Transcriptional Activities of Reovirus RNA Polymerase in Recoated Cores

INITIATION AND ELONGATION ARE REGULATED BY SEPARATE MECHANISMS*

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Diane L. Farsetta‡§, Kartik Chandran$§**, and Max L. Nibert‡§†††

From the ‡Department of Biochemistry, $§Institute for Molecular Virology, and ¶¶Cell and Molecular Biology Program, University of Wisconsin-Madison, Madison, Wisconsin 53706

The particle-associated reovirus polymerase synthesizes mRNA within only certain viral particle types. Reovirus cores, subviral particles lacking outer capsid proteins μ1, σ3, and σ1, produce mRNA and abortive transcripts. Reovirus virions, which contain complete outer capsids, cannot produce mRNA and produce few abortive transcripts. Recoated cores are virion-like particles generated by the addition of recombinant outer capsid proteins to cores. We used recoated cores to analyze transcriptional regulation by reovirus outer capsid proteins. Partially recoated particles, containing less than virion amounts of μ1 and σ3, synthesized mRNA at levels inversely proportional to outer capsid protein levels. Fully recoated cores exhibited undetectable mRNA synthesis levels, as did virions. However, recoated cores produced high levels of abortive transcripts. Recoated core abortive transcripts remained particle-associated and appeared to inhibit further abortive transcript production. Proteolysis of recoated cores removing μ1 and σ3 released accumulated abortive transcripts and relieved inhibition of mRNA and abortive transcript synthesis. These results suggest transcriptional elongation, but not initiation, is blocked by virion-like amounts of μ1 and σ3. Particle-associated abortive transcripts may down-regulate transcriptional initiation. Minor outer capsid protein σ1 had no demonstrable effect on transcriptional activities. Transcriptional regulation may ensure progeny virions do not compete with transcribing particles for ribonucleoside triphosphates.

Both cellular and viral RNA polymerases synthesize abortive transcripts in addition to full-length mRNA (discussed in Refs. 1–7). This leads to the conceptual division of transcription into two basic stages: initiation and elongation. Initiation alone yields abortive transcripts, whereas initiation followed by elongation yields full-length mRNA. A body of work identifying physical differences between initiating and elongating transcription complexes supports this two-stage model. For example, the carboxyl-terminal domain of cellular RNA polymerase II is unphosphorylated during initiation but highly phosphorylated during elongation (8–11). The phosphorylation state of P protein in the respiratory syncytial virus polymerase complex is important for the transition from initiation to elongation (12). Human immunodeficiency virus Tat protein is required for synthesis of full-length viral transcripts but not abortive transcripts (reviewed in Ref. 13). Certain mutations of bacteriophage T7 RNA polymerase render it capable of initiation but not elongation (14, 15). These findings suggest that RNA polymerases are regulated separately at initiation and elongation stages.

Mammalian orthoreovirus (reovirus) has been used as a model to study transcription since its particle-associated RNA-dependent RNA polymerase efficiently synthesizes large amounts of full-length mRNA (16–20). Other particle-associated viral enzymes modify viral transcripts through the addition of the eukaryotic 5′-cap structure, which was elucidated in reovirus (21, 22). Reovirus RNA polymerase also synthesizes abortive transcripts (23, 24), as do other viral and cellular RNA polymerases (see above). Reovirus abortive transcripts are predominantly two to four nucleotides long and are composed of the sequence 5′-GC(U)A, which is present at the extreme 5′ end of all reovirus mRNAs (23, 24). The conserved sequence at the 3′ terminus of reovirus mRNAs is 5′-UCAUC (25). For comparison, bacteriophage T7 abortive transcripts are two to seven nucleotides long (26).

Reovirus is a non-enveloped icosahedrally symmetric virus with two concentric protein capsids surrounding and protecting its segmented, double-stranded RNA genome (for reviews, see Refs. 25 and 27). In addition to 2 protein capsids and 10 segments of genomic double-stranded RNA, intact virions are reported to contain 2000 to 3000 single-stranded RNA oligonucleotides (oligos)1 of varying length and sequence (28–31). A study of immature progeny virions isolated from reovirus-infected cells indicated that synthesis of these particle-associated RNA oligos occurs at a late step in virion morphogenesis (32). It was hypothesized that the RNA oligos, which comprise both abortive transcripts and poly(A), are products of the reovirus

1 The abbreviations used are: oligo, oligonucleotide; r-core, recoated core; r-core + σ1, recoated cores containing σ1; T1L, type 1 Lang strain reovirus; T3D, type 3 Dearing strain reovirus; L/L, T1L σ1 and T1L σ3 proteins; D/L, T3D μ1 and T1L σ3 proteins; rNTP, ribonucleoside triphosphate.
polymerase after outer capsid assembly (29, 33, 34). Although there is evidence that RNA oligos are not required for infection (35), their significance has not been investigated further.

