Characterization of the Nucleoside Triphosphate Phosphohydrolase and Helicase Activities of the Reovirus \( \lambda 1 \) Protein*  

(Received for publication, January 31, 1997, and in revised form, May 12, 1997)  

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Previous studies have shown that the reovirus \( \lambda 1 \) core protein harbors a putative nucleotide-binding motif and exhibits an affinity for nucleic acids. In addition, a nucleoside triphosphate phosphohydrolase activity present in reovirus cores has been recently assigned to \( \lambda 1 \) using gene reassortment analysis. In this study, it was demonstrated that the recombinant \( \lambda 1 \) protein, expressed in the yeast Pichia pastoris, is able to hydrolyze nucleoside 5'-triphosphates or deoxynucleoside 5'-triphosphates. This activity was absolutely dependent on the presence of a divalent cation, Mg\(^{2+}\) or Mn\(^{2+}\). The protein can also unwind double-stranded nucleic acid molecules in the presence of a nucleoside 5'-triphosphate or deoxynucleoside 5'-triphosphate. These results provide the first biochemical evidence that the reovirus \( \lambda 1 \) protein is a nucleoside triphosphate phosphohydrolase/helicase and strongly support the idea that \( \lambda 1 \) participates in transcription of the viral genome.

Mammalian reoviruses are members of the Reoviridae family, and since their genome is made up of 10 segments of double-stranded RNA (dsRNA)\(^1\) and replicates in the cytoplasm, they must encode their own transcriptional and replication and repair (5–8). Helicases of prokaryotic, eukaryotic, and viral origins have been isolated and classified into defined superfamilies (9–14). These proteins are characterized by seven conserved motifs designated I, Ia, and II–VI (15). Motifs I and II are very well conserved and correspond to the A and B consensus sequences of a nucleotide-binding domain (16). Superfamily II includes an expanding group of DNA and RNA helicases that harbor a DEAD/DNA sequence in motif II (17). The sequences present in motifs III–V are less strictly conserved, and their roles are not clearly defined, whereas motif VI is supposed to be involved in nucleic acid binding given its high content of positively charged amino acids (13).

The \( \lambda 1 \) protein, a major component of the reovirus core, exhibits an affinity for dsRNA and dsDNA in filter binding assays and can also bind single-stranded RNA in gel retardation assays (18).\(^2\) Furthermore, analysis of gene reassortment has recently resulted in the assignment of NTPase activity present in reovirus cores to the L3 gene encoding \( \lambda 1 \) (19). In this study, we present a biochemical characterization of the enzymatic activities exhibited by the \( \lambda 1 \) protein encoded by the cloned L3 gene. The protein was expressed in the yeast Pichia pastoris and recovered by chromatography using the affinity of the protein for zinc, which is conferred by its zinc-finger motif. This protein was able to hydrolyze all NTPs and dNTPs with release of inorganic phosphate; however, different NTPs and dNTPs are utilized with various efficiencies. The protein can also unwind double-stranded nucleic acid molecules; this reaction requires the presence of a NTP or dNTP at a concentration consistent with a functional coupling between hydrolysis of nucleotide and helicase activity. These findings strongly support the idea that \( \lambda 1 \) participates as a helicase during transcription of the viral genome.

**EXPERIMENTAL PROCEDURES**

*Expression and Enrichment of \( \lambda 1 \)—The methylytrophic yeast *P. pastoris* strain GS115 (his4) was used for the expression of \( \lambda 1 \). All manipulations were performed according to the manufacturer's instructions (Invitrogen). The 5'-end of the cloned L3 gene encoding \( \lambda 1 \) was first reconstructed by polymerase chain reaction to remove homopolymers introduced in the original cloning procedure (20). The \( \lambda 1 \) expression plasmid pHIL-L3 was then constructed by inserting the complete L3 gene encoding \( \lambda 1 \) of the *P. pastoris* Yeast cells were transformed with linear (NotI-digested) DNA of pHIL-D2 or pHIL-L3 and selected on histidine-deficient medium. Transformants were identified, liquid cultures were prepared, and protein expression was induced by the addition of 0.5% methanol for 20 h at 30 °C. The yeast cells were recovered by centrifugation and disrupted with glass beads, and the lysates (1 ml) were submitted to affinity chromatography on zinc chelate affinity adsorbent columns (Boehringer Mannheim). The columns were washed extensively (100 ml) with wash

\(^*\) This work was supported in part by a grant from the Medical Research Council of Canada (to G. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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\(^1\) The abbreviations used are: dsRNA, double-stranded RNA; dsDNA, double-stranded DNA; NTP, nucleoside 5'-triphosphate; dNTP, deoxynucleoside 5'-triphosphate; NTPase, nucleoside triphosphate phosphohydrolase; dNTPase, deoxynucleoside triphosphate phosphohydrolase.

