

Transcriptional Activities of Reovirus RNA Polymerase in Recoated Cores

INITIATION AND ELONGATION ARE REGULATED BY SEPARATE MECHANISMS*

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The particle-associated reovirus polymerase synthesizes mRNA within only certain viral particle types. Reovirus cores, subviral particles lacking outer capsid proteins $\mu 1$, $\sigma 3$, and $\sigma 1$, 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 $\mu 1$ and $\sigma 3$, 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 $\mu 1$ and $\sigma 3$ 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 $\mu 1$ and $\sigma 3$. Particle-associated abortive transcripts may down-regulate transcriptional initiation. Minor outer capsid protein $\sigma 1$ 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

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¹ The abbreviations used are: oligo, oligonucleotide; r-core, recoated core; r-core + $\sigma 1$, recoated cores containing $\sigma 1$; T1L, type 1 Lang strain reovirus; T3D, type 3 Dearing strain reovirus; L/L, T1L $\mu 1$ and T1L $\sigma 3$ proteins; D/L, T3D $\mu 1$ and T1L $\sigma 3$ 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 (r-cores) 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+ $\sigma 1$ ² 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 (2%), neonatal bovine serum (2%) (Hyclone Laboratories, Logan, Utah), and penicillin (100 units/ml)-streptomycin (100 μ g/ml) (Irvine Scientific). Both type 1 Lang (T1L) and type 3 Dearing (T3D) reovirus strains were used in this study. Plaque assays to determine the infectivities of reovirus preparations were performed as described (43). *Trichoplusia ni* Tn High Five insect cells (Invitrogen, Carlsbad, CA) were grown in TC-100 medium (Life Technologies, Inc., Grand Island, NY) containing heat-inactivated fetal bovine serum (10%).

Virions and Cores—Purified T1L and T3D virions were obtained as described (44). Virion buffer contains 150 mM NaCl, 10 mM MgCl₂, and 10 mM Tris (pH 7.5). Cores were generated from virions by *in vitro* protease digestion and purified as described (44). Particle concentrations were estimated by A₂₆₀ (39, 42).

Expression of $\mu 1$ and $\sigma 3$ —Two different recombinant baculoviruses, each directing expression of both $\mu 1$ and $\sigma 3$ proteins, were used in this study. One baculovirus contained genes encoding wild-type T1L $\mu 1$ and T1L $\sigma 3$ proteins (*L/L*)². The second baculovirus contained genes encoding wild-type T3D $\mu 1$ and T1L $\sigma 3$ proteins (*D/L*). The vector used to generate *D/L* virus was constructed by replacing the T1L M2 gene (which encodes $\mu 1$) in the *L/L* pFastbac DUAL vector (Life Technologies, Inc.) with the T3D M2 gene. The T3D M2-pcDNA I construct (a generous gift of Leslie Schiff) was digested by *AseI* at a site in the vector just 3' to the M2 insert. The *AseI* overhangs were made blunt with the Klenow fragment of *Escherichia coli* DNA polymerase I as per manufacturer's instructions, and the construct was next digested with *NcoI*. *NcoI* cleaved the T3D and T1L M2 genes 195 base pairs into the insert. The 48 amino acids encoded by M2 upstream of the *NcoI* site are identical between T1L and T3D $\mu 1$ proteins. The resulting 2113-base pair-long T3D M2 fragment was ligated into the *L/L* pFastbacDUAL vector from which the T1L gene had been removed by digestion with *HindIII*, followed by Klenow treatment and *NcoI* digestion. Automated DNA sequencing at the University of Wisconsin Biotechnology Center DNA Facility (Madison, WI) verified the validity of all constructs. Klenow polymerase, T4 DNA ligase, and all restriction enzymes were from New England Biolabs (Beverly, MA).

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 T1L or T3D cores at a ratio of 380 μ g $\mu 1$ and 200 μ g $\sigma 3$ per 2.5×10^{12} cores. These amounts of $\mu 1$ and $\sigma 3$ represent a 2-fold excess of protein relative to the amounts needed to fully recoat the number of 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 described². 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 cores. Because the level of mRNA synthesis after activation is the only enzymatic property that differs between r-cores recoated at room temperature and those recoated at 37 °C (see "Results"), all mRNA data was generated with room temperature r-cores, whereas other data was generated with room temperature and/or 37 °C r-cores.