Reovirus virions can be proteolytically digested in vitro to remove the outer capsid (36), yielding transcriptionally active core particles (19, 37). The particle-associated RNA oligos are released upon conversion to cores (28, 35, 36, 38, 39). Only core particles synthesize full-length mRNA in vitro (16, 19, 40). However, both virions and cores are reported to synthesize abortive transcripts (23, 24). From these observations, it was hypothesized that the reovirus outer capsid blocks elongation but not initiation by the particle-associated polymerase (24).

The recent development of the in vitro reovirus recoated core system allows more direct testing of polymerase regulation. Recoated cores (rcores) are formed by the addition of recombinant major outer capsid proteins \( \mu_1 \) and \( \sigma_3 \) to cores (41). R-cores contain levels of \( \mu_1 \) and \( \sigma_3 \) that approximate those in virions (41), or 600 copies of each protein per particle (42). Both \( \mu_1 \) and \( \sigma_3 \) proteins are required to recoat cores; r-cores containing only \( \mu_1 \) or only \( \sigma_3 \) cannot be generated (41). R-cores containing minor outer capsid protein \( \sigma_1 \) (r-cores+\( \sigma_1 \)) in addition to \( \mu_1 \) and \( \sigma_3 \) can also be produced.\(^2\) Both r-cores (41) and r-cores+\( \sigma_1 \) resemble native virions with regard to protein composition, particle morphology, biophysical properties, and route of entry into cells. In this study, we used r-cores and r-cores+\( \sigma_1 \) to address the role of outer capsid proteins in transcriptional regulation. Our findings provide evidence that the particle-associated reovirus RNA polymerase is regulated separately at the initiation and elongation stages of transcription.

**EXPERIMENTAL PROCEDURES**

**Cells and Viruses**—Spinner-adapted murine L cells were grown in suspension in Joklik's modified minimal essential medium (Irvine Scientific, Irvine, Calif.) containing fetal bovine serum (10%) (Life Technologies, Inc., Grand Island, NY) containing heat-inactivated insect cells (Invitrogen, Carlsbad, CA) were grown in TC-100 medium (Life Technologies, Inc., Grand Island, NY). T. S. Dermody, T. S. Baker, and M. L. Nibert, submitted for publication.

**Transcriptional Activities of Reovirus Recoated Cores**

The D/L construct was used to generate recombinant baculovirus containing the dual expression cassette as per the Bac-to-Bac system (Life Technologies, Inc.). High titer baculovirus stocks were generated and utilized to produce large amounts of \( \mu_1 \) and \( \sigma_3 \) proteins, and cytoplasmic extracts of baculovirus-infected cells were prepared by lysis with Triton X-100 as described (41). Recoated Cores (R-cores) and Activated R-cores—To prepare r-cores, insect cell cytoplasmic extracts containing both \( \mu_1 \) and \( \sigma_3 \) (L/L or D/L) were incubated with purified TIL or T3D cores at a ratio of 380 \( \mu_\mu \) \( \mu_1 \) and 200 \( \mu_\mu \) \( \sigma_3 \) per 2.5 \( \times \) 10^14 cores. These amounts of \( \mu_1 \) and \( \sigma_3 \) represent a 2- to 3-fold excess of protein relative to the amounts needed to fully recoat the cores present. Incubation was either for 2 h at 37 °C or for 4 h at room temperature. R-cores+\( \sigma_1 \) were generated as described,\(^2\) R-cores were purified on two sequential CsCl density gradients as described (41). To prepare activated r-cores, r-cores were proteolytically digested in the manner described for cores but were not purified (45).

Experiments described under “Results” indicated that r-cores that had been recoated at 37 °C exhibited one-third to one-half of the mRNA synthesis levels of the parent cores after proteolytic activation (data not shown). Time course experiments demonstrated that the transcriptional enzymes in these particles do not lose activity with reaction time, due either to inefficient reinitiation of transcription or to reduction in elongation rates (data not shown). Rather, these activated r-cores exhibit consistent, inherently lower mRNA production than outer capsid-deficient cores.\(^2\)

**Partially Recoated Particles**—Partially recoated particles were generated similarly to r-cores, but higher ratios of particles to insect-cell cytoplasmic extract were used. Cores were incubated for 4 h at room temperature with a volume of insect cell lysate containing one-tenth to one-half the amount of \( \mu_1 \) and \( \sigma_3 \) outer capsid proteins utilized to generate r-cores. Particles were purified on two sequential CsCl density gradients, and the final gradient was fractionated to obtain a number of particle samples with varying levels of bound \( \mu_1 \) and \( \sigma_3 \). Partially recoated particle fractions were dialyzed into virus buffer and proteolytically activated as described for r-cores. Particle concentrations were estimated as for r-cores (see below). Levels of particle-bound \( \mu_1 \) and \( \sigma_3 \) were determined by SDS-polyacrylamide gel electrophoresis and densiometry, calculated from the \( \lambda \) to \( \mu_1 + \mu_1C \) protein band ratio, and compared with native virions to determine the percentage of outer capsid protein in solution.