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buffer (50 mM Tris-HCl, pH 8.0, and 150 mM NaCl), and the bound proteins were eluted sequentially with 1 ml of elution buffers, each made of 0.1 M sodium phosphate and 0.8 M NaCl and adjusted at pH 7.5, 7.0, 6.5, 6.0, or 5.5 (21). Protein dosage was performed using the Bradford protein assay (Bio-Rad).

**Western Blot Analysis**—Proteins were submitted to electrophoresis on SDS-10% polyacrylamide gels. Following electrophoresis, the proteins were transferred to a nitrocellulose filter and probed with a 1:1000 dilution method with unlabeled ATP. A similar experiment was performed using a goat anti-rabbit IgG conjugated to horseradish peroxidase and the Amersham enhanced chemiluminescence Western blotting kit.

**NTPase and dNTPase Assays**—Standard reactions (22) were performed in a buffer containing 30 mM Hepses-KOH, 2 mM MgCl₂, and 0.2 pmol of [γ-³²P]ATP (4500 Ci/mmol; ICN). Approximately 18 fmol (2.5 ng) of λ1 protein were used in each 15-μl reaction when an enriched λ1 fraction recovered from zinc affinity chromatography was used. The reactions were incubated at various temperatures and stopped by the addition of 0.1 μl EDTA at the times indicated. The ATPase activity was detected by the release of ³²P, from [γ-³²P]ATP. Reaction products (2 μl) were applied onto plastic-backed polyethyleneimine cellulose sheets (Aldrich) and separated by ascending chromatography in 0.375 m potassium phosphate buffer, pH 3.5. The sheets were then air-dried and subjected to autoradiography.

The Michaelis-Menten constant (Kₘ) was determined by the isotopic dilution method with unlabeled ATP. A similar experiment was performed with [γ-³²P]GTP (4500 Ci/mmol; ICN) and unlabeled GTP. The determination of Kₘ for dATP and dCTP was performed with 0.2 pmol of [α-³²P]dATP (3000 Ci/mmol, ICN) or [α-³²P]dCTP (3000 Ci/mmol; ICN) using isotopic dilution with unlabeled dATP and dCTP, respectively. In these two cases, deoxynucleoside triphosphate substrates were separated from deoxynucleoside diphosphate reaction products by polyethyleneimine cellulose chromatography in 0.8 M acetic acid and 0.9 M LiCl buffer.

The spots corresponding to the radiolabeled substrates or reaction products were identified following autoradiography; the corresponding regions were then excised from the polyethyleneimine cellulose sheets; and the radioactivity was measured by Cerenkov counting. For calculations, background values were first subtracted from product values by quantitation of radioactivity at the same level on chromatograms of control unincubated substrate. Thereafter, the ratio of generated products to total material was calculated by quantitation of both reaction products and residual substrate in each lane.

**Synthesis of Helicase Substrates**—The substrate for the DNA helicase assay was prepared by annealing the 17-base pair universal primer (5'-GTAAAAACGACGCTT-3') to single-stranded M13 DNA. Chain elongation was then performed with Sequenase (U. S. Biochemical Corp.) for 30 min at 37 °C in the presence of dGTP, dTTP, and [α-³²P]dTTP. The resulting substrate contained an extended radiolabeled oligomer of 22 nucleotides hybridized to M13 DNA and was purified by gel filtration (Sephadex G-25) to remove most of the unincorporated nucleotides.

