-glutamine, 100 U/ml penicillin, 100 μ g/ml streptomycin, and 25 ng/ml amphotericin B (Sigma). BHK-T7 cells were further maintained in 1 mg/ml Geneticin (InvivoGen, San Diego, CA) every second passage. L929 cells were cultured in minimum essential medium (MEM) (Sigma) supplemented with 10% FBS, 2 mM L-glutamine, 1% nonessential amino acids (Sigma), 1 mM sodium pyruvate (Sigma), 100 U/ml penicillin, 100 μ g/ml streptomycin, and 25 ng/ml amphotericin B. Ras-transformed NIH 3T3 (RasT) cells (ATCC) were maintained in DMEM with 10% newborn calf serum (Sigma), 2 mM L-glutamine, 1% nonessential amino acids (Sigma), 1 mM sodium pyruvate (Sigma), 100 U/ml penicillin, 100 μ g/ml streptomycin, 25 ng/ml amphotericin B, and 2 μ g/ml puromycin (Thermo Fisher Scientific, Waltham, MA).

Transient transfections. BHK-T7, BHK21, COS-7, and HEK293T cells were grown to approximately 80% confluence and transfected with TransIT-LT1 transfection reagent using twice the recommended DNA and LT1 reagent (Fig. 1 and 2). Twenty-four hours posttransfection, the medium was changed to include antibiotics. Forty-eight hours following transfection, cells were harvested for either RNA, flow cytometry analysis, or Western blot analysis or imaged for fluorescence.

Reverse genetics transfections. Recombinant reoviruses were generated using a plasmid-based reverse genetics system (Fig. 3 and 4) (35). Briefly, one well of a 24-well plate containing 85% confluent BHK-T7 cells was transfected with 4 plasmids carrying the 10 gene segments from T3D reovirus—pT7-L1-S1T3D, pT7-L3-M1T3D, pT7-M2-S2-S3-S4T3D, and pT7-L2-M3T3D (Addgene, Cambridge, MA)—and one plasmid expressing variations of T7RNAP, the capping enzyme NP868R, or both. Three microliters of TransIT-LT1 transfection reagent was used per microgram of plasmid DNA, with a total of 2.25 μ g of DNA added per well. Twenty-four hours posttransfection, the medium was changed to include antibiotics. To determine virus titers, cells were scraped into phosphate-buffered saline (PBS) 1 to 5 days posttransfection and freeze-thawed three times. Reovirus titers were obtained by titration of lysate on L929 cells. Briefly, L929 cells were infected for 1 h under standard growing conditions in serum-free (SF) medium. A 2% agar overlay was then added and maintained on the cells for 4 to 7 days.

TABLE 1 Primer sequences

Primer name	Sequence
T7p-GFP-F	TACGGGTACCTAATACGACTCACTATAG
T7p-GFP-R	TACGCATATGCCGCGGTTTTTTTTTTTTT
Ronin-F	GAATTCGAGCTCGGTACCAG
Ronin-R	GCAGACTCATCCACGGTAG
S1UnaG1-F	GGCGCCTCGCTCAGTTGCGCCTATCCTTG
S1UnaG1-R	CAAGGATAGGCGCAACTGAGCGAGGCGCC
S1UnaG2-F	ATGGTCTTTGTAGTCAGGACCGGGTTTTCTCCACGTCTC
S1UnaG2-R	GAGACGTGGAAGAAACCCCGGTCTGACTACAAAGACCAT
S4-RT-F	GGAACATTGTGAGAGCAGCA
S4-RT-R	GCAAGCTAGTGGAGGCAAGTC
M2-RT-F	ACGATGTCCCCACTATCAGC
M2-RT-R	GATTGCTTCGGCTATCTTCG
mGAPDH-RT-F	TGGCAAAGTGGAGATTGTTGCC
mGAPDH-RT-R	AAGATGGTGATGGGCTTCCCG
haGAPDH-RT-F	AACTTTGGCATTGTGGAAGG
haGAPDH-RT-R	GGATGCAGGGATGATGTTCT
UnaG-RT-F	CCTGGACACCCAAGTGAAGT
UnaG-RT-R	TGCACGTACACCAGCTTCTC

Large-scale virus preparations. T3D reovirus was prepared using L929 spinner cultures, extracted with Vertrel XF (Dymar Chemicals, Mississauga, Ontario, Canada), and purified through a cesium chloride gradient as previously described (87, 94, 95).

Reovirus neutralization. Polyclonal reovirus antibody (a generous gift from Patrick Lee, Dalhousie University) was added at a 1/50 volume, equivalent to cells 24 h after reverse genetics transfection. The medium was subsequently spiked with a 1/50 volume equivalent of reovirus antibody every 48 h until harvest.