**Protein Gel Electrophoresis and Densitometry**—Samples were subjected to SDS-polyacrylamide gel electrophoresis on 10% gels, and particle concentrations were determined by densitometry as described (41).

**Transcription Assays**—Transcription reactions (10 or 15 \( \mu_\mu \) total volume containing 2 \( \times \) 10^10 or 3 \( \times \) 10^10 particles, respectively) were prepared and transcription levels were quantitated by trichloroacetic acid precipitation, which precipitates RNA products of over approximately 50 nucleotides in length, followed by scintillation counting, as described (46).

**RNA Oligo Synthesis Reactions**—RNA oligo reactions were carried out similarly to transcription reactions, with the following exceptions: the divalent cation in the reaction was either 4 mmol MgCl_2 or 6 mmol MgCl_2, and the radiolabel used was 10 mCi/ml [\( ^32P \)]CTP (3000 Ci/mmol) (NEN Life Science Products, Boston, MA) (as opposed to other labeled ribonucleoside triphosphates [\( rNTP \)']s) (23, 24). Following incubation at temperature for time, reactions were boiled for 2 min prior to treatment with calf intestinal phosphatase (New England BioLabs) at 37 °C for 60 to 90 min. Abortive transcript levels were quantitated from high percentage acrylamide RNA-sequencing gels (below) by determining the volume of the abortive transcript bands (minus the volume of a comparable area of a lane in which no-particle control was run) visualized by phosphorimaging.

**RNA Electrophoresis**—Abortive transcripts were resolved on 20% acrylamide RNA-sequencing gels (19.1 acrylamide:bisacrylamide, 7 \( \mu_\mu \) urea, 1% TBE (90 mm Tris borate, 20 mm EDTA (pH 8.0)) with a 5% polyacrylamide gel. Oligo gels were cast at 7.5 cm thick, 16 cm wide, and 10 cm long. Gels were run at 600 V, 30 W, and 50 mA for 18 h prior to staining, and samples were run at 500 V, 30 W, and 30 mA until the xylene cyanol loading dye had migrated nearly two-thirds down the gel (approximately 4 h) (23, 24, 47).

Viral mRNAs were resolved on 1% agarose gels containing formaldehyde as described (48). Radiolabeled single-stranded RNA size markers were generated using the RiboMark labeling system, as per manu-

\(^2\) K. Chandran, X. Zhang, N. H. Olson, S. B. Walker, J. D. Chappell, T. S. Dermody, T. S. Baker, and M. L. Nibert, submitted for publication.
RESULTS

R-cores Do Not Produce Detectable Levels of Full-length Transcripts—When provided with all four rNTPs and a divalent cation, cores produce large amounts of full-length mRNA, but virions do not (16, 19, 40). Because virions and r-cores have similar biochemical, structural, and infectious properties (41), r-cores were expected to be as transcriptionally inactive as virions. It was conceivable, however, that in vitro recoating might not completely block the capacity of r-cores to synthesize viral mRNA. R-cores generated from either T1L or T3D cores, either T1L $\mu$1 or T3D $\mu$1, and T1L $\sigma$3 were analyzed for transcriptional activity (experiments described in subsequent sections were performed with r-cores generated from T1L cores, T1L $\mu$1, and T1L $\sigma$3, and results were confirmed with r-cores of different composition where noted). R-cores were found to be as inactive at mRNA synthesis as virions, as measured by incorporation of radiolabeled rNTPs into acid-precipitable material (Fig. 1B). Thus, the binding of stoichiometric (or virion-like) levels of $\mu$1 and $\sigma$3 outer capsid proteins to cores in our in vitro transcription system was sufficient to reduce viral mRNA synthesis to virion (or background) levels.

R-cores Can Be Activated to Produce Full-length mRNA—To address the concern that particle-associated transcriptional enzymes might suffer damage during the recoating process, we determined whether r-cores converted to cores by protease digestion (activated r-cores) synthesize full-length mRNA at high levels, as do parent cores. mRNA synthesis by parent virions and cores, r-cores, and activated r-cores (Fig. 1A) was compared. After proteolytic activation, r-cores (recoated at room temperature; see “Experimental Procedures”) were as highly active at full-length mRNA production as the parent cores (Fig. 1B). This was true of different r-core preparations containing either T1L or T3D $\mu$1 (data not shown). To ascertain whether activated r-cores produced the same transcripts as cores, radiolabeled mRNA synthesized in vitro by cores and activated r-cores was analyzed on agarose/formaldehyde gels. Core and activated r-core mRNAs comigrated in this and other gel systems (Fig. 1C and data not shown), providing evidence that the two particle types produced the same transcripts.

These results indicate that the recoating process does not damage reovirus transcriptional enzymes. The block to viral mRNA synthesis apparent in r-cores before proteolytic activation (Fig. 1B) thus appears to reflect a specific form of outer capsid-mediated transcriptional regulation.