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 virion 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 densitometry, calculated from the λ to ($\mu 1 + \mu 1C$) protein band ratio, and compared with native virions to determine the percentage of outer capsid substitution.

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 μ l total volume containing 2×10^{10} or 3×10^{10} particles, respectively) were performed, 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 mM MnCl₂ or 6 mM MgCl₂, and the radiolabel used was 10 mCi/ml [α -³²P]CTP (3000 Ci/mmol) (NEN Life Science Products, Boston, MA) (as opposed to other labeled ribonucleoside triphosphates (rNTPs)) (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 a 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 M urea, 1 \times TBE (90 mM Tris borate, 2 mM EDTA (pH 8.0)) with a 5% stacker. Oligo gels were cast to be 0.75 mm thick, 16 cm wide, and 20 cm long. Gels were pre-run at 600 V, 30 W, and 30 mA for at least 30 min before sample loading, 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.

facturer's instructions (Promega, Madison, WI).

Samples for both oligo and mRNA gels were disrupted by boiling for 2 min after the addition of an equal volume of 2× loading buffer containing 40 mM Tris (pH 8.0), 6 M urea, 10 mM EDTA, 1% SDS, 10% sucrose, and bromophenol blue and xylene cyanol dyes.

Thin-layer Chromatography—Oligo reactions were carried out as above except that the reaction volume was 20 μ l, and reactions contained 7×10^{10} particles, 2 μ Ci [α - 32 P]GTP (3000 Ci/mmol) (NEN Life Science Products), and 0.25 mM *S*-adenosyl-L-methionine. After incubation at 40 °C for 7 h, free label was removed by twice pelleting the particles by centrifugation at 16,000 $\times g$ for 1 h at 4 °C, removing the supernatant, and resuspending the particles in 20 μ l of virion buffer. Reaction then were boiled for 2 min. The samples were divided into two 10- μ l aliquots and prepared for thin-layer chromatography by either nuclease P1 digestion alone or both nuclease P1 digestion and calf intestinal alkaline phosphatase treatment. Either 1 μ g of nuclease P1 (Roche Molecular Biochemicals, Indianapolis, IN) or nuclease P1 with 10 units of calf intestinal alkaline phosphatase (New England Biolabs) and one-tenth volume 10× NEB Buffer 3 (New England Biolabs) were added to each aliquot, and reactions were incubated at 37 °C for 90 min. All reactions were phenol/chloroform-extracted before spotting onto 20 \times 20-cm polyethyleneimine-cellulose F TLC plastic sheets (EM Science, Gibbstown, NJ). TLC plates were developed in 1.2 M LiCl until the solvent front reached the top of the plate (approximately 90 min) (49).

Viral Plaque Assays—The particle to plaque-forming unit ratio of particle preparations was determined by plaque assay as described (43).

Computer Software—SDS-polyacrylamide gels were scaled uniformly and adjusted for optimal brightness and contrast in Photoshop 5.0 (Adobe Systems, San Jose, CA.). Abortive transcript bands on high percentage acrylamide RNA-sequencing gels were quantitated using Image Quant (Molecular Dynamics, Sunnyvale, Calif.). All figures were produced in Illustrator 7.0 (Adobe).

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 μ 1 or T3D μ 1, and T1L σ 3 were analyzed for transcriptional activity (experiments described in subsequent sections were performed with r-cores generated from T1L cores, T1L μ 1, and T1L σ 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 μ 1 and σ 3 outer capsid proteins to cores in our *in vitro* 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 μ 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