Plasmids. The T7p-GFP plasmid was generated by amplifying GFP from the template DNA plasmid peGFP (Addgene) using the T7p-GFP-F and T7p-GFP-R primers (Table 1). The resulting PCR fragment was digested with KpnI and SacII and ligated into the KpnI and SacII sites of plasmid pT7-S4T3D (T7p-S4) (a kind gift from Terry Dermody, University of Pittsburgh).

The Ronin plasmid was designed, and a linear double-stranded DNA fragment was synthesized from GeneArt Strings (Thermo Fisher Scientific). The fragment had a total size of 1,473 nucleotides. The fragment contains the 5' untranslated region of T3D S1 and the first 755 nucleotides from the T3D S1 gene sequence, a 2A self-cleaving peptide sequence (96), a 3× Flag tag, the codon-optimized sequence of UnaG, a small fluorescent protein from the Japanese eel (57), and the final 60 nucleotides from the S1 gene, as well as the 3' untranslated region. The fragment was amplified using primers Ronin-F and Ronin-R (Table 1) and digested with KpnI and SacII. The fragment was then ligated into the KpnI and SacII sites of the pBacT7-S1T3D vector (a generous gift from Terry Dermody). In order to create the plasmid UnaG, the 2A self-cleaving peptide of UnaG was rendered active as a result of several mutations being introduced by site-directed mutagenesis (Agilent, Santa Clara, CA). The mutations were introduced using primers S1UnaG1-F, S1UnaG1-R, S1UnaG2-F, and S1UnaG2-R (Table 1).

The sequences of T7RNAP and NP868R were synthesized and sequence verified by GeneART AG (Regensburg, Germany) (97), amplified by PCR, and cloned into the pCMVScript plasmid (Stratagene, La Jolla, CA) where the T7 ϕ 10 promoter sequence had been removed. The design of each plasmid was the same. They included an IE1 promoter/enhancer from human cytomegalovirus, a 5' untranslated region, a Kozak consensus sequence, the desired coding sequence, a 3' untranslated region, and a simian virus 40 (SV40) polyadenylation signal. T7RNAP with the leucine zipper EE1234L (LZip-T7RNAP), NP868R with the RR1234L leucine zipper sequence (LZip-NP868R), and C3P3 were generated by subcloning using restriction sites within the pCMVScript plasmid (97). The inactive C3P3 with the K-282-N mutation (97) was generated by site-directed mutagenesis (Agilent). The coding sequences for these plasmids were codon optimized for expression in human cells.

Each PCR was completed using the high-fidelity DNA polymerase iProof (Bio-Rad, Hercules, CA) according to the manufacturer's protocol. All ligations were completed with T4 DNA ligase (Thermo Fisher Scientific) as described by the manufacturer. The final plasmids were sequenced (McLab, San Francisco, CA) to ensure no errors were introduced during the cloning process. pcDNA3 was purchased from Addgene. The reovirus vectors pT7-L1-S1T3D, pT7-L3-M1T3D, pT7-M2-S2-S3-S4T3D, and pT7-L2-M3T3D were purchased from Addgene. The reovirus vector pT7-M2T3D (T7p-M2) was a kind gift from Terry Dermody.

Transfection/infection assays. BHK-T7 cells were transfected with pcDNA3, T7 RNA polymerase, or C3P3 and either the UnaG or Ronin construct using 2 times to 3 times the recommended amount of TransIT-LT1 reagent (Mirus) and DNA (Fig. 5). Twenty-four or 48 h posttransfection, BHK-T7 cells were infected with 1×10^5 to 2×10^5 PFU of wild-type reovirus for 1 h in SF medium. Following a 1-h adsorption, the infection medium was removed and replaced with complete BHK-T7 medium. For some samples, 6 h after infection of BHK-T7 cells, the medium was spiked with nocodazole (Sigma) to a final concentration of 10 μ M. The nocodazole was left on the cells for the remainder of the infection. Cells were scraped into the medium and collected between 1 and 3 days postinfection.