Full-length mRNA Synthesis by Partially Recoated Particles—Like virions, r-cores containing a full complement of major outer capsid proteins $\mu$1 and $\sigma$3 did not synthesize mRNA (Fig. 1B). To understand better how $\mu$1 and $\sigma$3 may block full-length transcript production, we generated partially recoated particles, which contain less $\mu$1 and $\sigma$3 than do virions or r-cores (see “Experimental Procedures”). Several density gradient fractions of partially recoated particles were isolated that varied in the average levels of bound $\mu$1 and $\sigma$3. Electron microscopy of partially recoated particles revealed core-like particles containing patches of outer capsid.3 Since partially recoated particles were well separated from cores on the density gradients, we are confident that the fraction with 10% of $\mu$1 and $\sigma$3 bound, for example, contained few, if any, cores. Partially recoated particles were found to exhibit mRNA synthesis levels inversely proportional to the amount of bound $\mu$1 and $\sigma$3 (Fig. 2). The relationship between mRNA synthesis and $\mu$1/$\sigma$3 binding was not linear, however. For example, particles with 30% $\mu$1/$\sigma$3 exhibited a 50% reduction in transcriptional activity, and particles with 40% $\mu$1/$\sigma$3 exhibited an 80% reduction in transcriptional activity (Fig. 2). Moreover, it ap-

3 K. Chandran, Y. Chen, and M. L. Nibert, unpublished observation.
pears as though a nearly complete outer capsid was required for transcriptional shut-off; particles with 75% of the outer capsid were slightly more transcriptionally active than virions (3% as opposed to 1.3%, Fig. 2). Additionally, there appeared to be a threshold of \( \mu 1/\alpha 3 \) binding required for transcriptional down-regulation. In fact, partially recoated particles with 10% \( \mu 1/\alpha 3 \) were even more active at mRNA synthesis than cores (Fig. 2). The basis of the transcriptional enhancement observed with low levels of \( \mu 1/\alpha 3 \) is unknown, but our findings with partially recoated particles provide strong evidence for the role of \( \mu 1/\alpha 3 \) outer capsid proteins in transcriptional regulation.

**R-cores Produce Large Amounts of Abortive Transcripts**

Having shown that r-cores are not competent for full-length transcript production (Fig. 1B), we examined them for abortive transcript synthesis. Virions, cores, and r-cores were incubated under conditions favoring abortive transcript production, and reaction products were examined on high percentage acrylamide RNA-sequencing gels. Virions were found to synthesize very low levels of abortive transcripts, in contradiction to earlier studies that documented virion abortive transcript synthesis at 20 to 30% of core levels (24) (Fig. 3A) (see “Discussion”). In the presence of Mn\(^{2+}\), both cores and r-cores produced large amounts of abortive transcripts (Fig. 3A). As described previously (23), cores produced fewer abortive transcripts during Mg\(^{2+}\)-containing reactions (Fig. 3A), whereas r-cores continued to synthesize high levels (Fig. 3A) (see “Discussion”).

By providing cores and r-cores with different subsets of rNTPs, we determined that r-cores synthesized RNA oligos of the same sequence as core-produced abortive transcripts, the predominant products being 5'-GC and 5'-GCU (Fig. 4). This was true of r-cores generated from either T1L or T3D cores and either T1L or T3D \( \mu 1 \) protein (data not shown). The high level of r-core abortive transcript production, along with undetectable mRNA production by r-cores, suggests that the reovirus polymerase is specifically blocked at a post-initiation step by the addition of virion-like levels of \( \mu 1/\alpha 3 \) outer capsid proteins to cores. Moreover, the difference in abortive transcript production by r-cores (high levels) and virions (low levels), despite their other similarities (41) (see above), indicates that the rate of transcriptional initiation differs between the two particle types.

**R-cores+\( \alpha 1 \) Behave Identically to R-cores in Full-length and Abortive Transcript Production**

One difference between virions and r-cores that may account for increased abortive transcript production by r-cores is that r-cores do not contain minor outer capsid protein \( \alpha 1 \). To address this possibility, r-cores+\( \alpha 1 \) were generated for comparison. Like both virions and r-cores (Fig. 1B), r-cores+\( \alpha 1 \) did not produce detectable amounts of full-length mRNA (data not shown). In the presence of Mn\(^{2+}\),
proteins

Reaction Time—(see below).

approached zero (Fig. 5

script synthesis decreased with reaction time and eventually

synthesis over the time course, the rate of r-core abortive tran-

Whereas cores maintained a steady rate of abortive transcript

parisons of transcriptional initiation in the two particle types.

by cores and r-cores were determined to permit further com-

ther T1L or T3D 

preparations generated from either T1L or T3D cores and ei-

addition, r-cores activated by proteolytic digestion consistently

all reaction components except rNTPs (data not shown). In

bars

(d)