Helicase assays—In the standard assay (23), the DNA and RNA substrates (0.3 pmol) were used in a total volume of 15 μl containing 25 mM Hepses-KOH, pH 7.5, 5 mM MgCl₂, 20 mM NaCl, 1 mM diethiothreitol, and 5 mM ATP. Approximately 18 fmol (2.5 ng) of λ1 protein were used in each 15-μl reaction when the enriched λ1 fraction recovered from zinc affinity chromatography was used; incubation was for 30 min at 37 °C. Components were added or removed in some reactions, as described below. The reactions were stopped by adding 0.5% SDS, 50 mM EDTA, and 40% glycerol. The reaction products were analyzed by electrophoresis on 15% (DNA) or 10% (RNA) nondenaturing Tris borate/EDTA-polyacrylamide gels. The gels were dried under vacuum and exposed for autoradiography.

**RESULTS**

**Expression and ATPase Activity of Recombinant λ1**—Sequence comparisons revealed that λ1 possesses two nucleotide-binding motifs normally present in NTPase (Fig. 1): a PRKTKGS sequence (A site) in the N-terminal region and a DEAD motif (B site). Furthermore, λ1 harbors the characteristic motifs found in members of the RNA/DNA helicase superfamily II, with slight variations occurring in certain motifs. To study these putative enzymatic activities, the λ1 protein encoded by the cloned L3 gene was expressed using the P. pastoris yeast expression system as described under “Experimental Procedures.” Enrichment of the protein and removal of endogenous yeast ATPases were then achieved by affinity chromatography,
The expression level of \( \lambda 1 \) protein expressed in \( P. \) pastoris was first tested for its ATPase activity (Fig. 2A). The same results were obtained from five independent negative controls and two separate cultures expressing recombinant \( \lambda 1 \) (data not shown). A summary of the \( \lambda 1 \) enrichment procedure is presented in Table I; the eluate fraction obtained at pH 7.5 was used in the rest of this work.

In an effort to further rule out any possible contamination with a copurifying yeast enzyme, thermoresistance of the NTPase activity was examined (Fig. 3). The approach used takes advantage of the fact that reovirus core enzymes are active at high temperature, whereas \( P. \) pastoris has an optimal growth temperature of 30 °C, and its enzymes are thus expected to be thermosensitive. Cell extracts from yeast cells harboring the control vector (pHIL-D2) or \( \lambda 1 \) expression vector (pHIL-L3) were thus examined for ATPase activity prior to or following heating at 42 °C for an extended period (5 h). This treatment essentially abolished the yeast endogenous ATPase activity (Fig. 3A), whereas part of the activity was retained in lysates from \( \lambda 1 \)-expressing yeast cells (Fig. 3B); the ATPase activity present in \( \lambda 1 \)-enriched fractions from zinc chelate affinity chromatography was completely resistant to thermal inactivation (Fig. 3D), whereas no activity was found in eluate fractions from yeast cells harboring the control vector pHIL-D2 (Fig. 3C).

Characterization of \( \lambda 1 \) NTPase/dNTPase Activity—The enzymatic activity of recombinant \( \lambda 1 \) found in elution fractions was then further investigated. This activity was absolutely dependent on the presence of the divalent cation \( Mg^{2+} \) or \( Mn^{2+} \), whereas the \( Ca^{2+} \), \( Cu^{2+} \), and \( Zn^{2+} \) cations were not effective cofactors (data not shown). The ATPase activity increased sharply with \( MgCl_2 \) concentration, reached a maximum at 2.5 mM, and was constant up to 10 mM (Fig. 4A). Similar results were obtained when \( MnCl_2 \) was substituted for \( MgCl_2 \) (data not shown).

To gain additional insight into the \( \lambda 1 \)-associated ATPase activity, the effect of temperature on the reaction was examined (Fig. 4B). The temperature optimum of the reaction, as judged by the maximum rate of ATP hydrolysis, was 42–50 °C; however, the activity was rapidly lost upon incubation at 50 °C. A 1-h incubation at 42, 37, and 30 °C resulted in similar rates of ATP hydrolysis, whereas the enzyme was less active at 25 °C.

The kinetics parameters of the reaction were determined at 37 °C and 10 min, at which time the reaction is still proceeding at its initial rate. The ATPase reaction velocity reached a plateau at \( \sim 7 \mu M \) ATP and exhibited a \( K_m \) of 1 \( \mu M \) as determined by a double-reciprocal plot (Fig. 4C). Identical results were obtained in three separate experiments.