RasT cells were infected with BHK-T7-transfected/infected lysates either directly or after purification of the virus. Briefly, for purification of BHK-T7-transfected/infected lysates, samples were freeze-thawed three times, and 500 μ l Vertrel (Dymar Chemicals) was added to each sample. Following 15 min of vortexing, samples were sonicated 3 times for 5 s each time at 30% amplitude and spun for 8 min at 10,000 rpm at room temperature, and the aqueous layer was transferred to a new tube. Following a 30-min treatment with DNase (Thermo Fisher Scientific) at room temperature, another 500 μ l of Vertrel was added, samples were vortexed for 30 min and spun for 15 min at 10,000 rpm, and the aqueous layer was transferred to a new tube. The sample was topped up with $2\times$ SF medium and spun at $108,000\times g$ for 90 min at 4°C (Beckman Coulter 120.1 rotor). The pellet was resuspended in 250 μ l PBS, and a 1/6 volume of a 50% solution of Capto core 700 slurry (Life Sciences, Pittsburgh, PA) was added (59). Samples were passed through an Illustra MicroSpin column (GE Healthcare) at $800\times g$ for 5 min to remove resin. The samples were then transferred to a new tube, and an equal volume of VP serum-free medium (Thermo Fisher Scientific) was added to each. The purity of virions was verified by silver staining (Bio-Rad) according to the manufacturer's directions, and samples were then used to infect RasT cells. The RasT cells were infected with lysates or purified samples for 1 h under standard growing conditions in SF medium. After 1 h, the infection medium was removed, the cells were washed three times in VP serum-free medium, and VP serum-free medium was added to the cells for the duration of the infection. At 20 to 36 h postinfection, the cells were viewed by microscopy and processed for flow cytometry, Western blot analysis, and qPCR.

Microscopy. Forty-eight hours posttransfection, cells were imaged for fluorescence using an EVOS FL Auto microscope (Thermo Fisher Scientific). Differential interference contrast (DIC) was used to confirm cell density and health. Images were assembled using Adobe Photoshop (Adobe, San Jose, CA).

Flow cytometry. Forty-eight hours posttransfection, cells were assessed for fluorescence using flow cytometry. Briefly, cells were detached from the plate using trypsin, collected by centrifugation, washed one time with PBS, and then fixed for 30 min at 4°C with 4% paraformaldehyde (Thermo Fisher Scientific). The cells were then washed with PBS, and cell fluorescence was detected by flow cytometry using an LSR Fortessa cell analyzer (BD Biosciences, Mississauga, Ontario, Canada), and the data were analyzed using FCS Express software (De Novo Software, Los Angeles, CA).

Western blotting. Cells were washed once with PBS and lysed in RIPA buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate with protease inhibitor cocktail [Sigma]) for 20 min at 4°C. The lysates were loaded onto 10% gels after the addition of $6\times$ Western sample loading buffer (375 mM Tris-HCl, pH 6.8, 30% glycerol, 10% sodium dodecyl sulfate, 1.43 M β -mercaptoethanol, and 0.03% bromophenol blue), and proteins were separated by SDS-PAGE and then transferred to a nitrocellulose membrane (Bio-Rad). The membranes were blocked for 1 h at room temperature in blocking buffer (3% bovine serum albumin [BSA] in TBST [50 mM Tris, 150 mM NaCl, 0.05% Tween 20]) and left overnight in primary antibody at 4°C. Primary antibodies included rabbit anti-reovirus to detect μ 1 and σ 3 (1:10,000; a generous gift from Patrick Lee, Dalhousie University), mouse monoclonal anti-GFP antibody (1:5,000; Covance, San Diego, CA), and mouse monoclonal anti- β -actin (1:5,000; Santa Cruz, Dallas, TX) to control for loading. Secondary antibodies were left on for 1 h at room temperature. A secondary antibody conjugated to Alexa Fluor 488 or 647 (1: 3,000; Jackson ImmunoResearch, West Grove, PA) was used to detect GFP and β -actin. A secondary antibody conjugated to horseradish peroxidase (1:10,000; Jackson ImmunoResearch) was used, along with a chemiluminescence substrate (ECL 2; Thermo Fisher Scientific) to detect reovirus proteins. Blots were imaged using an ImageQuant LAS 4010 imager (GE Healthcare Life Sciences), and analysis was performed using ImageQuant TL software (GE Healthcare Life Sciences).

RNA extraction and qRT-PCR analysis. RNA was extracted using TRI reagent (Sigma) and total RNA purification spin columns (GenElute mammalian total RNA miniprep kit; Sigma) as described by the manufacturer's protocol. An extra DNase step (Thermo Fisher Scientific) was included prior to loading the columns if a plasmid DNA transfection occurred prior to RNA purification. Moloney murine leukemia virus (MMLV) reverse transcriptase (Thermo Fisher Scientific), along with random primers, sense-specific GAPDH primers, and reovirus S4 or M2 gene-specific primers, was used to synthesize cDNA. The cDNA was used as a template for qPCR. qPCR was performed using Sybr Select master mix (Thermo Fisher Scientific) according to the manufacturer's directions using a CFX96 real-time system (Bio-Rad). The primers used for qPCR synthesis are described in Table 1 (the qPCR primers contain RT in their names). Data were analyzed using CFX Manager software (Bio-Rad), and standard curves were generated as previously described (98).

Accession numbers. The linear double-stranded DNA fragment was submitted to GenBank under accession number [KY496630](#).

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