(actions containing either dNTPs (d), white bars) or rNTPs (r), black

bars) and incubated at 37 °C for 6 h or with rNTPs but incubated on ice

((ice), gray bars). Particles were then dialyzed into virion buffer and

pelleted to remove reaction components. Samples (2 × 10^{11} particles/ 

reaction) were incubated in radiolabeled oligo reactions at 40 °C for 90

min. Abortive transcripts were quantitated as in A.

r-cores+α1 generated large amounts of abortive transcripts

(Fig. 3B), similar to r-cores and cores. Thus, the high level of

abortive transcripts produced by r-cores cannot be attributed to

their lack of α1. The similar transcriptional activities of r-cores

and r-cores-α1 indicate that the binding of major outer capsid

proteins α1 and α3 is sufficient to block the reovirus RNA

polymerase at a specific post-initiation step (see above). Addi-

tionally, the findings suggest that minor outer capsid protein

α1 plays little or no role in regulating the reovirus polymerase

(see below).

Aberrant Transcription Production by R-cores Decreases with Reaction Time—The kinetics of aberrant transcript production by cores and r-cores were determined to permit further comparisons of transcriptional initiation in the two particle types. Whereas cores maintained a steady rate of aberrant transcript synthesis over the time course, the rate of r-core aberrant transcript synthesis decreased with reaction time and eventually approached zero (Fig. 5A). The same trend was seen with r-core preparations generated from either T1L or T3D cores and either T1L or T3D α1 and with r-cores-α1 (data not shown). Most r-core preparations exhibited a significant decrease in initiation rate with reaction time regardless of whether Mn^{2+}

or Mg^{2+} was present during the oligo reaction. However, a minority of r-core preparations tested required Mg^{2+} for the down-regulation of initiation (see “Discussion”).

The decrease in r-core aberrant transcript synthesis rates with reaction time was not due to temperature-induced polymerase inactivation, since r-cores exhibited the same high, initial rate of aberrant transcript synthesis after preincubations with all reaction components except rNTPs (data not shown). In addition, r-cores activated by proteolytic digestion consistently exhibited a steady rate of aberrant transcript synthesis over reaction time courses, as did cores (data not shown). Therefore, the presence of α1 and α3 outer capsid proteins was associated with the decrease in aberrant transcript synthesis rates with reaction time.

R-core Modification Correlates with the Reaction-dependent Decrease in Aberrant Transcript Production—The decrease in r-core aberrant transcript synthesis with reaction time described above may be due to either a soluble inhibitor whose concentration increases with reaction time or a modification of the particles themselves. To determine which of these possibilities may be true, particles were placed in nonradioactive oligo reaction mixtures containing either all four rNTPs or all four deoxynucleoside triphosphates (dNTPs) (it was determined that reovirus particles cannot utilize dNTPs for nucleic acid synthesis (data not shown)). After pelleting and dialysis of particles to remove preincubation reaction components, particles were placed in a second, radioactive oligo reaction mixture to determine the effect of the preincubation on subsequent aberrant transcript synthesis. R-cores preincubated with rNTPs exhibited a decrease in subsequent aberrant transcript synthesis relative to r-cores preincubated with dNTPs (Fig. 5B). The low level of r-core aberrant transcript synthesis after rNTP preincubation approached the level of virions (Fig. 5B). However, r-cores preincubated with dNTPs exhibited a high level of subsequent aberrant transcript synthesis, as did r-cores preincubated with rNTPs on ice (Fig. 5B). Subsequent aberrant transcript synthesis by either virions or cores was unaffected by preincubation with rNTPs relative to dNTPs (Fig. 5B). In summary, these findings indicate that the decrease in r-core aberrant transcript synthesis with reaction time is not reversed by separating r-cores from other, soluble reaction components, is specific to r-cores, and requires rNTPs. Therefore, under conditions allowing r-core aberrant transcript synthesis, the reovirus polymerase is subject to a particle-based change in activity that substantially reduces transcriptional initiation.

Aberrant Transcripts Produced by R-cores Remain Particle-associated and Are Released upon Proteolytic Outer Capsid Removal—The experiments described above indicate that r-cores are functionally modified during rNTP-containing reactions that allow aberrant transcript synthesis. One possible modification is accumulation of aberrant transcripts within r-cores. Retention of aberrant transcripts by r-cores would be consistent with earlier observations that virions contain RNA oligos formed at a late step in particle morphogenesis (32). Additionally, r-core aberrant transcript retention would suggest a negative feedback mechanism whereby the synthesis and accumulation of RNA oligos inhibit further aberrant transcript production. To examine the possibility that newly synthesized aberrant transcripts are retained by r-cores, cores and r-cores were incubated in large oligo reactions. One aliquot of each reaction was set aside for determining the total level of aberrant transcript synthesis. The remaining portion of the r-core reaction was further divided in two, and one-half was proteolytically digested to remove the outer capsid (activated r-cores), whereas the other half was incubated at the same temperature without protease (r-cores). The activated r-core and r-core samples as well as the remaining portion of the core reaction were then purified on separate CaCl2 density gradients.