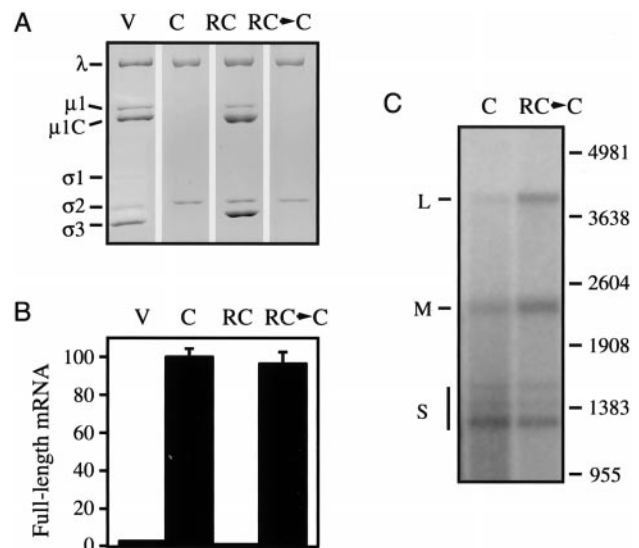


FIG. 1. mRNA synthesis by r-cores and r-cores proteolytically digested to cores (activated r-cores). A, representative protein gel of virions (V), cores (C), r-cores (RC), and activated r-cores (RC→C). Reovirus proteins are labeled to the left. All three λ proteins comigrate in standard gel systems, and μ 1 protein is present on viral particles as full-length μ 1 and μ 1C cleavage product. B, T1L virions and cores, r-cores generated from T1L cores, T1L μ 1, and T1L σ 3, and r-cores after proteolytic activation (2×10^{10} particles/reaction) were incubated in radiolabeled transcription reactions at 40 °C for 1 h. Transcription levels were quantitated by trichloroacetic acid precipitation and scintillation counting, and results are graphed relative to the mean core transcription level \pm S.D. C, T1L cores and activated r-cores, generated from T1L cores and containing T1L μ 1 and T1L σ 3 before digestion, were incubated in radiolabeled transcription reactions at 40 °C for 100 min (5×10^{10} particles/30- μ l reaction). 2 μ l of the resulting mRNA products were resolved on agarose/formaldehyde gels. The reovirus mRNA size classes are indicated to the left, and the migration of single-stranded RNA size markers indicated (in bases) to the right.

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 μ 1 and σ 3 did not synthesize mRNA (Fig. 1B). To understand better how μ 1 and σ 3 may block full-length transcript production, we generated partially recoated particles, which contain less μ 1 and σ 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 μ 1 and σ 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 μ 1 and σ 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 μ 1 and σ 3 (Fig. 2). The relationship between mRNA synthesis and μ 1/ σ 3 binding was not linear, however. For example, particles with 30% μ 1/ σ 3 exhibited a 50% reduction in transcriptional activity, and particles with 40% μ 1/ σ 3 exhibited an 80% reduction in transcriptional activity (Fig. 2). Moreover, it ap-

³ K. Chandran, Y. Chen, and M. L. Nibert, unpublished observation.

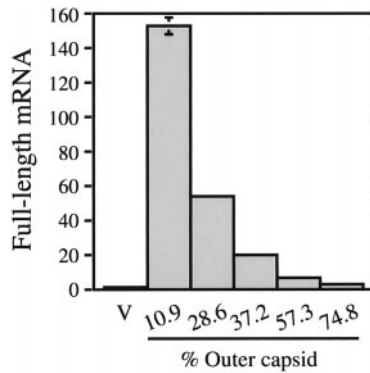


FIG. 2. mRNA synthesis by partially recoated particles. T1L virions (V) and partially recoated particles generated from T1L cores incubated with reduced levels of T3D $\mu 1$ and T1L $\sigma 3$ proteins (4×10^9 particles/reaction) were incubated in radiolabeled transcription reactions at 40°C for 2 h. Five CsCl density gradient fractions of partially recoated particles were assayed, and the level of outer capsid substitution of each fraction is indicated at the *bottom*. Transcription levels were quantitated by trichloroacetic acid precipitation and scintillation counting, and results are graphed relative to the mean transcriptional level of the same particles after proteolytic digestion to cores \pm S.D.

appears 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 $\mu 1/\sigma 3$ binding required for transcriptional down-regulation. In fact, partially recoated particles with 10% $\mu 1/\sigma 3$ were even more active at mRNA synthesis than cores (Fig. 2). The basis of the transcriptional enhancement observed with low levels of $\mu 1$ and $\sigma 3$ is unknown, but our findings with partially recoated particles provide strong evidence for the role of $\mu 1$ and $\sigma 3$ 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 $\mu 1$ and $\sigma 3$ 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 + $\sigma 1$ Behave Identically to R-cores in Full-length and