### Table I

**Summary of \( \lambda 1 \) protein enrichment**

| Chromatography step | Volume (ml) | Total protein (mg) | ATPase units/mg | Specific activity |
|---------------------|------------|-------------------|-----------------|------------------|
| pHIL-D2 crude lysate| 10         | 250               | 2540            | 10.2             |
| pHIL-D2 eluate fraction | 1         | 2.5               | 0               | 0                |
| pHIL-L3 crude lysate| 10         | 8000              | 3800            | 15.2             |
| pHIL-L3 eluate fraction | 1        | 2.5               | 500             | 150              |

The positions of the ATP substrate and inorganic phosphate product (Pi) are indicated.

Taking advantage of the presence of a putative zinc-binding motif on \( \lambda 1 \), the expression level of \( \lambda 1 \) was too low for detection by either Coomassie Blue or silver staining in the purified fractions (data not shown). However, immunoblotting analysis, using a monospecific anti-\( \lambda 1 \) antibody, did reveal the enrichment of an immunoreactive protein in the eluted fractions; this protein comigrated with the authentic \( \lambda 1 \) protein found in purified reovirions (Fig. 2A). The amount of \( \lambda 1 \) protein in these fractions was estimated to be \( \sim 0.5 \) ng/\( \mu l \) by comparisons with the signal obtained by immunoblotting using serial dilutions of purified reovirus as a standard. Protein dosage also revealed that enrichment of the protein was \( \sim 10 \)-fold compared with the crude lysate (Table I).
To determine the substrate specificity of the \(l_1\) protein, the hydrolysis of other ribonucleotides and deoxyribonucleotides was also tested. The protein exhibited a strong preference for adenosine nucleotides (ATP and dATP), which were hydrolyzed very efficiently. The enzyme displayed some activity on other NTPs and dNTPs tested, although the efficiency was different from one substrate to the other; the order of preference was dATP > ATP > GTP > dCTP according to the \(k_{\text{cat}}\) values obtained with these substrates (Table II).

RNA and DNA Helicase Activities of \(l_1\)—The ability of the \(l_1\) protein to unwind double-stranded nucleic acids was then investigated. The RNA helicase reaction catalyzed by the reovirus \(l_1\) protein was demonstrated by strand displacement of a partial RNA duplex as described under "Experimental Procedures." The \(l_1\)-associated RNA helicase activity required the presence of ATP, which could be substituted by either one of the NTPs and dNTPs tested, although the efficiency was different from one substrate to the other; the order of preference was dATP > ATP > GTP > dCTP according to the \(k_{\text{cat}}\) values obtained with these substrates (Table II).

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FIG. 3. Thermoresistance of \(l_1\)-associated NTPase activity. Crude lysates were obtained from yeast cells harboring the control vector pHIL-D2 or from yeast cells harboring the \(l_1\) expression vector pHIL-L3. Eluate fractions obtained at pH 7.5 from the zinc affinity chromatography procedure were also prepared from the same two lysates. These protein samples were heated at 42 °C for 5 h; an aliquot was kept on ice for the same time. Assays for ATPase activity were then performed at 37 °C using the standard procedure, and hydrolysis was measured at different times. A, crude lysate from control cells harboring pHIL-D2; B, crude lysate from yeast cells harboring pHIL-L3; C, eluate fraction from yeast cells harboring pHIL-D2; D, eluate fraction from yeast cells harboring pHIL-L3. [], control protein samples kept for 5 h on ice prior to use in the reaction; ■, protein samples heated at 42 °C for 5 h prior to the reaction.

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Additional controls were also performed to ensure that the helicase activity could not be due to a copurifying yeast protein. Crude lysate from control yeast cells was examined for the presence of helicase activity and was found to be negative. Furthermore, the enriched eluate from the zinc affinity column was also negative in the control cells, whereas the activity found in purified \(l_1\) eluate fractions was thermoresistant (Fig. 5C), as was the NTPase activity.