Equal numbers of unpurified and gradient-purified particles (cores, activated r-cores, and r-cores) were run on high percent-

age acrylamide RNA-sequencing gels to evaluate aberrant transcrip-
tion retention. R-cores retained a much higher percentage of produced aberrant transcripts than did cores (Fig. 6A). In fact, aberrant transcript retention values approximating 100% were consistently obtained with r-cores generated from T1L cores and either T1L or T3D α1 proteins (Fig. 6A and data not
Fig. 6. Association of abortive transcripts with particles after synthesis. A, TIL cores (C) and r-cores generated from TIL cores, T3D μ1, and TIL α3 (RC) (3 × 10^12 particles/reaction) were incubated in 300 μl of radiolabeled oligo reactions at 37 °C for 2 h. The r-core reaction was then divided into two and half was proteolytically digested to cores (RC–C). Portions of all three samples were then purified on CsCl density gradients. Equal numbers of total reaction (total) and post-centrifugation (CsCl) particles were run on high percentage RNA-sequencing gels, and RNA products were visualized by phosphorimaging. The mobilities of full-length and abortive transcripts are indicated at the right. The percentage of total abortive transcripts synthesized that copurify with particles is indicated at the bottom. B, r-cores generated from TIL cores, T3D μ1, and TIL α3 (9.1 × 10^12 particles/reaction) were incubated in a 750-μl radiolabeled oligo reaction at 37 °C for 2 h. Immediately after the reaction and 1, 3, 7, and 14 days later, aliquots were purified on CsCl density gradients. Equal numbers of total reaction and purified particles were run on high percentage RNA-sequencing gels, and the percentage of particle-associated abortive transcripts was determined as in A.

shown). Like cores, activated r-cores retained only a small percentage of the abortive transcripts produced by the parent r-cores in the preceding reaction (Fig. 6A). Therefore, abortive transcripts retained by r-cores were released upon proteolytic activation to core-like particles, just as virion-associated RNA oligos are released upon conversion to cores (24, 28, 35, 36, 38, 39). The experiment indicates that μ1 and α3 outer capsid proteins are associated with abortive transcript retention. Moreover, r-core-associated abortive transcripts appear to be likely mediators of the reduction in r-core abortive transcript synthesis with reaction time (see above).

To determine whether the association of abortive transcripts with r-cores was stable over time, r-cores were subjected to CsCl gradient purification at varying times after abortive transcript synthesis. The level of r-core-associated abortive transcripts showed no decrease more than a 2-week period, indicating that the association of abortive transcripts with r-cores is stable (Fig. 6B).

Abortive Transcripts Produced by R-cores Are Predominantly Uncapped—Previous studies of cores indicate that under conditions that promote 5′-mRNA capping, including the addition of methyl donor S-adenosyl-L-methionine, only 5–6% of abortive transcripts are capped and only half of those capped (2 to 3% of total) are methylated (23). Thin-layer chromatography revealed that under cap-promoting conditions, the predominant abortive transcript products of both virions and r-cores were uncapped and 5′-diphosphorylated (Fig. 7). This was true regardless of whether Mn^{2+} or Mg^{2+} was present during the oligo reaction (data not shown). These data suggest that the reovirus RNA triphosphatase (which removes the nascent mRNA 5′-γ-phosphate), but not the other three capping enzymes (guanylyltransferase and two methyltransferases), can efficiently modify abortive transcripts. These results agree with the previously reported observation that almost all the abortive transcripts produced by cores are uncapped (23) (see “Discussion”).

Abortive Transcripts Do Not Affect R-core Infectivity—The work described here identifies r-cores as novel tools that allow the characterization of virion-like particles in the absence or presence of abortive transcripts. In an attempt to determine the significance of virion-associated RNA oligos during reovirus replication, experiments were conducted to assess the effect of abortive transcripts on r-core infectivity. Plaque assays with murine L cells indicated that there was no difference in viral titer between r-cores that had been incubated in oligo reactions with dNTPs or rNTPs under conditions that result in the down-regulation of abortive transcript synthesis when rNTPs are present. The infectious titer of r-cores after dNTP versus rNTP-containing oligo reactions was determined in two separate experiments to be 5.7 × 10^6 versus 5.2 × 10^6 and 1.4 × 10^6 versus 1.3 × 10^6 particles/plaque forming unit, respectively. These data agree with previous evidence that cores, which lack RNA oligos, are infectious and give rise to progeny virions with the usual complement of RNA oligos (35). Also in agreement are findings that r-cores not incubated in RNA oligo reactions are infectious (41) and that r-cores + α1 not incubated in RNA oligo reactions not only are infectious but also show similar growth kinetics and infectious progeny yields as virions.

DISCUSSION

Outer Capsid-mediated Shut-off of Transcriptional Elongation—The well-established observation that cores, but not virions, produce full-length transcripts led to the hypothesis that
the reovirus outer capsid inhibits mRNA synthesis (24). Our finding that the addition of virion-like levels of μ1 and σ3 major outer capsid proteins to cores inhibits mRNA production (Fig. 1B) directly supports this hypothesis. Our findings further suggest that minor outer capsid protein r1 plays little or no role in regulating reovirus transcriptional activities.