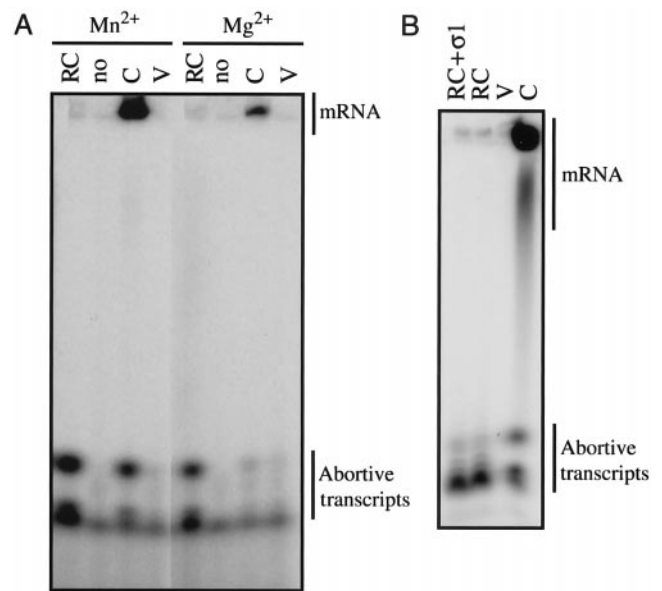


FIG. 3. Abortive transcript synthesis by virions, cores, r-cores, and r-cores + $\sigma 1$. A, T1L virions (V) and cores (C), r-cores generated from T1L cores, T1L $\mu 1$, and T1L $\sigma 3$ (RC), and buffer alone (no) assayed for abortive transcript synthesis. The divalent cation present during the oligo reaction was either 4 mM Mn^{2+} (left) or 6 mM Mg^{2+} (right). B, T1L virions and cores, r-cores, and r-cores + $\sigma 1$ generated from T1L cores, T1L $\mu 1$, T1L $\sigma 3$, and T1L $\sigma 1$ (RC + $\sigma 1$) assayed for abortive transcript synthesis. Experiments shown in A and B utilized 2×10^{10} particles incubated in radiolabeled oligo reactions at 40°C for 1 h. Reaction products were resolved on high percentage acrylamide RNA-sequencing gels and visualized by phosphorimaging. Positions of full-length mRNA and abortive transcripts are indicated to the right of each panel.

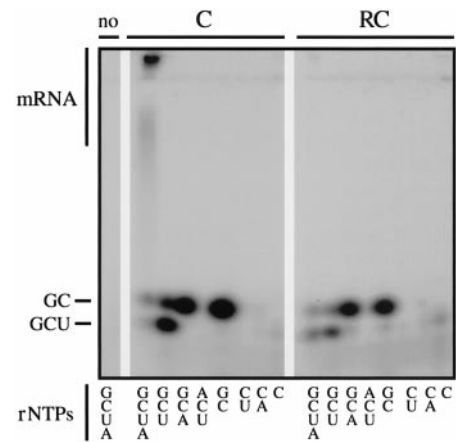


FIG. 4. Comparison of core- and r-core-produced abortive transcripts. T1L cores (C), r-cores generated from T1L cores, T3D $\mu 1$, and T1L $\sigma 3$ (RC) (3×10^{10} particles/reaction) or buffer alone (no) were incubated at 37°C for 90 min in radiolabeled oligo reactions containing the rNTPs listed at the *bottom*. Reaction products were resolved on a high percentage RNA-sequencing gel and visualized by phosphorimaging. The mobilities of mRNA and the two major abortive transcript products, 5'-GC and 5'-GCU, are indicated to the left. The faint signal present in the CTP-alone lane with both cores and r-cores proved upon further investigation to be nonspecific background.