DISCUSSION

Our understanding of the roles of individual core proteins in reovirus replication is still incomplete, although genetic information has been accumulated during the past few years (24, 25). Investigation of the biochemical activity of the reovirus \(l_1\) protein was inspired by sequence comparisons with other NTPase/helicase proteins. Although the \(l_1\) protein does not show any significant similarities to other proteins, it possesses the characteristic motifs found in the DEAD subfamily of the RNA/DNA helicase superfamily II. All these motifs are located in the amino-terminal third of \(l_1\); the affinity of the protein for nucleic acids was previously assigned to this region (18). Being one of the largest reovirus proteins, the \(l_1\) protein could not be easily expressed in bacterial expression systems. The recently described \(P.\) pastoris yeast expression system was instead used as a convenient way to produce recombinant \(l_1\). The expression levels obtained were low but sufficient for investigations of \(l_1\) catalytic activities. The reason for this low expression level is unknown, but might reflect toxicity of NTPase activity when overexpressed, thus resulting in the selection of cell clones expressing lower levels of the protein. Expression levels using \(P.\) pastoris have been reported to vary widely among proteins (Invitrogen). Since a truncated form of \(l_1\) encompassing the zinc-finger motif was previously expressed as a fusion protein in bacterial cells (18) and found to attach to zinc chelate columns,\(^3\) this procedure was thus adequate for \(l_1\) recovery. The zinc affinity chromatography pro-
procedure allowed us to efficiently reduce endogenous NTPase and helicase activities below the detection level of our assays while enriching both immunodetectable \( l_1 \) and its associated NTPase activity by \( \times 10 \)-fold. Thermoresistance was an additional proof that the NTPase activity measured could not be due to a contaminating yeast protein; although it is expected that NTPase activity could be abundant in yeast cells, those endogenous activities were completely inactivated upon extensive heat treatment.

All four NTPs and dNTPs tested were hydrolyzed by \( l_1 \), and it is most likely that all NTPs and dNTPs can be hydrolyzed since either one of them can substitute for ATP in the helicase assay. The efficiency of utilization appears to vary among the different NTPs and dNTPs, with the highest rate of hydrolysis for dATP, followed by ATP and other NTPs. However, it should be mentioned that even though all \( K_m \) values are in the micromolar range, they are lower for NTPs than for dNTPs. At a low concentration of substrates, as might be the case inside the viral core in vivo, ATP will thus likely be the preferred energy source.

A NTPase activity has been previously found in purified reovirus cores (19, 26, 27), and the activity has been recently assigned to \( l_1 \) using gene reassortment analysis (19). Interestingly, the core enzyme is capable of hydrolyzing either NTPs or dNTPs in the presence of \( Mg^{2+} \) or \( Mn^{2+} \) and also has an unusual temperature optimum of 51 °C (27, 28). The observation that dATP was more efficiently hydrolyzed relative to ATP at high substrate concentration was also made in previous studies (19, 27). The catalytic properties of the recombinant \( l_1 \) protein analyzed in this study are consistent with these previous findings and further support the idea that \( l_1 \) is the protein responsible for the NTPase activity observed in reovirus cores.

The recombinant \( l_1 \)-associated NTPase/dNTPase had an apparent temperature optimum of 50 °C, but the activity declined

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**TABLE II**

Kinetic analysis of alternative nucleoside triphosphate phosphohydrolysis

| Substrate | \( K_m \) | \( k_{cat} \) | \( V_{max} \) |
|-----------|---------|---------|---------|
| ATP       | 1.4     | 12.6    | 2.3 \( \times 10^{-13} \) |
| GTP       | 2.0     | 5.3     | 9.7 \( \times 10^{-14} \) |
| dATP      | 6.6     | 93.4    | 1.7 \( \times 10^{-12} \) |
| dCTP      | 7.1     | 1.3     | 2.3 \( \times 10^{-14} \) |

\( a \) Values for \( K_m \) and \( V_{max} \) were determined from Lineweaver-Burk plots of hydrolysis activity. Reactions were conducted with NTP and dNTP concentrations varying between 0.0125 and 15 \( \mu \)M.

\( b \) \( k_{cat} \) values were calculated from the purified \( l_1 \) protein concentration estimated by immunoblotting using serial dilutions of purified reovirus as a standard.
rapidly at this high temperature, most likely due to thermal inactivation of the enzyme. Recent studies indicated that A1 interacts with the λ3 (polymerase) and A2 (guanylyltransferase) core proteins (29, 30); such interactions might account for the higher stability of NTPase activity found in purified viral cores compared with the isolated recombinant A1 protein.