It is intriguing to speculate how outer capsid proteins may regulate the reovirus transcriptional machinery, located interior to the core shell (45, 50). A study of Reoviridae family member rotavirus identified a monoclonal antibody that greatly reduces mRNA synthesis when bound to otherwise transcriptionally active subviral particles (51). The bound antibody projected into a channel at the viral 5-fold axis, through which nascent transcripts are known to exit the particle (52). The authors suggested that transcription may stop when the initial nascent transcripts become tangled due to bound antibody blocking their exit through the 5-fold channel. If transcript tangling were occurring with reovirus r-cores, we would expect RNA products intermediate in length between abortive and full-length transcripts, as described in the rotavirus study (51). The absence of such RNAs (Figs. 3, 4, and 6) suggests that regulation of transcriptional elongation by reovirus outer capsid proteins is achieved through another mechanism.

We propose that the addition of stoichiometric levels of μ1 and σ3 to cores causes changes in the core shell and/or particle interior that block the transition from transcriptional initiation to elongation. This block could be manifested in several ways, including restriction of genomic double-stranded RNA template movement relative to the transcriptional machinery and inhibition of the polymerase from undergoing conformational or chemical changes that render it processive. As a result, the polymerase cannot proceed beyond the first two to four template nucleotides and produces only abortive transcripts. We do not currently know whether the dramatic narrowing of the λ2-lined channel at each 5-fold axis, shown to occur upon addition of μ1 and σ3 to cores (41), is necessary for transcriptional shut-off.

Our experiments with partially recoated particles indicate that most, but perhaps not all, of the outer capsid must be added to cores before transcriptional elongation is completely blocked (Fig. 2). Further study is needed to determine whether the intermediate levels of mRNA synthesis seen with partially recoated particles are due to a reduction in transcriptional rates, cessation of elongation from a subset of enzyme complexes within single particles, and/or cessation of all elongation from a subset of particles. Whichever is true, our results suggest that inhibition of transcriptional elongation is not an all-or-nothing phenomenon.

Previous studies of immature viral particles isolated from reovirus-infected cells suggested that the capacity of particles with incomplete outer capsids to synthesize full-length mRNA is important for reovirus replication (32, 53). The transcriptase particles identified in this manner contained complete genomes, all core proteins, reduced amounts of outer capsid proteins, and reovirus nonstructural protein μNS. Transcription by these and/or similar immature progeny particles provides the majority of viral mRNA present in infected cells (54, 55). Recent results from our lab suggest that μNS is involved in maintaining full-length transcript production by immature progeny particles by binding to particles and preventing completion of outer capsid assembly (56).

**Down-regulation of Transcription Initiation**—The initial, high level of r-core abortive transcript synthesis decreases with oligo reaction time (Fig. 5A), and this inhibition is maintained when r-cores are purified away from soluble reaction components (Fig. 5B). Down-regulation also requires rNTPs (Fig. 5B). These data suggest two possible mechanisms: 1) chemical modification, such as phosphorylation of protein(s), which requires a rNTP cofactor, and 2) interactions between newly synthesized, particle-associated (Fig. 6) abortive transcripts and viral proteins and/or genomic double-stranded RNA. Because there is presently no evidence for relevant chemical modifications and because the only known difference between virions (low initiation rates) and r-cores (high initiation rates) is the presence or absence of RNA oligos, respectively, we favor mechanism 2. Attempts using UV cross-linking to identify particle components that interact with abortive transcripts were inconclusive (data not shown).

The high level of r-core abortive transcript synthesis in the presence of Mg2+ (Fig. 3A) suggests that this activity of the reovirus polymerase is physiologically relevant. Although cores require Mn2+ to synthesize high levels of abortive transcripts (23) (Fig. 3A), the r-core polymerase is very active at initiation and abortive transcript production regardless of which divalent cation is present. Both viral and cellular RNA polymerases are known to exhibit increased activity and a wider range of activities in the presence of Mn2+, as compared with Mg2+ (57, 58). The capacity of r-cores to synthesize high levels of abortive transcripts and to down-regulate transcriptional initiation in the presence of Mg2+ suggests that this process may parallel progeny virion maturation in the host cytoplasm, which contains Mg2+, but not Mn2+, in millimolar amounts (see below).