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 $\sigma 1$. To address this possibility, r-cores + $\sigma 1$ were generated for comparison.² Like both virions and r-cores (Fig. 1B), r-cores + $\sigma 1$ did not produce detectable amounts of full-length mRNA (data not shown). In the presence of Mn^{2+} ,

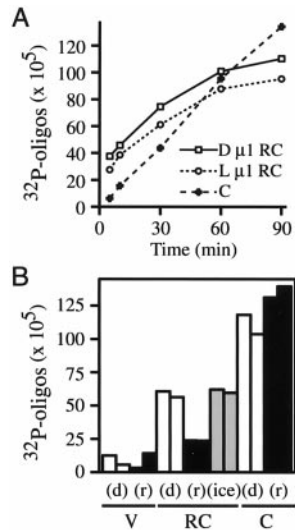


FIG. 5. Kinetics of abortive transcript production and effect of preincubation in oligo reactions upon subsequent abortive transcript synthesis. *A*, T1L cores (*C*) and r-cores generated with T1L cores, either T1L or T3D $\mu 1$, and T1L $\sigma 3$ (*L* $\mu 1$ RC and *D* $\mu 1$ RC, respectively) (3×10^{10} particles/reaction) were incubated in radiolabeled oligo reactions at 40 °C for the time indicated. Reaction products were resolved on high percentage RNA sequencing gels, visualized by phosphorimaging, and quantitated. *B*, T1L virions (*V*) and cores and r-cores generated from T1L cores, T1L $\mu 1$, and T1L $\sigma 3$ (*RC*) (1.5×10^{11} particles/reaction) were preincubated in nonradioactive 50- μ l oligo reactions 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^{10} particles/reaction) were incubated in radiolabeled oligo reactions at 40 °C for 90 min. Abortive transcripts were quantitated as in *A*.

r-cores+ $\sigma 1$ 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 $\sigma 1$. The similar transcriptional activities of r-cores and r-cores+ $\sigma 1$ indicate that the binding of major outer capsid proteins $\mu 1$ and $\sigma 3$ is sufficient to block the reovirus RNA polymerase at a specific post-initiation step (see above). Additionally, the findings suggest that minor outer capsid protein $\sigma 1$ plays little or no role in regulating the reovirus polymerase (see below).

Abortive Transcript Production by R-cores Decreases with Reaction Time—The kinetics of abortive 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 abortive transcript synthesis over the time course, the rate of r-core abortive 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 $\mu 1$ and with r-cores+ $\sigma 1$ (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 abortive transcript synthesis rates with reaction time was not due to temperature-induced polymerase inactivation, since r-cores exhibited the same high, initial rate of abortive 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 abortive transcript synthesis over

reaction time courses, as did cores (data not shown). Therefore, the presence of $\mu 1$ and $\sigma 3$ outer capsid proteins was associated with the decrease in abortive transcript synthesis rates with reaction time.

R-core Modification Correlates with the Reaction-dependent Decrease in Abortive Transcript Production—The decrease in r-core abortive 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 abortive transcript synthesis. R-cores preincubated with rNTPs exhibited a decrease in subsequent abortive transcript synthesis relative to r-cores preincubated with dNTPs (Fig. 5B). The low level of r-core abortive transcript synthesis after rNTP preincubation approached the level of virions (Fig. 5B). However, r-cores preincubated with dNTPs exhibited a high level of subsequent abortive transcript synthesis, as did r-cores preincubated with rNTPs on ice (Fig. 5B). Subsequent abortive 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 abortive 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 abortive transcript synthesis, the reovirus polymerase is subject to a particle-based change in activity that substantially reduces transcriptional initiation.

Abortive 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 abortive transcript synthesis. One possible modification is accumulation of abortive transcripts within r-cores. Retention of abortive 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 abortive transcript retention would suggest a negative feedback mechanism whereby the synthesis and accumulation of RNA oligos inhibit further abortive transcript production. To examine the possibility that newly synthesized abortive 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 abortive 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 CsCl density gradients.

Equal numbers of unpurified and gradient-purified particles (cores, activated r-cores, and r-cores) were run on high percentage acrylamide RNA-sequencing gels to evaluate abortive transcript retention. R-cores retained a much higher percentage of produced abortive transcripts than did cores (Fig. 6A). In fact, abortive transcript retention values approximating 100% were consistently obtained with r-cores generated from T1L cores and either T1L or T3D $\mu 1$ proteins (Fig. 6A and data not