The $K_m$ values for ATP and GTP hydrolysis calculated from Lineweaver-Burk plots were 1 and 2 μM, respectively. These $K_m$ values are in the same range observed for the well studied SV40 large T antigen, which is also a DNA and RNA helicase (31). To date, only three other viral helicases have been shown to possess both DNA and RNA helicase activities; they are SV40 large T antigen, vaccinia virus protein 18R, and hepatitis C virus protein NS3 (32–34). One cellular eukaryotic protein, nuclear DNA helicase II, also possesses the ability to unwind dsDNA and dsRNA substrates (35). The reovirus A1 protein shares with these four helicases the ability to hydrolyze all NTPs and dNTPs. However, SV40 large T antigen unwinds dsDNA when ATP is present, but unwinds dsRNA with UTP, CTP, or GTP as cofactor (32). The bound nucleotide seems to determine whether the T antigen acts as an RNA or DNA helicase. In contrast, the reovirus A1 protein and hepatitis C virus protein NS3 can use the energy provided by the hydrolysis of any NTPs or dNTPs for both their RNA and DNA helicase activities (34).

Many viral helicases have high basal NTPase activity in the absence of added nucleic acids. This is the case of the potyvirus-flavivirus-pestivirus proteins that have been recently characterized (36–39). In this respect, they differ from most cellular proteins of the NTPase/helicase superfamilies. For example, cellular protein 68 and eukaryotic translation initiation factor 2 and 4A exhibit almost no detectable ATPase activity in the absence of RNA (40, 41). Our results demonstrated that the reovirus A1 protein, like many viral NTPases, also exhibits NTPase and dNTPase activities in the absence of added nucleic acids. Although it cannot be completely excluded that nucleic acids can stimulate NTPase activity, the addition of various nucleic acids (single-stranded DNA, dsDNA, single-stranded RNA, and dsRNA) has failed to produce a significant change in $k_{cat}$ values for NTPase activity (data not shown).

The helicase activity of A1 was not detected at an ATP concentration of 0.1 μM and was maximal at 10 μM (data not shown). This is consistent with the $K_m$ value of 1 μM for ATPase activity and with a probable functional coupling between the two reactions where NTP or dNTP hydrolysis generates the energy required for unwinding of nucleic acids.

The significance, if any, of the DNA helicase activity of the A1 protein is currently not understood, but a previous report already demonstrated the A1 affinity for dsRNA and dsDNA in filter binding assays (18). As previously mentioned, the NS3 protein from hepatitis C virus is another example of an RNA virus protein with an associated DNA helicase activity. Like the reovirus A1 protein, the role of the DNA helicase activity in the multiplication cycle or pathogenesis of hepatitis C virus is not yet established (34).

In addition to earlier genetic evidence that assigned transcriptase activity to λ3, more recent biochemical evidence has confirmed that this protein possesses RNA polymerase activity (29). However, the protein appears to be unable by itself to transcribe its natural dsRNA substrate. This has led to the idea that additional proteins are involved in transcription. The A1-associated RNA helicase activity reported in this study strongly suggests an involvement of this protein in the transcription of the double-stranded reovirus genome. Although it is possible that normal transcription and replication of the viral genome require the formation of a well structured core or the participation of cellular protein(s), this report is the third case, with the previous examples of A2 and A3 (29, 42), where an actual enzymatic activity could be demonstrated on a solubilized reovirus protein. To our knowledge, this report of A1 helicase activity is the first demonstration of such an activity in a mammalian dsRNA virus.

The reovirus core also contains enzymes that modify the 5′-end of newly synthesized mRNAs by adding a cap structure similar to the one present on cellular mRNAs (43, 44). An RNA triphosphatase activity (polynucleotide phosphohydrolase) is involved in the formation of the reovirus cap structure by releasing inorganic phosphate from the 5′-triphosphate end of the nascent mRNAs (1). The nature of the core protein involved in this process is currently unknown, but the A1 protein, with its ability to release the terminal phosphate from free nucleotides, is certainly an attractive candidate to exert a similar activity on polynucleotides harboring a 5′-triphosphate end.

Acknowledgments—We thank Carole Danis for expert technical assistance and Dr. Pierre Belhumeur for suggesting the use of the yeast expression system. We also thank Dr. Michael R. Roner for the generous gift of the anti-A1 antibody.

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