Since the transition from virion to core (28, 35, 36, 38, 39) or r-core to activated r-core (Fig. 6A) releases RNA oligos, there appear to be three possible locations for the particle-associated abortive transcripts: 1) between the outer and inner capsids; 2) inside the λ2 turret, which is closed in virions (59) and r-cores (41) but open in cores (50, 59) and presumably open in activated r-cores; and 3) within the particle interior and capable of outward diffusion only after the outer capsid is removed. Hypothesis 1 would require abortive transcripts to migrate from the particle interior, where they are presumably synthesized, to another site in order to regulate polymerase activity. Although this is conceivable, it seems inefficient and therefore unlikely. The lack of significant amounts of virion and r-core 5’-capped abortive transcripts (Fig. 7) argues against hypothesis 2, since λ2 contains both guanylyltransferase- (50, 60–63) and methyltransferase- (50, 64–66) capping active sites, and reovirus can cap small nucleotide and dinucleotide substrates (24). We therefore hypothesize that r-core–associated RNA oligos are located as described in hypothesis 3. Hypothesis 3 allows abortive transcripts produced in the particle interior to remain there and to down-regulate initiation through interactions with the polymerase, other proteins in the transcriptional complex, and/or genomic RNA. Experiments are under way to test these possibilities.

It seems remarkable that rNTPs can move into the particle interior to serve as substrates for abortive transcript synthesis but that the 5’-diphosphorylated dinucleotide and trinucleotide abortive transcript products (5’-ppGpC and 5’-ppGpCPU, where p denotes a phosphoryl group) are quantitatively retained within r-cores (Fig. 6). This suggests that abortive transcripts are retained in particles through a mechanism other than simple size exclusion. Two possible and not mutually exclusive mechanisms for abortive transcript retention are strong, sequence-specific interactions with particle components and different compartmentalization of abortive transcripts after synthesis. By determining where in r-cores the abortive transcripts are located, we hope to gain a better understanding of the mechanisms of both oligo retention and the down-regulation of transcriptional initiation.
Abortive Transcription Production by Virions—A previous report indicated that virions synthesize abortive transcripts at 20–30% of core levels (24). From Fig. 3 and other data not shown, we calculate virion abortive transcription production to be less than 1% that of cores in our experiments. All of the different strains and plaque isolates of virions we tested exhibited this low level of abortive transcript synthesis over a range of conditions, including those used in the previous study (24). Since the quantity of RNA oligos per reovirus virion can vary significantly depending on the viral strain and of the cell line used to test such hypotheses.

We propose that abortive transcripts arise from leak from virions, and as a result, older virion preparations may synthesize more abortive transcripts as it aged with extended storage at 4 °C (data not shown). This suggests that RNA oligos may slowly leak from virions, and as a result, older virion preparations may synthesize more abortive transcripts. We propose that virions characterized in the previous study (24) showed higher levels of abortive transcript synthesis because they contained lower initial levels of RNA oligos than did our virions.

Potential Roles of Abortive Transcripts in the Viral Replication Cycle—Our r-c core infectivity experiments indicate that particle-associated abortive transcripts have no significant effect on viral titer. However, RNA oligos are present in all reovirus virions regardless of the viral strain and of the cell type used for propagation (30, 31). This conservation suggests that the kinetics of r-c core abortive transcript synthesis (Fig. 5A) and the decrease in r-c core abortive transcript production after RNA oligo reactions (Fig. 5B) suggest that one function of particle-associated abortive transcripts may be to down-regulate transcriptional initiation. Initiation down-regulation may be advantageous to keep the reovirus polymerase in a near-inactive state until outer capsid removal or alteration signals that the viral particle is in the appropriate cell compartment for mRNA synthesis. Such regulation would also ensure that, in an infected cell containing both progeny virions and actively transcribing progeny particles, virions would not compete with transcriptase particles for rNTPs. Similar regulation at the level of transcriptional elongation is found in bacteria to control phage replication and assembly (reviewed in Ref. 68). Particle-associated RNA oligos may conceivably have other functions during reovirus replication. Oligos released from infecting particles and/or synthesized by progeny particles may contribute to the inhibition of cellular protein synthesis (69, 70), similar to what is hypothesized for vaccinia virus (71, 72). Another potential function of abortive transcripts may be structural stabilization of virions. If abortive transcripts perform any of these functions, their absence may not affect viral titer but may affect the kinetics of replication or other parameters. The reovirus in vitro recombining system can now be used to test such hypotheses.

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64. Koonin, E. V. (1993) J. Gen. Virol. 74, 733–740
65. Seliger, L. S., Zheng, K., and Shatkin, A. J. (1987) J. Biol. Chem. 262, 16289–16293
66. Luongo, C. L., Contreras, C. M., Farsetta, D. L., and Nibert, M. L. (1998) J. Biol. Chem. 273, 23773–23780
67. Farrell, J. A., Harvey, J. D., and Bellamy, A. R. (1974) Virology 62, 145–153
68. Uptain, S., Kane, C., and Chamberlin, M. (1997) Ann. Rev. Biochem. 66, 117–172
69. Gomatos, P. J., and Tamm, I. (1963) Biochim. Biophys. Acta 72, 651–653
70. Zweerink, H. J., and Joklik, W. K. (1970) Virology 41, 501–518
71. Bablanian, R., and Banerjee, A. K. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 1290–1294
72. Lu, C., and Bablanian, R. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 2037–2042