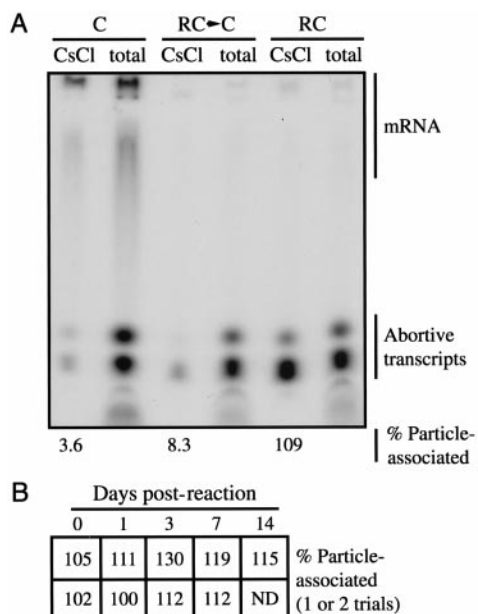


FIG. 6. Association of abortive transcripts with particles after synthesis. *A*, T1L cores (C) and r-cores generated from T1L cores, T3D μ 1, and T1L σ 3 (RC) (3×10^{12} particles/reaction) were incubated in 300 μ l of radiolabeled oligo reactions at 37 °C for 2 h. The r-core reaction was then divided 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 T1L cores, T3D μ 1, and T1L σ 3 (9.1×10^{12} particles/reaction) were incubated in a 750- μ l 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). This experiment indicates that μ 1 and σ 3 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

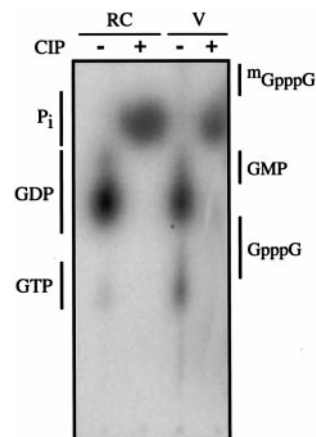


FIG. 7. Chromatographic analysis of abortive transcript 5' structures. Virions (V) or r-cores generated from T1L cores, T3D μ 1, and T1L σ 3 (RC) (7×10^{10} particles/reaction) were incubated in oligo reactions containing [α - 32 P]GTP at 40 °C for 7 h. Reactions were divided into two and treated with either nuclease P1 alone, to isolate abortive transcript 5' structures for analysis, or nuclease P1 in addition to calf intestinal alkaline phosphatase (CIP), to hydrolyze unblocked 5'-phosphate groups. Calf intestinal alkaline phosphatase treatment is indicated at the top, underneath the particle type. The mobility of nonradiolabeled markers developed on the same plate is indicated to both sides; to the left, for markers with which samples comigrated, and to the right, for other markers. Not shown is a no particle control, which comigrated with GTP in the absence of calf intestinal alkaline phosphatase treatment and comigrated with inorganic phosphate (P_i) after calf intestinal alkaline phosphatase treatment.

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+ σ 1 not incubated in RNA oligo reactions not only are infectious but also show similar growth kinetics and infectious progeny yields as virions.²

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 $\mu 1$ and $\sigma 3$ major outer capsid proteins to cores inhibits mRNA production (Fig. 1B) directly supports this hypothesis. Our findings further suggest that minor outer capsid protein $\sigma 1$ 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 $\mu 1$ and $\sigma 3$ 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 $\lambda 2$ -lined channel at each 5-fold axis, shown to occur upon addition of $\mu 1$ and $\sigma 3$ 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 μ NS. 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 μ NS 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 Transcript 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 Mg^{2+} (Fig. 3A) suggests that this activity of the reovirus polymerase is physiologically relevant. Although cores require Mn^{2+} 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 Mn^{2+} , as compared with Mg^{2+} (57, 58). The capacity of r-cores to synthesize high levels of abortive transcripts and to down-regulate transcriptional initiation in the presence of Mg^{2+} suggests that this process may parallel progeny virion maturation in the host cytoplasm, which contains Mg^{2+} , but not Mn^{2+} , 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 $\lambda 2$ 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 $\lambda 2$ 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 Transcript 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 transcript 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 even within the same virion preparation (67), it seems reasonable that two different laboratories may produce virions with different average levels of RNA oligos, perhaps due to differences in particle preparation and/or storage conditions. In support of this hypothesis, we observed that a given virion preparation became capable of synthesizing progressively 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-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 function. The kinetics of r-core abortive transcript synthesis (Fig. 5A) and the decrease in r-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* recoating system can now be used to test such hypotheses.

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